Excitatory amino acid transporters (EAATs) mediate two distinct transport processes, a stoichiometrically coupled transport of glutamate, Na\(^+\), K\(^+\), and H\(^+\), and a pore-mediated anion conductance. We studied the anion conductance associated with two mammalian EAAT isoforms, hEAAT2 and rEAAT4, using whole-cell patch clamp recording on transfected mammalian cells. Both isoforms exhibited constitutively active, multiply occupied anion pores that were functionally modified by various steps of the Glu/Na\(^+\)/H\(^+\)/K\(^+\) transport cycle. Permeability and conductivity ratios were distinct for cells diazylized with Na\(^-\)- or K\(^-\)-based internal solution, and application of external glutamate altered anion permeability ratios and the concentration dependence of the anion influx. EAAT4 but not EAAT2 anion channels displayed voltage-dependent gating that was modified by glutamate. These results are incompatible with the notion that glutamate only increases the open probability of the anion pore associated with glutamate transporters and demonstrate unique gating mechanisms of EAAT-associated anion channels.

Excitatory amino acid transporters (EAATs)\(^*\) mediate the removal of glutamate from the synaptic cleft in the central nervous system and the uptake of glutamate in kidney and intestinal transporters (1–3). Five structurally distinct subtypes of mammalian glutamate transporters, EAAT1–EAAT5, have been identified in recent years (4–9). Each of these isoforms exhibits two separate transport processes: a stoichiometrically coupled countertransport of one glutamate with three Na\(^+\), K\(^+\), and H\(^+\), and a pore-mediated anion conductance. We studied the anion conductance associated with two mammalian EAAT isoforms, hEAAT2 and rEAAT4, using whole-cell patch clamp recording on transfected mammalian cells. Both isoforms exhibited constitutively active, multiply occupied anion pores that were functionally modified by various steps of the Glu/Na\(^+\)/H\(^+\)/K\(^+\) transport cycle. Permeability and conductivity ratios were distinct for cells diazylized with Na\(^-\)- or K\(^-\)-based internal solution, and application of external glutamate altered anion permeability ratios and the concentration dependence of the anion influx. EAAT4 but not EAAT2 anion channels displayed voltage-dependent gating that was modified by glutamate. These results are incompatible with the notion that glutamate only increases the open probability of the anion pore associated with glutamate transporters and demonstrate unique gating mechanisms of EAAT-associated anion channels.

Glutamate Modifies Ion Conduction and Voltage-dependent Gating of Excitatory Amino Acid Transporter-associated Anion Channels\(^*\)

Received for publication, July 23, 2003, and in revised form, September 18, 2003
Published, JBC Papers in Press, September 23, 2003, DOI 10.1074/jbc.M307990200

Nico Melzer, Alexander Biela, and Christoph Fahlke

From the Institute of Physiology, RWTH Aachen, 52057 Aachen, Germany and Centro de Estudios Científicos, Avenida Prat 514, Valdivia, Chile

EXPERIMENTAL PROCEDURES

Expression of hEAAT2 and rEAAT4 in tsA201 and HEK293 Cells—A pcDNA3.1-hEAAT2 construct was generated by subcloning the coding region of hEAAT2 (16) (kindly provided by Dr. M. Hediger, Harvard Medical School, Boston) into a pcDNA3.1 vector (Invitrogen) using flanking NotI restriction sites. The pcDNA3.1-rEAAT4 construct was kindly provided by Dr. J. Rothstein, The Johns Hopkins University, Baltimore (17). Transient transfection of tsA201 cells using the Ca\(^{2+}\)-dependent lipofection method (Bio-Rad) was performed as described previously (18). To identify cells with a high probability of expressing recombinant transporters, cells were cotransfected with a plasmid encoding the CD8 antigen and incubated 5 min with polystyrene microbeads precoated with anti-CD8 antibodies (Dynal, Great Neck, NY) (19). Only cells decorated with microbeads were used for electrophysiological recordings. By adjusting the CD8/EAAT cDNA ratio, we optimized our conditions so that almost every cell with beads exhibited a current component with characteristic properties shown in Fig. 1. For each construct, two independent recombinants were examined and exhibited indistinguishable functional properties. Oligodendrocyte cell lines stably expressing hEAAT2 were obtained by selection for resistance to the aminoglycoside antibiotic geneticin (G418, Roche Applied Science) as described previously (18). The stable cell lines so obtained exhibited electrical properties indistinguishable from transiently transfected tsA201 cells. Untransfected tsA201 cells displayed a negligible endogenous anion current component (current amplitude at -175 mV: Cl\(^-\), -54 ± 6 pA; NO\(_3\), -63 ± 13 pA; SCN\(^-\), -56 ± 10 pA; at +125 mV: Cl\(^-\), 40 ± 3 pA; NO\(_3\), 31 ± 3 pA; SCN\(^-\), 211 ± 13 pA, n > 5 cells) that was not modified by the addition of glutamate.

Whole-cell Recordings—Whole-cell patch clamp recordings were performed using an Axopatch 200B (Axon Instruments, Union City, CA) or EPC10 (HEKA Electronics, Lambrecht, Germany) amplifier. Borosilicate pipettes were pulled with resistances of 1.0–2.2 megohms. More than 80% of the series resistance was compensated by an analog procedure resulting in calculated voltage errors <5 mV. Currents were filtered at 5 kHz and digitized with a sampling rate of 50 kHz using a Digidata (Axon Instruments) or an ITC-16 (HEKA) AD/DA converter. Cells were clamped to 0 mV for at least 2 s between test sweeps. The solutions of the standard solutions are as follows: extracellular (in mM) 140 NaCl, 4 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 5 HEPES, pH 7.4; intracellular (in mM) 115 NaCl, 2 MgCl\(_2\), 5 EGTA, 10 HEPES, pH 7.4. For certain experiments, NaCl in the intra- or extracellular solution was equimolarly substituted with NaNO\(_3\), NaSCN, KCl, KNO\(_3\), or KSCN. Substrate-containing external solutions were made by adding 0.5 mM l-glutamate. Anion currents were recorded always under con-
ditions that abolish stoichiometric glutamate current, i.e., in the absence of external glutamate or internal K⁺ (20). For determination of anion permeability and conductivity ratios for external anions, cells were moved into the stream of solutions containing various sodium salts (in mM) 140 NaX, 4 KCl, 2 CaCl₂, 1 MgCl₂, 5 HEPES, pH 7.4 (X denotes Cl⁻, I⁻, NO₃⁻, and SCN⁻). To test for a concentration dependence of biionic reversal potentials (Fig. 4C), measurements were performed on cells internally dialyzed with (in mM) 177-X sodium glutamate, 5 EGTA, 10 HEPES, pH 7.4, in an external solution containing (in mM): Y NaScN, 150-Y sodium glutamate, 5 HEPES, pH 7.4 (ratio YX = 117:150). For the experiments shown in Fig. 5, solutions with various external [SCN⁻] were made by substituting NaSCN in the external standard solution with equimolar NaCl as the Cl⁻ conductance of EAAT2 is negligible compared with SCN⁻ (Fig. 2). For all these experiments, we used external and/or internal agar salt bridges, made from a plastic tubing filled with 3 mM KCl in 0.3% agar, to connect the Ag/AgCl electrode. Offset potentials determined at the end of each experiment, and junction potentials either calculated using the JPCalc software (Dr. P. Barry, University of South Wales, Sydney, Australia (21)) or directly measured (22) were used to correct results.

Data Analysis—Data were analyzed with a combination of pClamp (Axon Instruments, Union City, CA) and SigmaPlot (Jandel Scientific, San Rafael, CA) programs. Current amplitudes were used without subtraction procedure, and all summary data are shown as means ± S.E. For statistical evaluation the Student's t test was used.

To obtain current-voltage relationships in various internal or external anions, isochronal current amplitudes were determined 1 ms after the voltage step. Current ratios given in Fig. 4 were determined by dividing isochronal current amplitudes obtained from the same cell after application of 0.5 mM l-glutamate by the corresponding value before application of external substrate. For the determination of normalized current amplitudes as a mean of channel selectivity (Tables I and II), we divided current amplitudes in various external anions by the corresponding value determined from the same cell in a NO₃⁻-based external solution, rather than in a standard external solution to minimize errors introduced by dividing by the rather small current amplitudes of cells expressing EAAT2 in external Cl⁻. Reversal potentials were used for further analysis when the cell exhibited a whole-cell conductance that was at least three times larger than the mean value for untransfected cells under identical conditions. Permeability ratios were calculated from reversal potential measurements under biionic conditions using the Goldman-Hodgkin-Katz equation as described (18) (Tables I and II). Affinity constants (Kᵢ) (Fig. 5) were calculated by fitting isochronal current amplitudes versus [SCN⁻] after a Lineweaver-Burk transformation and fitting a linear function with a least square routine with Excel (Microsoft) and Sigma Plot (Jandel Scientific). Kᵢ values were determined from individual cells, in the absence and in the presence of l-glutamate, and all Kᵢ values provided are means ± S.E. of 7 individual cells. The voltage dependence of the selectivity (Fig. 7A) was determined by plotting the normalized instantaneous current amplitude at +90 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 value (R(V) = Iᵩ/(1 + e(V−V₅₀)/S) + PᵢNµb). To describe the time course of current inactivation, a sum of two exponentials and a time-independent value were fit to data recorded during a series of voltage steps from a holding potential of 0 mV (Fig. 7B). To describe the recovery from inactivation, we used a pulse protocol consisting of a 150-ms conditioning pulse to +160 mV, a hyperpolarizing prepulse (to potentials between −180 and −120 mV) of variable duration, and a fixed test pulse to +160 mV (Fig. 7C). The dependence of peak current amplitude on the prepulse duration was fit with a single exponential giving the time constant of recovery from inactivation (Fig. 7D).

Noise Analysis—Non-stationary noise analysis (23, 24) was performed using an EPC10 amplifier (HEKA Electronics, Lambrecht, Germany) equipped with a 16-bit AD/DA converter as described (18). Currents were recorded at 10 kHz and digitally recorded with a sampling rate of 50 kHz. Cells were held at the current reversal potential, and a series of 300 records was recorded by pulsing to a certain voltage. Pairs of subsequent records were then subtracted using the PulseTools software (HEKA Electronics, Lambrecht, Germany) to compute the experimental non-stationary ensemble variance (25). The variance determined in cells expressing EAAT4 was significantly larger than the background noise observed in non-transfected cells, and we observed a clear correlation between current and noise amplitudes determined at different cells. The amplitude of a current noise generated by the opening and closing of ion channels depends on the unitary current amplitude (I), the number of channels (N), and the absolute open probability (P) of the underlying channels (23, 24) as shown in Equation 1.

\[ \sigma^2 = NI^2P(1 - P) \]  

To determine the single channel amplitude at a certain potential, the background variance \( \sigma^2_B \) measured at the current reversal potential was subtracted from variances determined at various time periods after a voltage step to positive potentials (+120 to +160 mV). The subtracted variances were sorted into current bins (25) and plotted versus the corresponding mean current amplitude (Fig. 6). Under these experimental conditions the absolute open probability changes with time as given by Equation 2.

\[ P(t) = \frac{I(t)}{N} \]  

yielding Equation 3.

\[ \sigma^2(t) = \sigma_B^2 - \sigma(t) = \frac{I(t)}{N} \]  

A fit of Equation 3 to the variance versus mean current (24) plot allowed us to determine the unitary current amplitude and the number of anion channels. By using these two values we calculated the absolute open probabilities (P) at the holding potential by extrapolating the instantaneous macroscopic current amplitude \( I_{\text{inst}} \) and dividing this amplitude by the number of channels (N) and the single channel amplitude (I) as shown in Equation 4.

\[ P = \frac{I_{\text{inst}}}{N I} \]  

RESULTS

EAAT-associated Anion Currents Are Active in the Absence of Transporter Substrate—Fig. 1 shows representative recordings from tsA201 cells expressing EAAT2 and EAAT4. Cells were intracellularly dialyzed with a potassium-free solution and extracellularly perfused with glutamate-free solutions with either Cl⁻ (Fig. 1, A and D), NO₃⁻ (Fig. 1, B and E), or SCN⁻ (Fig. 1, C and F) as main anions. These conditions eliminated the GluNa⁺/H⁺/K⁺ current component (20) and allowed us to record anion currents in isolation. For cells expressing EAAT2, currents in external Cl⁻ were small (current amplitude at +125 mV: 0.11 ± 0.02 nA, n = 16) (Fig. 1A). Substitution of Cl⁻ by NO₃⁻ (Fig. 1B) or SCN⁻ (Fig. 1C) increased the anion influx at positive potentials and also the chloride efflux at negative potentials. Cl⁻ currents in cells expressing EAAT4 were larger than those in cells expressing EAAT2 (current amplitude at +125 mV: 0.27 ± 0.05 nA, n = 12) (Fig. 1D). Again, external NO₃⁻ (Fig. 1E) and SCN⁻ (Fig. 1F) enhanced the current amplitudes at negative and positive potentials as compared with recordings in external Cl⁻. Under all external anion compositions EAAT2- and EAAT4-associated anion currents rose instantaneously upon voltage steps to negative and positive potentials. The two isoforms differed in the time course of current responses to depolarizing voltage steps. Whereas EAAT4-associated currents decayed in a voltage- and time-dependent manner, such current relaxations could not be observed in cells expressing EAAT2 (Fig. 1).

Fig. 2A shows the voltage dependence of the absolute EAAT2-associated current amplitudes for external Cl⁻, I⁻, NO₃⁻, and SCN⁻ in the absence of external glutamate. EAAT2 displayed a SCN⁻ > NO₃⁻ > I⁻ Cl⁻ conductivity sequence (Table I). Fig. 2B presents averaged isochronal current amplitudes for cells intracellularly dialyzed with solutions containing Cl⁻, NO₃⁻, and SCN⁻ illustrating a higher conductivity also for intracellular polyatomic anions compared with Cl⁻ (Table II). Permeability ratios from the reversal potentials obtained in these experiments revealed for EAAT2 a relative anion permeability sequence of P_I < P_SCN < P_NO3 < P_Cl for external anions (Table I), and a P_SCN > P_NO3 > P_Cl sequence for internal anions.
EAAT2- and EAAT4-associated anion currents differ in the relative anion conductivities as well as in the relative anion permeabilities (Tables I and II). Similar experiments with EAAT4 (Fig. 2, C and D) demonstrated an SCN⁻/NO₃⁻ conductivity and permeability sequence for external as well as for internal anions (Tables I and II).

**Table I**

<table>
<thead>
<tr>
<th>Anion</th>
<th>External Conductivity (nS/µM⁻¹)</th>
<th>Internal Conductivity (nS/µM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl⁻</td>
<td>0.3 ± 0.06</td>
<td>0.3 ± 0.06</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>4.9 ± 0.28</td>
<td>4.9 ± 0.28</td>
</tr>
<tr>
<td>SCN⁻</td>
<td>8.8 ± 0.40</td>
<td>8.8 ± 0.40</td>
</tr>
</tbody>
</table>

**Table II**

<table>
<thead>
<tr>
<th>Anion</th>
<th>External Permeability (µM⁻¹)</th>
<th>Internal Permeability (µM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl⁻</td>
<td>0.1 ± 0.06</td>
<td>0.1 ± 0.06</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>4.0 ± 0.24</td>
<td>4.0 ± 0.24</td>
</tr>
<tr>
<td>SCN⁻</td>
<td>8.3 ± 0.40</td>
<td>8.3 ± 0.40</td>
</tr>
</tbody>
</table>

Transitions in the Glutamate Transport Cycle Modify EAAT-associated Anion Pathways—Application of external glutamate enhanced EAAT-associated anion currents and modified the underlying anion channels. Fig. 3 shows EAAT4-associated current amplitudes in the absence and in the presence of glutamate under two ionic compositions: NaCl-based internal and NaNO₃-based external solutions (Fig. 3, A and C) and NaNO₃-based internal and NaCl-based external solutions (Fig. 3, B and D). For both conditions, application of external glutamate caused a 2-fold increase of the anion current amplitude (Fig. 3, C and D, and Fig. 4A). Similar increases of the anion current amplitude by glutamate are observed in cells expressing EAAT2 (Fig. 4B). This demonstrates that there is a substantial constitutive activity of EAAT anion channels in the absence of glutamate.
Glutamate Modifies Selectivity and Gating of EAAT-Anion Channels

Table II
Relative anion permeabilities for internal anions
△, statistical significance of differences in permeability ratios between perfusion with and without glutamate based on a Student’s t test. * and ** indicate statistically significant differences based on a Student’s t test (*, p < 0.05; **, p < 0.01). N, the number of cells.

<table>
<thead>
<tr>
<th>Internal Na⁺</th>
<th>E₁ (mV)</th>
<th>Pₓ/Pₓ</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAAT2</td>
<td>NO₃</td>
<td>26.5 ± 7.5</td>
<td>3.7 ± 0.9₆</td>
</tr>
<tr>
<td></td>
<td>SCN</td>
<td>72.3 ± 7.2</td>
<td>26.2 ± 6.0₆₉</td>
</tr>
<tr>
<td>EAAT4</td>
<td>NO₃</td>
<td>33.6 ± 2.1</td>
<td>5.9 ± 0.5₆</td>
</tr>
<tr>
<td></td>
<td>SCN</td>
<td>37.9 ± 5.1</td>
<td>6.5 ± 1.5₆</td>
</tr>
<tr>
<td>EAAT2</td>
<td>NO₃</td>
<td>41.0 ± 8.4</td>
<td>6.8 ± 1.6₆</td>
</tr>
<tr>
<td></td>
<td>SCN</td>
<td>95.5 ± 8.3</td>
<td>67.2 ± 16.5₆</td>
</tr>
<tr>
<td>EAAT4</td>
<td>NO₃</td>
<td>49.6 ± 3.3</td>
<td>9.3 ± 1.4₆</td>
</tr>
<tr>
<td></td>
<td>SCN</td>
<td>61.3 ± 4.9</td>
<td>15.9 ± 3.0₆</td>
</tr>
</tbody>
</table>

EAAT4-associated anion currents display distinct time and voltage dependences for various anionic conditions. For NaCl-based internal and NaNO₃-based external solutions, there was a time-dependent current decrease at positive potentials (Fig. 3A). In contrast, for NaNO₃-based internal and NaCl-based external solution, currents activated upon membrane hyperpolarization (Fig. 3B). Glutamate caused a change of the time and voltage dependence of currents for both anion conditions. Current inactivation was less pronounced in the presence of glutamate in external NO₃⁻ (Fig. 3A), and for internal NO₃⁻ a time-dependent current decrease at negative potentials appeared after glutamate application (Fig. 3B). EAAT2 anion currents did not exhibit time-dependent changes of the current amplitude for external (Fig. 1) or for internal NO₃⁻ (data not shown).

For EAAT2 and EAAT4, the glutamate-induced current increase depended on the anion composition. For cells intracellularly dialyzed with a NaCl-based internal solution, these values were highest for NO₃⁻ and smaller for SCN⁻ and Cl⁻ (Fig. 3A). Moreover, for various ionic conditions application of glutamate caused a significant shift of the reversal potential (Fig. 3, C and D, insets, Tables I and II). These shifts were not due to glutamate permeation through the anion pore, as glutamate caused shifts to more negative under certain conditions (Fig. 3C, inset) and shifts to more positive potentials under others (Fig. 3D, inset). They rather indicate an alteration of the relative anion permeability of EAAT2- and EAAT4-associated anion pathways by glutamate (Fig. 4B).
Glutamate Modifies Selectivity and Gating of EAAT-Anion Channels

Other steps in the glutamate transport cycle (20, 26–28) also altered the selectivity of the anion pores. Internal solutions without K⁺ prevent the occupation of K⁺-bound states and might therefore result in a distinct distribution of transporter states than under standard conditions (20). If all anion-conducting states displayed the same anion permeability, the reversal potentials determined with the two distinct internal solutions would be identical. In contrast, differences in measured reversal potentials indicate that distinct conformations of EAAT transporters are associated with different anion permeabilities. For EAAT2 and EAAT4, the anion permeability ratios for certain external anions measured in the absence of glutamate depended on the intracellular cation. The relative permeability of EAAT2 for external I⁻ and for internal SCN⁻ was significantly different for cells dialyzed with Na⁺ or K⁺ (Table I). For EAAT4, distinct anion permeabilities for external SCN⁻ and NO₃⁻ but for none of the tested internal anions were observed (Table I). These results indicate that various conformational states of EAAT2 and EAAT4 are associated with distinct anion selectivities.

EAAT2 and EAAT4 Exhibit Multiply Occupied Anion-selective Pores—The EAAT-associated anion conductance exhibited several properties that appear unusual for a pore-mediated process: an increase of Cl⁻ efflux upon application of more permeant external anions (Fig. 2) comparable with a trans-acceleration phenomenon, a change of permeability and conductivity upon addition of glutamate (Figs. 3 and 4, Tables I and II), and the dependence of anion selectivity on the intracellular cation (Tables I and II). These observations could cast some doubt on the notion that the EAAT-anion conductance is carrier-mediated anion transport. Moreover, it indicated that the anion pores of EAAT2 and EAAT4 contain more than one carrier and thus do not occur in a fixed stoichiometry and excluded a process: an increase of Cl⁻ efflux upon application of more permeant external anions (Fig. 2) comparable with a trans-acceleration phenomenon, a change of permeability and conductivity upon addition of glutamate (Figs. 3 and 4, Tables I and II), and the dependence of anion selectivity on the intracellular cation (Tables I and II). These observations could cast some doubt on the notion that the EAAT-anion conductance is carrier-mediated anion transport. Moreover, it indicated that the anion pores of EAAT2 and EAAT4 contain more than one carrier and thus do not occur in a fixed stoichiometry and excluded a

External Glutamate Modifies the Transfer of Anions through EAAT-associated Pores—The observed changes of anion conductance (Fig. 4) suggest that certain steps in anion permeation through the EAAT anion pores are altered by external substrate. To test which of these steps are primarily affected by external glutamate, we determined the effects of varying the external [SCN⁻] on reversal potentials and on the measured Kᵢ values at positive potentials (Fig. 5C). It is also in agreement with the unchanged dissociation constants at negative potentials (Fig. 5D). Most likely, at negative potentials the k⁺trans rate constant (describing a transition against the electrostatic gradient) is substantially smaller than at positive potentials. Its contribution to the dissociation constants at negative membrane potentials will therefore be negligible. Thus, a glutamate-induced change of k⁺trans will only little affect the Kᵢ values at negative potentials, in agreement with the experimental results (Fig. 5D). The glutamate-induced acceleration of the k⁺trans rate constant predicts an increase of the unitary current amplitude, providing a novel explanation for the increase of the macroscopic current amplitude in the presence of substrate.

Unitary Properties of EAAT4-associated Anion Channels—In external I⁻, NO₃⁻, and SCN⁻, EAAT4-associated anion currents displayed prominent time- and voltage-dependent current relaxations in both the absence and the presence of external glutamate (Figs. 1 and 3). These transient currents are not capacitive charge movements; they are changes of ionic current

Fig. 5. Effect of changes of [SCN⁻] on isochronal current amplitudes in tsa201 cells expressing EAAT2. A, mean current amplitudes for various external [SCN⁻] in the absence of external glutamate. SCN⁻ was substituted equimodularly with Cl⁻ – B, mean current amplitudes for various external [SCN⁻] in the presence of 0.5 mM external glutamate. C and D, voltage dependence of the Kᵢ for SCN⁻ in the absence (●) and in the presence (○) of external glutamate. Means ± S.E. from 7 cells.
Glutamate Modifies Selectivity and Gating of EAAT-Anion Channels

amplitudes as illustrated by the following three pieces of evidence: the amplitude differed for distinct anions (Figs. 1 and 2), the reversal potential shifted upon alterations of the anion composition (Fig. 3), and integrals of the transient currents elicited by steps to positive potentials were always distinct from the integral determined after stepping back to the holding potential. The observed current relaxations therefore can be caused by two distinct mechanisms: time-dependent changes of the open probability of the anion channel or decreases of the unitary current amplitude. We employed non-stationary noise analysis to distinguish between the two possibilities.

Fig. 6 shows variance analysis from a tsA201 cell expressing EAAT4 dialyzed with NaCl-based internal solution in a NaNO₃-based external solution. Fig. 6, A and B, illustrates the time course of the average current for a voltage step from the current reversal potential (−53 mV) to a test potential of +120 mV and the corresponding time course of the variance. Although the current amplitude decreased with time after the voltage step, the noise increased. Such a time dependence of the current amplitude and the noise ($\sigma^2 = N_i^2 P(1 - P) = (I^2/N)(1/P - 1)$) indicates that the EAAT4-associated current relaxations are due to changes of the number of open channels. Gating of EAAT4 occurs by the same mechanism as gating of ion channels.

To obtain the unitary current amplitude and the absolute open probability of the underlying channels, the variance-mean current plot in Fig. 6C was fitted to Equation 3. From this fit, the unitary current amplitude, $57 \pm 7 \text{ fA} (n = 9)$ at +120 mV, and the number of channels in the cell were obtained. By dividing the instantaneous current amplitude by the product of the unitary current amplitude and the number of channels, we then calculated the absolute open probability at the holding potential ($0.84 \pm 0.06, n = 9$)). Measurements were repeated in several cells at three different test potentials (Fig. 6D).

Because the open probability of EAAT4 is distinct from zero at the end of the test pulse, the variance-mean current plot is not a complete parabola. However, as the fitted parabola assumed its maximum within the experimentally determined variance-mean current plot, the fits were well defined. We obtained reproducible results for the unitary current amplitude and the open probabilities at the beginning and the end of the voltage step in several cells. All these results demonstrated that our non-stationary noise analysis reliably determined the unitary current amplitude of EAAT4. The high absolute open probability of EAAT4 anion channels in glutamate-free solutions demonstrates that a mere increase of the open probability cannot explain the glutamate-induced increase of the macroscopic current amplitude. Obviously, glutamate also increases the unitary current amplitude of EAAT4 anion channels.

Gating of EAAT4-associated Anion Channels—We next studied time- and voltage-dependent opening and closing transitions of EAAT4-associated anion channels in cells dialyzed with NaCl-based internal solution in a NaNO₃-based external solution. In the absence of glutamate, steady-state inactivation can be described with a single Boltzmann distribution with a midpoint of inactivation at 75.4 ± 9.2 mV ($n = 7$) and a voltage-dependent minimum open probability of 0.19 ± 0.02 ($n = 7$) (Fig. 7A). The significant minimum open probability can be assigned to an incomplete inactivation of EAAT4 anion channels by noise analysis (Fig. 6C). Current amplitudes decayed in a biexponential time course with two time constants of about 5 and 60 ms (Fig. 7B). Stepping back to negative potentials after a positive test step caused a monoexponential increase of the amplitude of the currents elicited by a following step to +160 mV (Fig. 7, C and D).

Voltage-dependent gating of EAAT4 anion channels is modified by external glutamate. Addition of external glutamate increased the minimum open probability to 0.60 ± 0.05 ($n = 7$) and shifted the midpoint of inactivation to 106.3 ± 3.2 mV ($n = 7$) (Fig. 7A). Before application of glutamate, currents decayed in a biexponential time course at positive potentials (Fig. 7B). After application of 0.5 mM glutamate, the current decay became monoexponential within a time constant of about 5 ms and

![Figure 6](image-url)

**Fig. 6.** Variance analysis for EAAT4 anion channels. A, mean current trace obtained from 300 current responses to a test step to +120 mV from a holding potential of −53 mV. B, time course of the variance (solid line). C, variance versus current plot fitted to the function $\sigma^2 = i(t) - i(t)^2/N$ (dashed line). D, voltage dependence of the unitary current amplitudes for three different test potentials (○). The dashed line illustrates the current-voltage relationship of the instantaneous macroscopic current from 6 cells measured under identical conditions scaled down to superimpose on the single channel currents. All data were obtained from cells dialyzed with a standard intracellular solution and extracellularly perfused with NaNO₃-based solution.

![Figure 7](image-url)

**Fig. 7.** Time- and voltage-dependent changes of EAAT4-associated anion currents. A, voltage dependence of the relative open probability of the EAAT4 anion channels in the absence (○, $n = 7$) and in the presence of 0.5 mM glutamate (□, $n = 7$) and in the presence of 0.5 mM glutamate (□, $n = 31$) and in the presence (○, $n = 31$) of 0.5 mM glutamate. C, current response of a tsA201 cell expressing EAAT4 to a pulse protocol consisting of a pulse to +160 mV followed by a voltage step to −120 mV of variable duration and a fixed test step to +160 mV. D, voltage dependence of the time constants of the recovery from inactivation in the absence (○, $n = 7$) and in the presence of 0.5 mM glutamate (○, $n = 5$). All data were obtained from cells dialyzed with a standard intracellular solution and extracellularly perfused with NaNO₃-based solution.
a significantly increased relative late current amplitude, i.e. a significant increase of the ratio of the late by the instantaneous current amplitude at positive potentials (Fig. 7, A and B). Furthermore, glutamate decreased the time constants of recovery from inactivation (Fig. 1D). The glutamate-induced gating alterations will result in an increase of the EAAT4 anion conductance under various conditions. In the absence as well as in the presence of external glutamate, membrane depolarization might cause an inactivation of EAAT-associated anion channels. In the presence of glutamate, this inactivation will be less complete and recovered faster after returning to the resting potential causing a decreased input resistance and membrane length constant.

**DISCUSSION**

**EAAT-associated Anion Currents**—The small background anion permeability of our expression system enabled us to measure directly EAAT-associated anion currents in the absence as well as in the presence of external substrate. For both isoforms, anion currents were significantly different from currents observed in untransfected cells. Application of external glutamate caused a 2-fold increase of the anion current amplitude (Fig. 4A) indicating that EAAT-associated anion channels are active in the absence of substrate and that this tonically active anion conducance is a substantial component of the activated anion current (Figs. 3 and 4). The existence of a glutamate-independent anion conducance has been reported (15, 31, 32); however, previous estimates of the relative amplitude of this current amplitude for EAAT1 and EAAT3 were much lower (between 0.05 and 0.2 (15, 32)). This difference likely arises from the methods used to measure the glutamate-independent anion currents. Earlier experiments employed blocking agents to estimate the glutamate-independent anion conducance. It appears possible that block of the anion conducance by these agents is incomplete resulting in an underestimation of the substrate-independent conducance. In contrast, we determined current amplitudes without subtraction procedures. As anion currents are potentially contaminated by currents conducted by endogenous anion channels, our estimate of the tonically active EAAT-associated anion currents might be above the real value. For measurements with Cl⁻ as the only permeant anion, currents associated with EAAT2 are quite small, and indeed the glutamate-induced current increase determined under these conditions is smaller than the values for other anions (Fig. 4A). However, for other experimental conditions, the contribution of endogenous anions currents appears to be negligible for the following experimental results. The substrate-independent anion currents in EAAT2 and EAAT4-expressing cells differ in selectivity (Table I) and display a distinct time and voltage dependence of the current amplitude (Fig. 1), demonstrating that the anion currents are conducted by distinct heterologously expressed anion channels. Moreover, if a significant component of the substrate-independent anion current were conducted by endogenous channels, one would expect that the relative glutamate-induced anion current increases differ for varying expression levels of the EAAT protein, which was not observed in our experiments with I⁻, NO₃⁻, or SCN⁻ as permeant anions.

**Anion Selectivity of EAAT-associated Pores**—For EAAT2 and EAAT4, larger and polyatomic anions display a higher permeability and conductivity than Cl⁻. The mechanisms underlying this particular anion selectivity of EAAT-associated anion channels are currently not very well understood. However, a comparison with a different class of anion-selective channels, the CIC family of voltage-gated chloride channels, allows us insights into the processes that are involved in anion permeation and selection between different anions. CIC channels, similar to every anion channel tested so far, exhibit pores with anion-binding sites that prefer large and polyatomic anions (34). There are two classes of CIC channels that differ in the selectivity between anions, isoforms with a Cl⁻ > NO₃⁻ and isoforms or mutant channels exhibiting an NO₃⁻ > Cl⁻ permeability and conductivity sequence. These two classes of CIC channels do not differ in the selectivity of binding but in the absolute binding interaction energy for anions (34). For example, in CIC-1, a CIC isoform with a Cl⁻ > NO₃⁻ anion permeability and conductivity sequence, binding of anions is tight. Therefore, larger and polyatomic anions swell longer than small anions at the anion-binding sites. This reduces the permeability of these anions and causes the block of chloride current (35, 36). In contrast, in CIC-4, exhibiting a NO₃⁻ > Cl⁻ permeability sequence, the interaction energy is substantially lower. Association steps to the binding sites are rate-limiting and thus permeability follows the binding selectivity of the anion-binding sites (18). For EAAT2 and EAAT4, we found no indication of block by permeating anions. Apparently, for both anion pores, anion binding is less tight than in CIC channels resulting in the observed SCN⁻ > NO₃⁻ > I⁻ > Cl⁻ conductivity sequence.

**EAAT4-associated Anion Channels Have a Small Unitary Conductance and a High Open Probability in Substrate-free Solution**—By using non-stationary noise analysis, we determined unitary current amplitudes of EAAT4 anion channels in the fA range (Fig. 6D). By taking the distinct voltages and anion composition into account, these results are significantly larger than published unitary current amplitudes for EAAT1 (15) and smaller than those reported for a native glutamate transporter in photoreceptors from the tiger salamander (12) and heterologously expressed EAAT5 (33). We do not know the reasons for these differences. They might be caused by isoform-specific differences in anion conducance, but they could also be due to the distinct experimental procedures employed. The earlier results were obtained by applying various concentrations of glutamate assuming that the open probability in the absence of substrate is close to zero. If the studied glutamate transporter-associated anion channels exhibited comparatively large open probabilities in the absence of substrate as EAAT4, it could be that EAAT current amplitudes and variances have been subtracted by these procedures.

**A Substrate-modified Anion Pore in EAAT2 and EAAT4**—For EAAT2 and EAAT4, glutamate application alters the current reversal potentials under various asymmetric anion conditions (Figs. 3 and 4 and Tables I and II) indicating changes of the selectivity of the EAAT-associated anion channels. Experiments on cells dialyzed with either K⁺-containing or K⁺-free solution demonstrated that not only binding of glutamate but also other steps in the glutamate uptake cycle modify the anion selectivity of the EAAT anion pore. The finding that conformational changes of the glutamate carrier domain cause alterations of the anion selectivity filter suggests a close spatial proximity of glutamate transport and anion pore.

Comparing dissociation constants for SCN⁻ in the absence and in the presence of glutamate revealed that the Kᵩ values at positive voltages were increased by application of substrate but unaffected in the negative range (Fig. 4), in agreement with the notion that glutamate increases the rate constant for transversing the pore. These results suggest that glutamate causes an augmentation of the unitary current amplitude. This idea is supported by the high absolute open probability of EAAT4 anion channels in the absence of glutamate (0.84 ± 0.06, n = 9 at −53 mV). An increase of the open probability alone cannot explain the 3-fold increase of the macroscopic current amplitude by glutamate under these conditions (Figs. 3A and 4A).
A Glutamate-modified Voltage-gated Anion Channel—So far, EAAT-associated anion channels have been thought to be gated by a tight coupling to the glutamate carrier domain. Opening and closing were assumed to be governed by transitions in the glutamate uptake cycle resulting in gating features that are very similar to ligand-gated channels. Our results demonstrate certain discrepancies to this concept. EAAT-associated anion channels exhibit a substantial open probability in the absence of substrate, gating of EAAT4 depends on the independent open probability (Fig. 5) and accelerating the recovery from inactivation (Fig. 5). In the presence as well as in the absence of substrate, gating of EAAT4 depends on the anion composition (Fig. 3, A and B), quite similar to other anion channels, such as CIC-type (37, 38) or volume-activated anion channels (39).

Our results suggest a new view of EAAT-associated anion channels. They function as voltage-dependent anion channels much like conventional anion channels, but the functional properties are altered by the glutamate carrier domain. This modification not only alters transitions between conducting and non-conducting states but also affects pore properties, in clear contrast to almost all known examples of a modification of an ion channel by a ligand-bound receptor.

Acknowledgements—We thank Dr. M. Hediger and Dr. J. Rothstein for providing expression constructs for hEAAT2 and rEAAT4; Dr. Patricia Hidalgo and Dr. J. P. Johnson for critical review of the manuscript and helpful discussions; Dr. Louis DeFelice for helpful discussions; and Hannelore Heidtmann and Barbara Poser for excellent technical assistance.

REFERENCES