Noise analysis to study unitary properties of transporter-associated ion channels

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Excitatory amino acid transporters (EAATs) do not only mediate secondary-active glutamate uptake but also function as anion channels. We recently used macroscopic current recordings and noise analysis to determine unitary current amplitudes of anion channels associated with a neuronal EAAT isoform, EAAT4. We found that, at symmetrical NO₃⁻, EAAT4 anion channels exhibit a single channel conductance of ~1 pS in the absence as well as in the presence of glutamate. These results indicate that glutamate increases EAAT4 anion currents by modifying exclusively open probabilities, however, leaves unitary current amplitudes unaffected. Noise analysis has been developed for ion channels with a single conductance state and thus switch back and forth between a closed and a single open state. This analysis can also be used for equally spaced subconductance states but usually fails to distinguish transitions between various conductance levels.

Excitatory amino acid transporters (EAATs) are crucial for termination of glutamatergic synaptic transmission and for maintenance of low resting glutamate levels. EAATs transport glutamate as stoichiometrically coupled co-transporters of one L-glutamate, three sodium ions and one potassium ion while one proton is counter-transported. However, EAAT glutamate transporters do not only mediate coupled transport of L-glutamate and co-substrates but also pore-mediated anion permeation. EAAT anion currents are small in the absence of L-glutamate and increase upon application of the transport substrate in a concentration-dependent manner. Moreover, in many but not in all EAATs, anion channel activity requires the presence of external Na⁺. Anion currents closely follow transitions within the glutamate uptake cycle and there are—with the exception of the anion dependence of certain isoforms—no indications for gating transitions outside the transport cycle.

EAAT anion channel gating can be described by a kinetic scheme that is based on the glutamate transport cycle in which anion channel opening is associated with certain states. To account for the substrate dependence of macroscopic currents, different transporter states might either...
Results and Discussion

Figure 1 shows two kinetic schemes that account for the substrate-dependent gating of EAAT-associated anion channels and the tight coupling to the glutamate transport cycle. Glutamate uptake is initiated by association of three Na\(^+\), one H\(^+\) and one glutamate to the outward-facing conformation of the transporter. Subsequent translocation moves a large portion of the transporter to the inside allowing release of the substrates to the cytoplasm. After association of K\(^+\) retranslocation completes the uptake cycle. Since pH-buffered solutions and internal Na\(^+\) instead of K\(^+\) were used in our experiments, we decided to simulate ensembles of transporter-associated ion channels and to study their properties by noise analysis.
experiments, the kinetic schemes for both models were simplified by omitting H⁺ binding, while lumping together several binding steps to a single reaction and by allowing internal Na⁺ to permit transporter re-translocation²² (Fig. 1A and D). In model 1 the different states are associated with distinct unitary currents (Fig. 1A and Table 1), whereas in model 2 opening of the anion channels occurs from certain states with defined opening and closing states (Fig. 1D and Table 1). We thus attributed opening and closing rates to channel modes branching from each state (R_i-R_j) of the transport cycle (Fig. 1D and Table 1).

For both models we used a variation of a Dynamic Monte Carlo method²⁶,²⁷ to simulate microscopic and macroscopic EAAT4 anion currents. Figure 1B and E show simulated single-channel recordings at two voltages (±200 mV) and in the absence (top) or in the presence (bottom) of a saturating concentration of L-glutamate (20 mM). For model 1, the simulated single-channel amplitudes i were smaller in the absence (Fig. 1B and upper part) than in the presence of L-glutamate (lower part). At negative voltages the L-glutamate-free channel is open most of the time, while depolarization results in rapid flickering between open and closed states and lowered absolute open probability \( p_o \) (Fig. 1B and upper part). With L-glutamate larger single channel amplitudes appear in addition to the small-conductance channel openings that are also present under glutamate-free conditions. Open probabilities are comparable at positive and at negative voltages in the presence of substrate (Fig. 1B and lower part). Model 2 includes identical unitary current amplitudes with and without L-glutamate (Fig. 1D and E). In the absence of L-glutamate, membrane hyperpolarization favors channel openings, whereas absolute \( p_o \) is small at positive voltages (Fig. 1E and upper part). Application of L-glutamate strongly increases the probability of channel opening. With L-glutamate, \( p_o \) is larger at ±200 mV than at -200 mV (Fig. 1E and lower part).

We next performed simulations on a multi-channel level (\( N = 10,000, t = 10 \) s) at ±100 mV in the presence of saturating L-glutamate and employed noise analysis on simulated current recordings. Figure 2 shows power spectra determined using fast Fourier transformation for both kinetic schemes. Both power spectra can be well fit with a sum of two Lorentzians, equivalent to a decay of the corresponding autocorrelation function governed by two exponential components²⁸ (Fig. 2A and B, dashed lines). The acquired corner frequencies \( f_c^{\pm} \) are in accordance with values obtained by fast Fourier transformation of electrophysiological recordings from WT EAAT4 (\( f_c^{\pm} = 1.2 \) Hz, \( f_c^{\pm} = 103.9 \) Hz).²³

| Table 1. Simulation parameters |
|-----------------------------|
| No. | Forward reaction | Backward reaction | \( z_0 \) |
|-----|------------------|------------------|------|
| 1   | 23.70 x 10^1 M^s^-1 | 275.0 x 10^1 M^-s^-1 | 0.33 |
| 2   | 75.0 x 10^1 M^s^-1 | 10.1 x 10^1 M^-s^-1 | 0.33 |
| 3   | 16.3 x 10^1 M^s^-1 | 0.8 x 10^1 M^-s^-1 | 0.33 |
| 4   | 1.66 x 10^1 M^-s^-1 | 14.6 x 10^1 M^-s^-1 | 0.51 |
| 5   | 249.0 x 10^1 M^-s^-1 | n. d. | |
| 6   | 34.5 x 10^1 M^-s^-1 | 1.38 x 10^1 M^-s^-1 | 0.56 |
| 7   | 24.8 x 10^1 M^-s^-1 | 3.32 x 10^1 M^-s^-1 | |
| 8   | 1.4 x 10^2 M^-s^-1 | 1.0 x 10^2 M^-s^-1 | |
| 9   | 14.4 x 10^1 M^-s^-1 | 842.0 x 10^1 M^-s^-1 | 0.27 |

State-specific anion conductances in fS (model 1)

Table: State-specific opening/closing rates and conductance (model 2)

| To | TiNa3: | TiNa2: | TiNa2G: | TiK (Na): |
|----|-------|-------|---------|-----------|
| 93 | 0     | 48    | 25       | 0         |
| 87 | 17    | 809   | 0        | 914       |

Rate constants of the transport process and channel gating at 0 mV. Electrogenic reactions are defined by \( \bar{z} \) value corresponding to the product of the charge. The fraction of the electric field determines the charge movement across the membrane. Clockwise transitions in the model scheme are denoted as “forward reactions.” For model 1, conductance values \( \gamma \) are given for each state within the transport cycle. Opening and closing rates \( \sigma \) and \( \zeta \) correspond to model 2.

Thus be ignored in EAAT4 anion channel noise analysis.

Simulated macroscopic currents for model 1 are small and inwardly rectifying in the absence of L-glutamate (Fig. 3A and upper part), but much larger and non-rectifying in the presence of L-glutamate. At positive voltages the currents inactivate (Fig. 3A and upper part), while depolarization induces a deactivation (Fig. 3A and lower part). We analyzed these simulated currents by stationary noise analysis.²⁰,²³,²⁹,³⁰ Steady-state currents \( \langle \sigma \rangle \) and mean current amplitudes were measured at every voltage step of the simulations, and \( \sigma^2 - I_{\bar{V}}V^2 \) ratios were plotted against \( I_{\bar{V}}^2V^2 \) for each voltage pulse (Fig. 3B). The linear transformation of the data enables a calculation of the unitary properties by linear regression of the obtained values.²³,²⁹,³⁰ The number of channels \( N \) is given by the slope \( \langle \sigma \rangle \) of the fitted straight, whereas the \( y \)-axis
Figure 3. Whole-cell current simulation of model 1. (A) Simulated current traces of model 1 in the absence (top) and in the presence (bottom) of 20 mM L-glutamate ($t = 200$ ms, $F_s = 10$ kHz, $N = 10,000$). (B) Representative stationary noise analyses of simulated currents of model 1 for the L-glutamate-free (black squares) and the L-glutamate-bound channel (white diamonds) (-L-glu: $N = 14,285$; $\gamma = 63$ fS, +L-glu: $N = 4,523$, $\gamma = 1,089$ fS). (C) Voltage dependence of the open probability for simulated channels in the absence (black squares) and in the presence of 20 mM L-glutamate (white diamonds). For comparison, experimentally determined relative open probabilities of WT EAAT4 from (23) were normalized to the maximal open probability of the simulated channels in the presence of L-glutamate and given as solid lines. (D) Estimated channel numbers $N_n$ from analysis of model 1 using different time intervals $\Delta t$ interval $= [0.01, \ldots, 1.5$ s]. Analysis was performed in the absence (black squares) and in the presence (white diamonds) of 20 mM L-glutamate. (E) Estimated single channel amplitudes $i_s$ from analysis of different time intervals $\Delta t$ interval at -100 and -150 mV as in (D). Analysis was performed in the absence (black squares) and in the presence (white diamonds) of 20 mM L-glutamate.

Figure 2. Fourier analysis of simulated whole-cell currents. (A and B) Power density spectra from simulated EAAT4 anion currents using model 1 (A) or model 2 (B) ($N = 10,000$, $F_s = 10$ kHz, $t = 10$ s, 20 mM L-glutamate) showing the frequency dependence of the Lorentzian noise (black circles) and transporter noise (white circles) for model 1 (A) and model 2 (B). Fits with double Lorentzian functions are given as dashed lines. Corner frequencies $f_{c,1,2}$ for models 1 and 2 are indicated in the graphs.
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identical single channel conductance with and without L-glutamate (−L-glu: $\gamma = 801$ fS, +L-glu: $\gamma = 839$ fS) without changing the absolute number of channels (−L-glu: $N = 14,288$, +L-glu: $N = 9,945$) (Fig. 4B). Absolute open probabilities were determined by dividing simulated macroscopic current amplitudes by unitary current amplitudes and the number of channels (Fig. 3C). The so-obtained values are similar to experimental data obtained from tail-pulse analyses of original current recordings from WT EAAT4 at saturating L-glutamate, but significantly deviate in its absence (Fig. 3C).

To test the influence of the analyzed time intervals on the results of noise analysis we simulated current responses to 30 voltage jumps (2 s) from 0 mV to voltages between -200 and +200 mV. We then analyzed different time intervals (between 0.01 and 1.5 s) of the simulated currents and compared the resulting numbers of channels and unitary amplitudes. Plotting the number of channels $N$ (Fig. 3D) or the unitary amplitude $i$ (Fig. 3E) versus the length of the time interval allows assessment of the validity of the noise analysis in dependence on the used time length interval. For model 1, intervals longer than 1,000 ms are necessary to obtain the correct number of channels $N$ (Fig. 3D). The values for the unitary amplitudes $i$ increased constantly with $\Delta t_{\text{interval}}$ and did not saturate at the theoretical values (Fig. 3E).

Noise analysis of currents simulated based on model 2 (Fig. 4A) correctly revealed identical single channel conductance with and without L-glutamate (−L-glu: $\gamma = 801$ fS, +L-glu: $\gamma = 839$ fS) without changing the absolute number of channels (−L-glu: $N = 9,270$, +L-glu: $N = 8,248$) (Fig. 4B) and glutamate-increased absolute open probabilities (Fig. 4C). Comparison of the calculated voltage dependence of the open probability of simulated channels with experimental data\textsuperscript{23} shows optimal compatibility between these two data sets (Fig. 4C). The accuracy in determining the number of channels increases with duration (Fig. 4D). This analysis demonstrates that the theoretical number of channels $N$ can be estimated in presence of L-glutamate from a $\Delta t_{\text{interval}} < 100$ ms (Fig. 4D). The reliability of the unitary amplitude $i$ reaches its highest precision at longer $\Delta t_{\text{interval}} (> 0.5$ s) (Fig. 4E).
Our simulations eliminate many doubts about the use of noise analysis for EAAT-associated anion channels. The influence of transporter shot noise is negligible for channels with amplitudes in the range as observed for EAAT4 (Fig. 2). Power spectra of EAAT anion channels provide information on voltage-dependent transitions of the uptake cycle (Fig. 2). Lastly and most importantly, model 1 and model 2 predict substantial differences in EAAT4 anion current noise that can be detected by the noise analysis we performed in our recent paper.\textsuperscript{19} Noise analysis is thus capable of distinguishing between changes in anion channel amplitude or open probability as basis of substrate-dependent gating. Experimental data\textsuperscript{23} and the here presented simulations demonstrate that EAAT4 anion channels exhibit only a single conductance state. Opening and closing of EAAT4 anion channels occur from different states of the uptake cycle with distinct rate constants. This notion is in full agreement with a stable anion conduction pathway that is opened and closed by conformational changes underlying coupled transport.

**Methods**

**Simulation of ion channels.** Microscopic and macroscopic EAAT4 anion currents were simulated using a variation of a Dynamic Monte Carlo method, the so-called Gillespie Algorithm.\textsuperscript{26,27} We first determined the probabilities for each state occupancy at 0 mV by solving kinetic equations of the transitions within the transport process in the MATLAB environment (MathWorks) (Table 1). A cumulative sum of these probabilities was calculated and the initial state was chosen using random numbers uniformly distributed between 0 and 1. The propensities of switching to one of the neighbors is given by the fraction of the rate constant leading to this state divided by the sum of all rates leading away from the current state, e.g., $k_{\text{on}}(k_{\text{on}} + k_{\text{off}} + r_{\text{off}})$ for transitions from state $r_{\text{on}}$ to $r_{\text{off}}$. The direction of the next reaction is then determined using a second random number and the cumulative sum of the distinct propensities. The lifetime of the current state is given by, with being the sum of all rate constants leading away from state $r$. The transporter state was updated accordingly and the procedure was repeated until simulation time exceeded the desired limit. Corresponding anion currents were calculated by assigning single-channel open probabilities $p_{\text{it}} = 0$ to “transport states” $r_{\text{on}}$, $r_{\text{off}}$, and $p_{\text{it}} = 1$ to “channel states” $r_{\text{ch}}$, $r_{\text{ch}}^{-1}$ binning these data into blocks of length $1/F_s$ with a sampling frequency of $F_s = 10$ kHz and subsequent calculation of time-weighted averages of open probabilities within each block. We then summarized these data from all channels and calculated total anion current as $I = P_{\text{it}} \cdot V_{\text{mem}} \cdot \gamma$ with $V_{\text{mem}}$ being the membrane voltage, $P_{\text{it}}$ the macroscopic open probability and $\gamma$ the single-channel conductance (931 fS). We assumed a linear $i-V$-curve based on the presumption of a symmetrical anion distribution and experimental measurements.\textsuperscript{23}

**Simulation of transporter-noise.** To test the influence of shot-noise generated by electrogenic steps within the uptake cycle we simulated current noise due to electrogenic as $i = z d N_{\text{aq}} \cdot e_{\text{aq}} \cdot F_s$ that gives the current evoked by the transition from $r_{\text{on}}$ to $r_{\text{off}}$, with an associated effective charge movement $z d N_{\text{aq}}$ (Table 1), where $F_s$ denotes the sampling frequency.\textsuperscript{24}

**Noise analysis of simulated anion currents.** Power spectrum analysis of simulated ion currents was performed on the steady-state phase of 10-s-long current traces simulated at +100 mV in the MATLAB environment. Corner frequencies ($f_{\text{c}}$) were calculated from fitting power spectra to biphasic Lorentzian functions as described (Fig. 2A and B).\textsuperscript{23} Validity of noise analysis was assessed by calculation of absolute channel numbers $N$ and unitary amplitudes $i$ at -100 and -150 mV using stepwise increased time intervals ($\Delta t_{\text{interval}}$) within the steady-state phase of simulated current traces (Figs. 3D–E and 4D–E). Voltage dependencies of relative open probabilities of EAAT4-associated anion channels were calculated from experimentally determined instantaneous tail current amplitudes ($I_{\text{ch}}$) at +135 or -135 mV after preceding 200 ms steps to certain test voltages (Figs. 3G and 4C), as described in reference 23.
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