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Ion Fluxes Associated with Excitatory Amino Acid Transport

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Summary

Flux of substrate and charge mediated by three cloned excitatory amino acid transporters widely expressed in human brain were studied in voltage-clamped Xenopus oocytes. Superfusion of L-glutamate or D-aspartate resulted in currents due in part to electrogenic Na^+ cotransport, which contributed 1 net positive charge per transport cycle. A significant additional component of the currents was due to activation of a reversible anion flux that was not thermodynamically coupled to amino acid transport. The selectivity sequence of this ligand-activated conductance was $NO_3^- > I^- > Br^- > Cl^- > F^-$. The results suggest that these proteins mediate both transporter- and channel-like modes of permeation, providing a potential mechanism for dampening cell excitability, in addition to removal of transmitter.

Introduction

Reuptake of neurotransmitters is mediated by specific membrane proteins that couple the electrochemical gradients of additional cotransported ions to drive the concentrative influx of transmitter (for review, see Lester et al., 1994). The molecular mechanisms underlying this coupling process are unknown. Voltage-clamp studies have revealed substrate-independent ion fluxes mediated by some cloned neurotransmitter transporters, including those for 5-hydroxytryptamine (Mager et al., 1994), γ-aminobutyric acid (Cammack et al., 1994), and glutamate (Vandenberg et al., 1995), suggesting the presence of channel-like properties in these molecules. Uptake of the excitatory amino acid neurotransmitters glutamate and aspartate in brain synaptosomes is associated with influx of Na^+ and efflux of K^+ and OH^- (Kanner and Sharon, 1978; Erecinska et al., 1983). Voltage-clamp recording in retinal photoreceptors and glial cells has been used to isolate currents associated with excitatory amino acid uptake that contribute significantly to these cells' electrical properties (Brew and Attwell, 1987; Tachibana and Kaneko, 1988; Schwartz and Tachibana, 1990; Eliasof and Werblin, 1993). A stoichiometry proposed for glutamate uptake involves cotransport of $2Na^+$:$1Glu^-$ with countertransport of 1 K^+ and 1 OH^-, resulting in translocation of 1 net positive charge (Bouvier et al., 1992).

Isolation of cDNA clones from rat and rabbit has revealed a mammalian gene family of glutamate transporters (Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992). Three homologous excitatory amino acid transporter (EAAT) subtypes are widely expressed in human brain (EAAT1, EAAT2, and EAAT3; Arriza et al., 1994). A kinetic study of one of the human transporters (EAAT2; Wadiche et al., 1995) demonstrated that the number of charges translocated per transport cycle varies according to the membrane potential, in contrast to the result expected for a simple transport model involving a fixed stoichiometry. Data are presented here that explain the basis for this variable stoichiometry by demonstrating that members of this transporter family mediate thermodynamically uncoupled Cl^- currents activated by the molecules they transport.

Results

Steady-State Currents Activated by Excitatory Amino Acids

The voltage dependence of the currents mediated by EAAT1, EAAT2, and EAAT3 was examined by clamping oocytes expressing the transporters at potentials between +60 and −30 mV and superfusing them with the transport substrate D-aspartate (Arriza et al., 1994) at 100 μM. At negative membrane potentials, amino acid superfusion induced inward currents in oocytes expressing all three transporter subtypes (Figures 1A, 1C, and 1E). The amino acid–dependent current mediated by EAAT2 did not reverse at potentials up to +60 mV (Figures 1C and 1D). Surprisingly, however, amino acid superfusion induced currents that reversed at positive membrane potentials in oocytes expressing EAAT1 (E_{rev} = 9.3 ± 0.7 mV; n = 46; Figures 1A and 1B) or EAAT3 (E_{rev} = 38.0 ± 2.7 mV; n = 28; Figures 1E and 1F). The outward currents were most likely not due to reverse transport of accumulated substrate because they were observed in response to the first application of amino acid, when the membrane was clamped at depolarized potentials (Figure 1). In addition, varying external concentrations of amino acid affected the amplitudes of the currents without changing the reversal potential (Figure 2). In the absence of Na^+ (choline substitution), neither inward nor outward currents were induced by amino acid superfusion in any of the three transporter subtypes (n = 4). The application of 1 mM L-glutamate or D-aspartate to water-injected oocytes did not activate a detectable current (n = 6).

A Component of the Transporter Current Is Carried by Cl^-

The amino acid–dependent outward current observed at positive potentials in oocytes expressing EAAT1 or EAAT3 was abolished when external Cl^- ions (Cl^-_{out}) were replaced by gluconate ions, with little effect on the inward currents (Figures 3A–3C). This result suggested the possibility that the outward currents mediated by EAAT1 and EAAT3 were carried by Cl^-. This was tested by examination of the reversal potential of the amino acid–dependent currents mediated by EAAT1 and EAAT3 as a function of the extracellu-

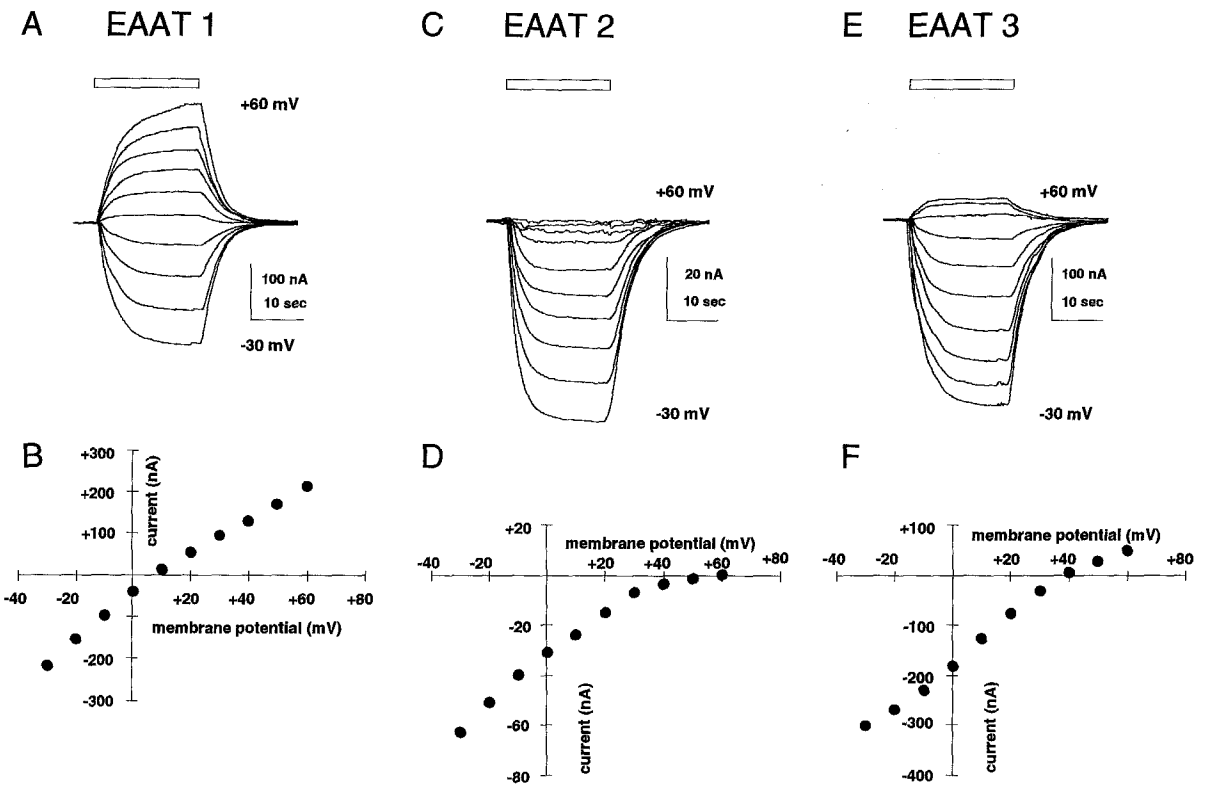

Figure 1. Currents Mediated by Human Excitatory Amino Acid Transporters

Currents induced by bath application (indicated by bar) of 100 μM D-aspartate to Xenopus oocytes expressing EAAT1 (A), EAAT2 (C), and EAAT3 (E). The corresponding steady-state current–voltage relations are shown in (B), (D), and (F). Currents are recorded at potentials from −30 to +60 mV and are offset to align holding currents. Note outward currents observed for EAAT1 and EAAT3.

lar Cl^- concentration ($[Cl^-]_{out}$). The reversal potential of the EAAT1 and EAAT3 currents shifted 54.1 ± 1.8 mV (n = 5) and 53.7 ± 4.3 mV (n = 5) per decade change in $[Cl^-]_{out}$, respectively (Figure 3D). Although the magnitudes of the reversal potential shifts were close to predictions for a Cl^--selective electrode, the absolute values of the reversal potentials were significantly more positive than the value of E_{Cl}, the Cl^- equilibrium potential (see below). This result suggested that other ions in addition to Cl^- carried a portion of the transporter-mediated current activated by excitatory amino acids.

To examine the properties of the transporter currents further, intracellular Cl^- (Cl^-_{in}) was depleted by dialysis of EAAT1-expressing oocytes for 16–24 hr at 17°C in Cl^--free medium (gluconate substitution); these oocytes were compared with matched oocytes incubated in ND96. E_{Cl} was measured before and after Cl^-_{in} depletion by measurement of reversals of endogenous Ca^{2+}-dependent Cl^- channels

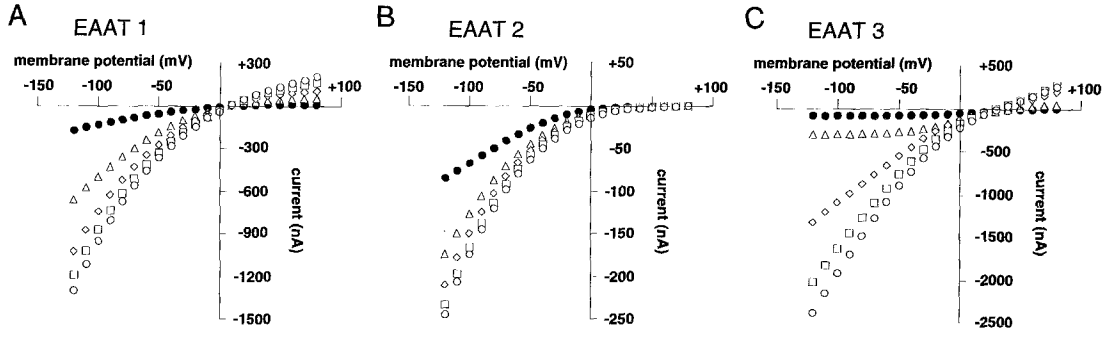

Figure 2. Concentration and Voltage Dependence of Excitatory Amino Acid Currents

Steady-state current–voltage relations of representative oocytes between +80 and −120 mV, obtained by subtraction of control currents from the corresponding currents in the presence of varying concentrations of D-aspartate. Xenopus oocytes expressing EAAT1 (A), EAAT2 (B), and EAAT3 (C). The reversal potential (EAAT1 and EAAT3) does not shift as a function of [D-aspartate]. D-aspartate doses were 10 μM (closed circles), 30 μM (triangles), 100 μM (diamonds), 300 μM (squares), and 1 mM (open circles).

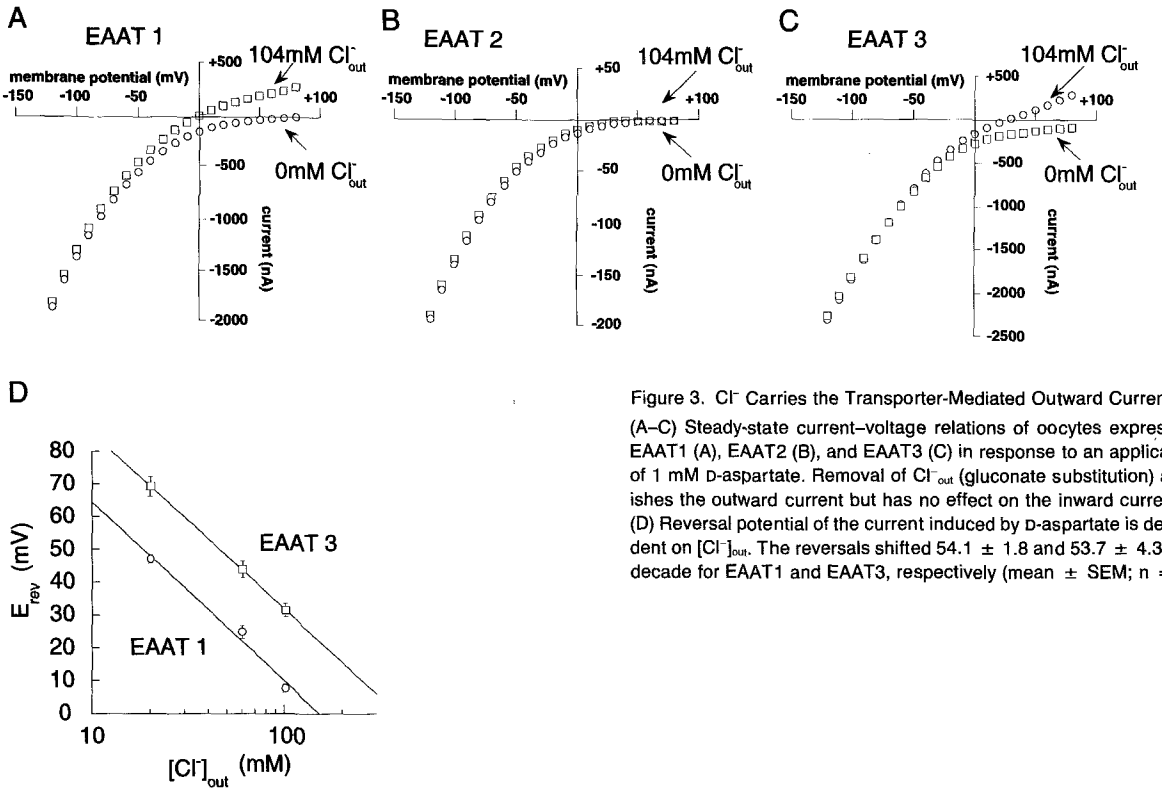

Figure 3. Cl^- Carries the Transporter-Mediated Outward Current

(A–C) Steady-state current–voltage relations of oocytes expressing EAAT1 (A), EAAT2 (B), and EAAT3 (C) in response to an application of 1 mM D-aspartate. Removal of Cl^-_{out} (gluconate substitution) abolishes the outward current but has no effect on the inward current.

(D) Reversal potential of the current induced by D-aspartate is dependent on $[Cl^-]_{out}$. The reversals shifted 54.1 ± 1.8 and 53.7 ± 4.3 mV/decade for EAAT1 and EAAT3, respectively (mean ± SEM; n = 5).

(see Experimental Procedures). With 104 mM $[Cl^-]_{out}$, E_{Cl} was shifted from −17 ± 1 mV (n = 4) to −81 ± 3 mV (n = 3), a value corresponding to a change in intracellular concentration from 53 to 4 mM. Comparison of currents activated by D-aspartate in the presence of Cl^-_{out} revealed that the inward current was significantly reduced following dialysis of Cl^-_{in}, with little effect on the outward current (Figure 4A). The reversal of the current was shifted from 12.5 ± 2.9 mV (n = 5) to 7.5 ± 0.9 mV (n = 5). When both Cl^-_{in} and Cl^-_{out} were substituted with gluconate, the remaining excitatory amino acid–induced current exhibited an exponential dependence on membrane potential (e-fold/75 mV) and did not reverse at potentials up to +80 mV (Figure 4B). The Cl^--dependent component of the excitatory amino acid–dependent current was resolved by subtraction of current–voltage recordings in the nominal absence of Cl^-_{in} and Cl^-_{out} from recordings in the presence of normal $[Cl^-]_{in}$ and $[Cl^-]_{out}$ (Figure 4C). The reversal of this difference current was ~10 mV more negative than the measured value of E_{Cl}, which is likely due to incomplete dialysis of Cl^-_{in}.

Because Xenopus laevis oocytes express high levels of Ca^{2+}-activated Cl^- channels (Robinson, 1979; Barish, 1983), the possibility was examined that the transporters might activate endogenous Cl^- channels, e.g., via Ca^{2+} permeating the transporter or by second messenger–mediated intracellular Ca^{2+} release. However, microinjection of oocytes expressing EAAT1 with 10 nmol of BAPTA, a quantity sufficient to block receptor-mediated intracellular Ca^{2+} release ($[BAPTA]_i \approx 10$ mM; Kavanaugh et al., 1991), had no effect on the excitatory amino acid–dependent current–voltage relations (n = 5). In addition, the oocyte Cl^- channel blocker niflumic acid (100 μM; White and Aylwin, 1990) and the nonselective blocker SITS (1 mM; Greger, 1990) had no effect on the current induced by 1 mM D-aspartate, nor did removal of extracellular Ca^{2+} (n = 4).

Thermodynamics of Transport

The above results suggest that the transporters mediate a reversible Cl^- current in addition to an inward current presumably associated with electrogenic cotransport (Figure 4). To test the idea that the excitatory amino acid–dependent current remaining in the absence of Cl^- reflects electrogenic amino acid flux, its voltage dependence was compared with that of the EAAT1-mediated [^{3}H]D-aspartate uptake. The [^{3}H]D-aspartate flux displayed a voltage dependence similar to that of the D-aspartate-activated current in the absence of Cl^- (e-fold/75 mV; Figure 4B; Figure 5A, inset). Time integration of currents associated with flux of radiolabeled amino acid in recording buffer containing Cl^- revealed that the ratio of the flux of charge to that of amino acid varied with membrane potential (Figure 5A). The quantity of charge translocated per transport cycle ranged from approximately +3.5 e_o at −100 mV to approximately −2.5 e_o at +25 mV (Figure 5B). Thus, amino acid flux is not directly proportional to the net ionic flux.

To investigate the influence of the Cl^- electrochemical gradient on uptake of amino acid, flux of [^{3}H]D-aspartate mediated by EAAT1 was measured under isopotential conditions with the Cl^- electrochemical gradient directed either outwardly or inwardly by varying $[Cl^-]_{out}$ (Figure 5C).

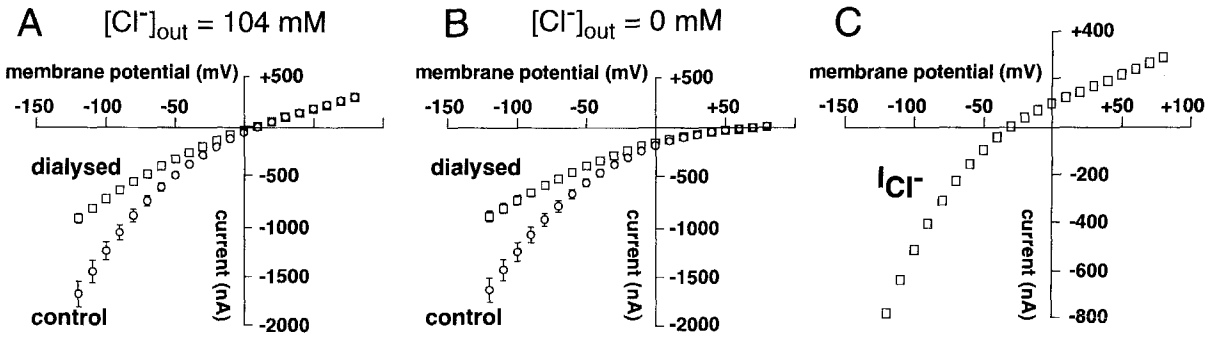

Figure 4. Depletion of Cl^-_{in} Reveals Two Currents

(A) Currents activated by 300 μM D-aspartate in oocytes expressing EAAT1 dialyzed to remove Cl^-_{in} (gluconate substitution; n = 5) compared with undialyzed cells (n = 6; $[Cl^-]_{out}$ = 104 mM).

(B) Currents activated by 300 μM D-aspartate in dialyzed cells compared with undialyzed cells ($[Cl^-]_{out}$ = 0 mM).

(C) Cl^--dependent component of the transport current revealed by subtraction of the mean current–voltage curves recorded in the presence (control; A) and absence (dialyzed; B) of Cl^-.

At 0 mV, with an inwardly directed Cl^- gradient ($[Cl^-]_{out}$ = 104 mM), uptake during a 100 s pulse of 100 μM [^{3}H]D-aspartate was 0.48 ± 0.06 pmol/s (n = 9). With an outwardly directed Cl^- gradient at the same membrane potential ($[Cl^-]_{out}$ = 0 mM), flux of [^{3}H]D-aspartate was unchanged (0.47 ± 0.06 pmol/s; n = 8). This result demonstrates that the driving force responsible for amino acid influx is not related to the Cl^- electrochemical gradient, suggesting that the Cl^- and amino acid fluxes occur independently.

Assuming that influx of excitatory amino acid is driven thermodynamically by electrogenic ion cotransport, the above results suggest that the net current reflects the sum of the inward current from amino acid flux (I_{AA}) and the current arising from the reversible and thermodynamically uncoupled amino acid–activated Cl^- conductance (I_{Cl}). To test this hypothesis further, the quantity of charge translocated per molecule of amino acid was compared in the presence and absence of Cl^- by measuring the time integrals of currents resulting from a 100 s pulse of 100 μM [^{3}H]D-aspartate. At −80 mV, the number of fundamental charges per molecule of D-aspartate was 2.4 ± 0.02 (n = 4), 2.0 ± 0.07 (n = 6), and 2.7 ± 0.3 (n = 5) for EAAT1, EAAT2, and EAAT3, respectively. After depletion of Cl^-_{in}, superfusion of the same concentration of label in Cl^--free buffer resulted in a reduction in the quantity of charge translocated per molecule of D-aspartate to 1.4 ± 0.1 (n = 6), 1.1 ± 0.04 (n = 5), and 1.2 ± 0.04 (n = 4), respectively. The flux of radiolabel in nominal Cl^--free conditions was not significantly changed relative to uptake in the presence of Cl^-. Thus, with Cl^-_{in} depleted and in the absence of Cl^-_{out}, there is a translocation of ~ 1 net positive charge associated with D-aspartate flux. In the presence of Cl^-, less than 1 charge was translocated per molecule

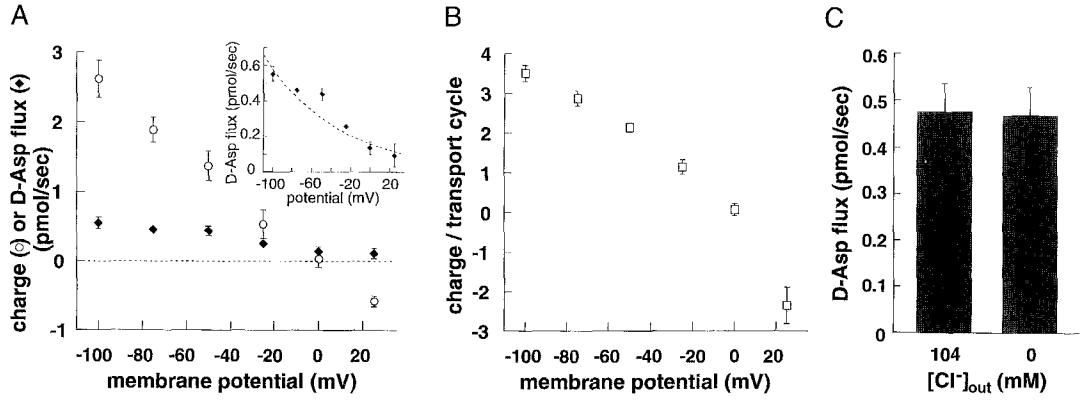

Figure 5. The Quantity of Charge Translocated per Transport Cycle Varies with Membrane Potential owing to a Thermodynamically Uncoupled Cl^- Flux

(A) Amino acid uptake and charge translocation were simultaneously measured during a 100 s application of 100 μM [^{3}H]D-aspartate to voltage-clamped oocytes expressing EAAT1. (Inset) Voltage dependence of labeled amino acid flux; superimposed exponential (e-fold/75 mV) derived from fit of transport current under nominal Cl^--free conditions (see Figure 4B).

(B) Quantity of charge translocated per transport cycle varies with the membrane potential.

(C) Under isopotential conditions (0 mV), amino acid uptake is not affected by changing the Cl^- electrochemical gradient.

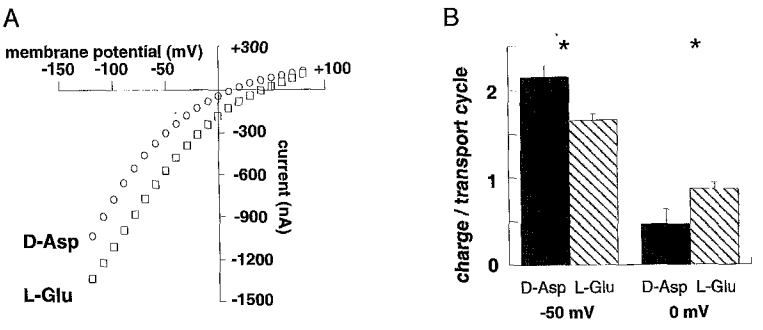

Figure 6. Reversal Potential and Relative Amplitude of Cl^- Flux Is Substrate Dependent

(A) Voltage dependence of EAAT1-mediated currents induced by application of 100 μM D-aspartate (circles) or 100 μM L-glutamate (squares). Reversal potentials differ by 37 ± 3.5 mV (n = 8).

(B) Charge translocation per cycle of transport measured for D-aspartate (closed bars) and L-glutamate (hatched bars) while clamping cells below (−50 mV) or above (0 mV) E_{Cl}.

of amino acid at potentials positive to E_{Cl}, whereas at potentials negative to E_{Cl}, more than 1 charge was translocated. At E_{Cl}, ~1 charge was translocated (Figure 5B). The results are consistent with the interpretation that translocation of 1 net positive charge is intrinsically coupled to uptake of a molecule of D-aspartate, but additional charge transfer arises from the uncoupled flux of Cl^- through the transporter.

Gating and Selectivity of the Cl^- Permeation Pathway

The reversal potential predicted for a theoretical current reflecting the sum of a current flowing through a perfect inward rectifier and a reversible Cl^- conductance is dependent on the relative magnitude of each component (see Discussion and Figures 8A–8C). The reversal potential of the EAAT1-mediated current activated by L-glutamate was more positive than that activated by D-aspartate, by 37.0 ± 3.5 mV (n = 8; Figure 6A). This result suggests that the flux of Cl^- per transport cycle gated by D-aspartate was greater than that gated by L-glutamate, since the D-aspartate-induced current reverses closer to E_{Cl}. To test this possibility, the number of charges translocated per molecule of [^{3}H]D-aspartate was compared with that of [^{3}H]L-glutamate at potentials positive and negative to E_{Cl}. At −50 mV, ~33 mV more negative than E_{Cl}, the ratio of charge flux to amino acid flux was greater for D-aspartate than for L-glutamate; the converse was true at 0 mV, ~17 mV more positive than E_{Cl} (Figure 6B). These results are consistent with translocation of either amino acid being coupled to movement of 1 charge, with the magnitude of the Cl^- flux induced by D-aspartate being greater.

The permeation of anions other than Cl^- through the transporter was examined by recording EAAT1 currents activated by 100 μM D-aspartate under bi-ionic conditions, with substituted test ions outside at 100 mM and physiological concentrations of Cl^-_{in} (~53 mM; see Experimental Procedures). The amplitudes and the reversal potentials of the currents varied among the ions tested (Figure 7). The general selectivity sequence, reflected in both the current reversal potentials and the outward current amplitudes, was $NO_3^- > I^- > Br^- > Cl^- > F^-$. A precise Goldman–Hodgkin–Katz analysis of the relative anion permeabilities based on the reversal potential is not possible because the inwardly rectified substrate flux leads to a different zero current equation at each potential.

Discussion

Electrogenic uptake of excitatory amino acids is mediated by a family of membrane proteins that cotransport Na^+ (Kanner and Sharon, 1978; Stallcup et al., 1979) and countertransport OH^- (Erecinska et al., 1983; Bouvier et al., 1992) and K^+ (Kanner and Sharon, 1978; Amato et al., 1994). If transport were tightly coupled to translocation of these inorganic ions, application of amino acid to one membrane face would be expected to result in a unidirectional current with a polarity determined by the stoichiometry of the cotransported ions. For a cotransport stoichiometry of $1AA^-:2Na^+$ with countertransport of 1 OH^- and 1 K^+ (Bouvier et al., 1992), a net charge of +1 would accompany influx of each molecule of glutamate or aspartate regardless of membrane potential. Results from a recent study of EAAT2 kinetics demonstrate, however, that the net charge accompanying translocation of glutamate varies according to membrane potential (Wadiche et al., 1995), and the present study demonstrates that under appropriate conditions the current associated with excitatory amino acid influx can indeed reverse polarity.

These results can be explained by a model involving activation of a Cl^- conductance in parallel with the conductance associated with amino acid flux. In this model, the net excitatory amino acid–dependent current represents the sum of the Cl^- current (I_{Cl}) and the electrogenic transport current (I_{AA}). Although the Cl^- current is activated by transport substrates, the Cl^- electrochemical gradient is not thermodynamically coupled to transport, since amino acid flux is unaffected by the direction of the Cl^- driving force (see Figure 5C). Furthermore, excitatory amino acid influx occurs in the absence of Cl^-. From measurements of the quantity of charge translocated with each molecule of amino acid in the presence of Cl^-, it was ascertained that the net positive charge translocated into the cell per transport cycle was $>1\ e_o$ at membrane potentials negative to E_{Cl}, while at potentials positive to E_{Cl}, it was $<1\ e_o$. At the equilibrium potential for Cl^-, ~1 positive charge accompanied each molecule of amino acid entering through the transporter (see Figure 5B). In addition, the voltage dependence of radiolabeled amino acid influx was similar to that of the amino acid–dependent current in Cl^--free conditions (e-fold/75 mV; see Figure 4B; Figure 5A). Together, these results suggest that translocation of excitatory amino acid is coupled to translocation of 1 net positive charge.

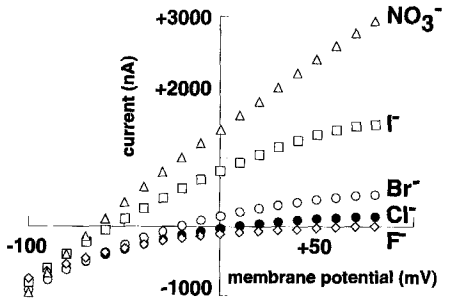

Figure 7. Anion Selectivity of EAAT1

Currents induced by 100 μM D-aspartate in a representative oocyte expressing EAAT1 with bath solutions containing various test ions as 100 mM Na^+ salt plus gluconate salts of Ca^{2+} (1.8 mM), Mg^{2+} (1 mM), and K^+ (2 mM).

The above model predicts that the reversal potential of the total current is independent of amino acid concentration only if the concentration dependence for activation of I_{AA} and I_{Cl} is the same (Figures 8B and 8C). Thus, the observed concentration independence of the transporter current reversal potentials (see Figure 2) is consistent with both I_{Cl} and I_{AA} arising from excitatory amino acid binding to a single site. Each transporter subtype exhibited intrinsic (expression level–independent) differences in the reversal potential of the net current activated by excitatory amino acids, which would be unlikely if the Cl^- current were mediated by a distinct molecular species. Furthermore, classical Cl^- channel blockers did not affect the transporter-mediated currents, supporting the hypothesis that the transporters directly mediate both currents. The reversal potential of the net current is predicted to shift with changes in $[Cl^-]_{out}/[Cl^-]_{in}$, but the magnitude of this shift, as well as the absolute value of the reversal potential, will be influenced by the relative magnitude of I_{AA} and I_{Cl} (Figure 8C). In general, the greater the contribution of I_{AA} to the net current at the reversal potential, the less effect changing the Cl^- gradient will have on the reversal potential. Similarly, the greater the relative magnitude of I_{Cl}, the closer will be the net current reversal potential to E_{Cl}. Thus, the difference in reversal potentials for the L-glutamate- and D-aspartate-activated currents in EAAT1 may be accounted for by differences in ligand efficacy for activation of the Cl^- current, leading to a difference in flux of Cl^- per transport cycle (see Figure 6). In addition to substrate-dependent changes in reversal potentials, the transporters displayed subtype-specific differences in the reversal potentials (ranging from +9 mV in EAAT1 to +38 mV in EAAT3 to >80 mV in EAAT2 for D-aspartate currents). In addition to possible intrinsic differences in activation of the Cl^- current, other reasons for differences in reversal potentials between subtypes could include differences in the voltage dependence of substrate flux or in intrinsic rectification of I_{Cl}. Interestingly, a recently cloned human cerebellar transport subtype, EAAT4, mediates an excitatory amino acid–induced current that is carried predominantly by Cl^- and reverses close to E_{Cl} (Fairman et al., 1995). In all three subtypes examined in the present study, a significant percentage (50%–73%) of the total current activated by D-aspartate was carried by Cl^- at −80 mV, based on measurements of the net quantity of charge translocated per transport cycle, assuming a charge of +1 e_o per cycle due to coupled cotransport.

The selectivity of the ligand-gated anion conductance was revealed by substitution experiments that demonstrated the sequence $NO_3^- > I^- > Br^- > Cl^- > F^- >>$ gluconate$^-$. An absence of anomolous mole fraction behavior observed between I^- and Cl^- (data not shown) is consistent with a single anion binding site in the transporter pore, although more rigorous tests will be required to rule out a multi-ion permeation pathway. This selectivity sequence is identical to that of a neuronal Cl^- channel, which displays complex conductance properties that appear to result from anion–cation interactions in the channel pore (Franciolini and Nonner, 1987, 1994). As the excitatory amino acid–activated Cl^- current through the transporter required Na^+, it is possible that Na^+ and Cl^- interact in the pore of the transporter, although binding of Na^+ may simply be required prior to excitatory amino acid binding (Wadiche et al., 1995). Unlike currents mediated by the neuronal Cl^- channel, in which the reversal potential is affected by cation concentrations (Franciolini and Nonner, 1987), reducing $[Na^+]_{out}$ from 101 to 20 mM reduced the excitatory amino acid–activated current amplitude 53% ± 8% (-80 mV; n = 4), without changing the reversal potential.

Na^+- and glutamate-dependent currents with properties similar to those described here have been reported in vertebrate photoreceptor cells (Sarantis et al., 1988; Tachibana and Kaneko, 1988; Eliasof and Werblin, 1993). In addition, fluctuation analyses of currents in photoreceptors of turtle (Tachibana and Kaneko, 1988) and salamander (Larsson et al., 1994, Biophys. J., abstract) suggest the presence of a small conductance channel activated by glutamate transporter substrates. The activation of a Cl^- conductance concomitant with transport would provide a potential mechanism to offset the depolarizing action of transmitter reuptake and dampen cell excitability. The molecular mechanisms underlying this current remain to be elucidated. The thermodynamic independence of the Cl^- and glutamate fluxes suggests that these ions do not simultaneously permeate a single-file pore. Therefore, unless the transporter has a "double-barreled" ion pathway to allow independent permeation, a gating mechanism must exist for switching between Na^+-coupled amino acid translocation and Cl^- permeation. One potential mechanism for such a switch involves the bound glutamate molecule itself constituting a critical part of the selectivity site required for Cl^- permeation (e.g., by contributing a positively charged α-amino group with which the anion could interact in transit). In such a model (see Figures 8D and 8E), the mean time that glutamate is bound to the transporter before unbinding or being translocated through the pore would determine the mean lifetime of the anion-conducting state. Variations in the microscopic kinetics of this gating process could lead to differences in the relative amplitudes of amino acid and anion fluxes for different transporter

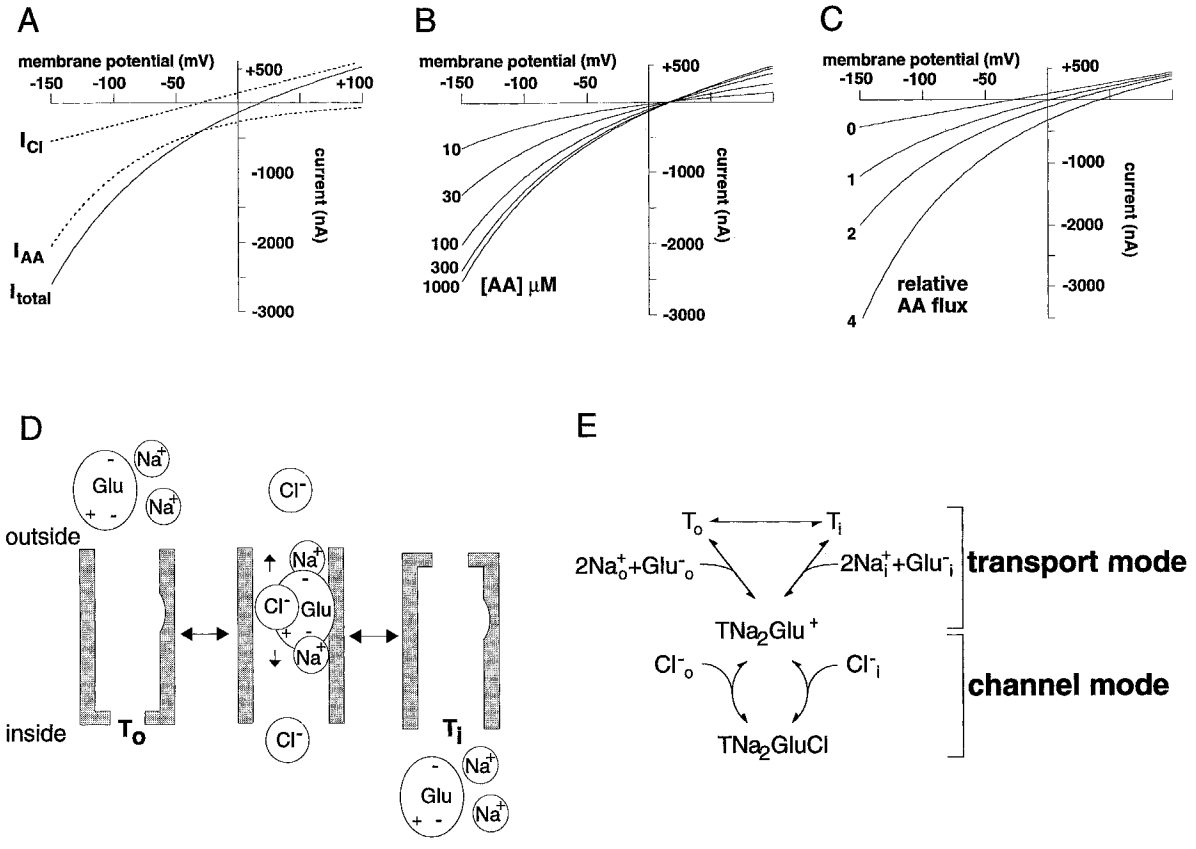

Figure 8. Excitatory Amino Acid Transporter Modes

(A) Model of total transporter current (solid line) as the sum of a reversible Cl^- current (I_{Cl}) and an electrogenic current from coupled flux (I_{AA}; see Experimental Procedures).

(B) The predicted reversal potential of the net current is independent of amino acid concentration when the concentration dependence of I_{AA} and I_{Cl} is the same.

(C) The absolute reversal potential is dependent on the amino acid flux relative to that of Cl^-. The curves shown represent the effect of scaling the intrinsic transporter turnover rate by the indicated factor while holding the Cl^- conductance constant.

(D and E) Cartoon (D) and corresponding kinetic scheme (E) representing modes of transporter operation. The alternating access model requires a state transition for glutamate permeation ($T_o \leftrightharpoons T_i$), while Cl^- permeation requires only that glutamate be bound. For simplicity, a partial reaction cycle is shown that omits the countertransport step for K^+ and OH^- (or HCO_3^-) as proposed by Bouvier et al. (1992).

subtypes and substrates such as those observed in the present study.

Experimental Procedures

Expression and Electrophysiological Recording

Capped mRNAs transcribed from the cDNAs encoding the human brain glutamate transporters EAAT1–EAAT3 (Arriza et al., 1994) were microinjected into stage V-VI Xenopus oocytes (50 ng/oocyte), and membrane currents were recorded 3–6 days later. Recording solution (ND96) contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES (pH 7.4). In Na^+ or Cl^- substitution experiments, ions were replaced with equimolar choline or gluconate, respectively. Two microelectrode voltage-clamp recordings were performed at 22°C with a Geneclamp 500 interfaced to an IBM compatible PC using a Digidata 1200 A/D controlled using the pCLAMP 6.0 program suite (Axon Instruments), and to a Macintosh using a MacLab A/D (ADInstruments). The currents were low pass–filtered between 10 Hz and 1 kHz and digitized between 20 Hz and 5 kHz. Microelectrodes were filled with 3 M KCl solution and had resistances of <1MΩ. Offset voltages in Cl^- substitution experiments were avoided by the use of a 3 M KCl–agar bridge from the recording chamber to a 3 M KCl reservoir containing an Ag/AgCl electrode. Current–voltage relations were determined either by measurement of steady-state currents in response to bath application of substrates or by off-line subtraction of control current records obtained during 200 ms voltage pulses to potentials between −120 and +80 mV from corresponding current records in the presence of substrate. The Cl^- reversal potential was determined in oocytes expressing EAAT1 and in uninjected cells by measuring the reversal potential of currents mediated by Ca^{2+}-activated Cl^- channels endogenous to Xenopus oocytes following activation with 1 μM A23187 (Barish, 1983).

^{3}H-Labeled Amino Acid Flux

Current measurements were made during superfusion of 100 μM [^{3}H]D-aspartate (0.42 Ci/mmol; Amersham) or [^{3}H]L-glutamate (1 Ci/mmol; Amersham) onto oocytes voltage-clamped at various potentials for 100 s. Following washout of the bath (<20 s), oocytes were rapidly transferred into a scintillation tube, lysed, and measured for radioactivity. In control experiments, no significant efflux of radiolabel was detected during this time in oocytes injected with 100 pmol of [^{3}H]D-aspartate (final concentration ≈ 100 μM). Currents induced by ^{3}H-labeled amino acids were recorded using Chart software (ADInstruments) and integrated off-line, followed by correlation of charge transfer with radiolabel flux in the same oocytes. All data are expressed as mean ± SEM.

Modeling

The following expression was used to model a current resulting from

the sum of a reversible Cl^- current and an inwardly rectifying transport current as a function of voltage:

$$I_{tot}(E) = \{[EAA]/([EAA] + K_{0.5})\}\{(E - E_{Cl})(g_{Cl}) - (tN)(F)\exp(-E\mu)\},$$

where $I_{tot}(E)$ is the total membrane current as a function of voltage; [EAA] is the concentration of excitatory amino acid; $K_{0.5}$ is the concentration of amino acid that activates 50% of each current; (g_{Cl}) is the Cl^- conductance due to the channel mode of the transporter, assumed for simplicity to be ohmic; (E_{Cl}) is the equilibrium reversal potential for Cl^-; F is the Faraday constant; t is the turnover rate of the transporter at 0 mV for a given amino acid; N is the number of moles of transporter expressed; and μ is a Boltzmann factor determining the voltage dependence of the transporter turnover rate.

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