Dynamic Equilibrium between Coupled and Uncoupled Modes of a Neuronal Glutamate Transporter

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Running Title:

Selective Modification of Glutamate Flux

Abbreviations used:

MTSET      [2-(trimethylammonium)ethyl]methane thiosulfonate
MTSEA      (2-aminoethyl)methane thiosulfonate
Glt-1       glutamate transporter-1
EAAC-1      excitatory amino-acid carrier-1
GLAST-1     glutamate/aspartate transporter-1
EAAT        excitatory amino-acid transporter
Glu         glutamate
DTT         1,4-dithiothreitol
Summary

In the brain, the neurotransmitter glutamate is removed from the synaptic cleft by (Na$^+$ + K$^+$)-coupled transporters by an electrogenic process. Moreover, these transporters mediate a sodium- and glutamate-dependent uncoupled chloride conductance. In contrast to the wild type, uptake of radiolabelled substrate by the I421C mutant is inhibited by the membrane impermeant [2-(trimethylammonium)ethyl]methane thiosulfonate and also by other sulfhydryl reagents. In the wild type and the unmodified mutant, substrate-induced currents are inwardly rectifying and reflect the sum of the coupled electrogenic flux and the anion conductance. Remarkably, the I421C mutant modified by sulfhydryl reagents exhibits currents that are non-rectifying and reverse at the equilibrium potential for chloride. Strikingly, almost 10-fold higher concentrations of D-aspartate are required to activate the currents in the modified mutant as compared to untreated I421C. Under conditions where only the coupled currents are observed, the modified mutant does not exhibit any currents. However, when the uncoupled current is dominant, sulfhydryl reagents cause more than a 4-fold stimulation of this current. Thus, the modification of the cysteine introduced at position 421 impacts the coupled but not the uncoupled fluxes. Although both are activated by substrate, they behave as independent processes that are in dynamic equilibrium.
Introduction

The major excitatory neurotransmitter in the brain is glutamate. Transporters remove the transmitter from the synaptic cleft, so that its extracellular concentrations are maintained below neurotoxic levels (1–5). Moreover, glutamate transporters play an important role in limiting the duration of synaptic excitation (6–9). Glutamate transport is an electrogenic process (10–12) involving cotransport with sodium and hydrogen ions, followed by countertransport of potassium (13–15). The stoichiometry of the process has been determined to be 3Na⁺:1H⁺:1K⁺:glutamate (3,16).

The five known eukaryotic glutamate transporters GLT-1 (17), GLAST-1 (18), EAAC-1 (19), EAAT-4 (20) and EAAT-5 (21) have an overall identity of around 50%. The homology is significantly higher in the carboxyl-terminal half. Glutamate transporters have a non-conventional topology containing two reentrant loops, two transmembrane domains as well as an outward facing hydrophobic linker region (22–24), although there remains some controversy on some of the features of this topology (25). Two adjacent amino acid residues of GLT-1, located in transmembrane domain 7 and conserved in all other glutamate transporters, tyrosine-403 and glutamate-404 are required for potassium binding (15,26). Two other residues, located in reentrant loop II and transmembrane domain 7 respectively, have been found to be determinants of sodium specificity (27,28). Arginine-447 of the EAAC-1 transporter, located in transmembrane domain 8 and also fully conserved in the other glutamate transporters, appears to be a determinant for the binding of the gamma-carboxyl group of glutamate and is also important for potassium interaction (29).

In addition to the ion-coupled glutamate translocation, glutamate transporters mediate a thermodynamically uncoupled chloride flux. It is activated by two of the molecules they transport: sodium and glutamate (20,30). This indicates the existence of a tight link between gating of the anion conductance and permeation of glutamate. It has been suggested that this capacity for enhancing chloride permeability may serve to off-set the depolarization which
occurs during glutamate transport (20). In a very recent study we have reported that in EAAC-1, lithium can replace sodium in the coupled uptake, but not in its capacity to gate the glutamate-dependent anion conductance (28). This suggests that the conformation gating the anion conductance may be different from that during substrate translocation. If this idea is correct, it should be possible to selectively perturb one of the two fluxes mediated by the glutamate transporters.

We have engineered externally accessible cysteines into EAAC-1 with the aim of introducing fluorescent maleimides to monitor conformational changes during transport. Analysis of one of the mutants with a cysteine residue introduced in the hydrophobic linker region revealed that the coupled uptake in this mutant is very sensitive to sulfhydryl reagents. Strikingly, the substrate-induced anion-conductance is not affected at all under these conditions. We have exploited the sulfhydryl- modified mutant to reveal novel properties of the substrate gated anion-conducting state and its relation to the coupled transport cycle.
Experimental Procedures

*Generation and subcloning of mutants* - The rabbit glutamate transporter EAAC-1 (19), with nine histidines added (29), in the vector pBluescriptSK(-) (Stratagene), was used as a parent for site-directed mutagenesis as described earlier (31,32). Restriction enzymes *Pin*AI and *Pfl*MI were used to subclone the mutations into the construct containing the his-tagged wild type EAAC-1 residing in either pBluescriptSK(-) or in pOG2 (29). The latter is an oocyte expression vector containing a 5’-untranslated *Xenopus* β-globin sequence and a 3’-poly(A) signal. The coding and non-coding strands were sequenced between the above two restriction sites.

*Expression and uptake in HeLa cells* - HeLa cells were cultured in supplemented Dulbecco's modified Eagle's Medium (Biological Industries), infected with a recombinant vaccinia/T7 virus vTF7-3 (33), transfected with 0.5µg DNA/well using lipofectamine (2µg/well) (Roche Molecular Biochemicals) and D-[2,3-3H]aspartic acid uptake was measured as previously described (28). For experiments using the sulphydryl reagent MTSET (Toronto Research Chemicals), culture medium was removed and the cells were washed twice with 1ml choline uptake solution (sodium replaced with equimolar choline), then incubated with 200µl uptake solution containing 500µM MTSET for 2 min and then washed again, twice in 1ml choline uptake solution (37 C) before the 10 min uptake assay was initiated by adding 200µl radioactive sodium uptake solution at 37 C. Cells that did not receive MTSET were subjected to the same incubation and washing procedures. From dose-response experiments the concentration of MTSET giving half maximal inhibition was determined to be 5 µM (data not shown) - 100-5000 times this concentration has been used on HeLa cells and oocytes in initial experiments to rule out the possibility that MTSET reacts with wild-type EAAC1.
Capped run-off cRNA transcripts were made from transporter constructs in pOG2, linearised with SacI, using mMessage mMACHINE (Ambion) and Xenopus laevis oocytes were prepared using Collagenase Type1A (Sigma) or Blendzyme 3 (Roche Molecular Biochemicals) and injected as previously described (28). Oocytes were maintained either in modified Barth’s saline (mM); NaCl 88, KCl 1, MgSO4 1, NaHCO3 2.4, CaCl2 1, Ca(NO3)2 0.3, HEPES 10, pH 7.5, with freshly added sodium pyruvic acid 2, theophylline 0.5 and supplemented with 10000 U/l penicillin, 10mg/l streptomycin, 50mg/l gentamycin or in ND96 recording solution (see below) supplemented with 1.5% heat inactivated horse serum, 0.5 mM theophylline and 2.5 mM pyruvic acid.

Oocyte electrophysiology - Currents were measured from Xenopus laevis oocytes using conventional two-electrode voltage clamp recording as previously described (28). Penetrated oocytes were gravity-perfused with ND96 recording solution (in mM); NaCl 96, KCl 2, CaCl2 1.8, MgCl2 1, HEPES hemisodium 5, pH 7.5. Sodium chloride and HEPES hemisodium was replaced with equimolar choline chloride and free acid HEPES or replaced with equimolar lithium chloride and free acid HEPES in sodium replacement experiments, pH was adjusted to pH 7.5 with Tris-OH in these solutions. In experiments using the highly permeant thiocyanate anion (Fig. 6) the 2mM KCl was replaced by 20mM KSCN and the sodium chloride concentration was varied by replacing NaCl with equimolar choline chloride. In these experiments the extracellular grounding electrode was placed in 2M KCl and connected to the recording chamber with an agar/2M KCl bridge. Before applying either of the sulfhydryl reagents MTSET (Toronto Research Chemicals) or beta-maleimido proponic acid (Sigma), the flow was stopped and the reagent added directly into the bath under voltage clamp. Before applying the reducing agent 1,4-Dithiothreitol (DTT) directly into the bath, the oocyte was unclamped, grounding electrode removed and the flow stopped. MTSET and/or
DTT was allowed to react for 2 min followed by ~10 min washout (in initial experiments using higher MTSET and DTT concentrations the washout time was 20 min). The net current was measured before MTSET application, after MTSET application and after DTT application, in that order. Both the MTSET and the beta-maleimido proponic acid stock solutions were made in the recording solution to which it was added (in the case of maleimide pH was adjusted to 7.5 with NaOH) and kept on ice for up to 2 hours and the DTT was made in 1mM sodium acetate (pH 5.2). The final concentrations in the bath for MTSET and DTT were 2.5-25mM and 50mM, respectively in initial experiments and later 1mM and 10mM, respectively as estimated from the measured chamber volume in the presence of an oocyte. Unless stated otherwise, oocytes were perfused in recording solution with or without 1mM D- or L-aspartate.

Data analysis - Data have been normalised to account for variability in expression level within and between different oocyte batches. Steady-state net currents defined as (I_{ND=D-Asp} - I_{ND}) were analysed using Clampfit6.05 and Origin6.1 (Microcal). The current-voltage relations shown were obtained by averaging the net currents at each holding potential from 218 to 248ms. For representational purposes, the traces of the net currents shown in Figures 2 and 4 have been low-pass filtered off-line using the boxcar algorithm (Clampfit). Concentrations of D-aspartate or sodium giving half-maximal currents (EC50) were determined with the generalised Hill equation (28) using a fixed maximum current (Imax) and the determined EC50 and Hill number (nH) were used to generate the curves shown. Residuals were minimised with the Levenberg-Marquardt method. The build-in functions of Origin6.1 were used for statistical testing. Data are mean +/- S.E. of 3-10 oocytes from 2-4 different batches.
Results

*Inhibition of D-[3H]-aspartate uptake in I421C by MTSET*- HeLa cells expressing the I421C-EAAC-1 mutant exhibit similar rates of D-[3H]-aspartate uptake to those in which the wild type is expressed (Fig. 1). In contrast to the wild type however, this uptake is almost fully inhibited by preincubation of the cells with 500 µM of the impermeant sulfhydryl reagent MTSET (Fig. 1). The introduction of a serine or an alanine residue at this position does not render EAAC-1 sensitive to MTSET (Fig. 1). These observations indicate that the inhibition of uptake in I421C-EAAC-1 is due to the introduced cysteine and renders unlikely the possibility that the introduction of a cysteine at position 421 causes the transporter to expose an endogenous cysteine that could mediate the inhibition by the sulfhydryl reagent. When I421C-EAAC-1 is treated with concentration of MTSET low enough (5 µM) to retain significant residual activity, the major impact is on maximal uptake (\( V_{\text{max}} \)): 66 +/- 6 % inhibition (n = 3). The apparent affinity for substrate (\( K_m \)) is essentially unchanged 27 +/- 5 and 25 +/- 4 µM (n =3) for untreated and MTSET treated I421C, respectively. This indicates that the inactivation of uptake by MTSET is due to a progressive reduction of functional transporters.

*Modification of the substrate-gated currents by sulfhydryl reagents*- it is well established that in sodium containing media, addition of acidic amino acid substrates to oocytes expressing cloned glutamate transporters, results in the activation of a current representing the sum of two currents. The first is a non-reversing inwardly rectifying current (reflecting the coupled cycle), whereas the second is a reversing non-rectifying anion conductance that is thermodynamically uncoupled (20,21,30). The reversal potential of the combined current depends on the acidic amino acid substrate; L-aspartate gives rise to reversal at less positive potentials than L-glutamate and D-aspartate (28,30). Analysis of the
D-aspartate induced currents of oocytes expressing I421C-EAAC-1 in the presence and absence of MTSET reveals a remarkable result: Instead of currents which are inward at the range of membrane potentials tested (−100 mV to +40 mV), preincubation with 1 mM MTSET results in currents that are almost symmetrical around the holding potential of -25mV (Fig. 2). Treatment of the MTSET modified mutant transporters with dithiothreitol, which is expected to restore the cysteine at the 421 position, also restores the voltage dependence of the D-aspartate induced currents (Fig. 2). Similar results have been obtained when L-aspartate induced currents are monitored (data not shown). Other sulfhydryl reagents such as MTSEA and beta-maleimido propionic acid, modify the currents of I421C in a similar way as MTSET. As expected DTT is able to restore the current modified by MTSEA but not by beta-maleimido propionic acid (data not shown). Plotting of the average current/voltage relation (I/V-plot) of six oocytes shows that MTSET treatment of I421C, changes the behavior of the substrate-induced currents from inwardly rectifying to ohmic and reversing at -25mV (Fig. 3B). DTT restores the inwardly rectifying currents (Fig. 3B). In contrast, the I/V relations of WT-EAAC-1 are not affected by MTSET (Fig. 3A). It is of interest to note that the reversal potential of the D-aspartate induced currents of the MTSET treated I421C transporters is around −25 mV (Fig. 3B), very close to the reversal potential of chloride (30). Moreover, the I/V relation in this mutant is reminiscent of those in GLT-1 and EAAC-1 mutants that are locked in the exchange mode (15,26,29) and therefore only exhibit the uncoupled chloride conductance.

Further evidence for the idea that the currents observed with MTSET modified I421C transporters, solely represent the anion conductance, is provided in Fig. 4. We have very recently found that in the presence of lithium, EAAC-1 transporters mediate the coupled current only (28). If the currents from MTSET modified I421C solely are due to the substrate-induced anion conductance, one would predict that in lithium media, no current will be observed at all. Since net D-aspartate currents in lithium medium are rather small, we have
displayed those obtained with L-aspartate. The latter gives rise to larger currents in lithium media (28). As can be seen in Fig. 4, MTSET treated I421C transporters do not exhibit any significant L-aspartate induced currents in lithium media. Again, the effect of MTSET can be reversed by DTT.

Characterisation of the substrate-gated anion conductance - the selective elimination of the coupled current in I421C by MTSET allows us to study the substrate activated gating of the anion conductance in isolation from substrate translocation. One prediction is that the reversal potential of the current should be independent of the concentration of D-aspartate, but this would not be expected for the size of the current. Indeed, at increasing D-aspartate concentrations, the reversal potential remains the same (Fig. 5B). On the other hand, the size of the current — representing the opening of more chloride channels, increases with the increasing D-aspartate concentration (Fig. 5B). The dependence of the current of untreated I421C-EAAC1 transporters on the D-aspartate concentration is shown in Fig. 5A. It is inwardly rectifying and similar to that of wild-type EAAC-1 with or without MTSET treatment (data not shown). Also the affinity of untreated I421C-EAAC-1, around 25µM, is similar to that of the wild type with or without the sulfhydryl reagent (data not shown, 19). Analysis of the D-aspartate concentration dependence of the currents reveals that in MTSET treated I421C, the apparent affinity for this substrate is lowered by almost 10-times as compared to the untreated mutant transporters (Fig. 5C). The Hill number for D-aspartate is not changed (t-test at the 0.05 significance level) 1.1 +/-0.1 and 1.2 +/- 0.2 at -100mV and +40mV respectively before treatment and 0.9 +/- 0.1 and 1.0 +/- 0.2 after treatment with MTSET. In both cases this apparent affinity is voltage independent — at least at negative potentials where a direct comparison is feasible (Fig. 5C).

We have taken advantage of the I421C mutant to address the question if - as is the case for coupled flux - multiple sodium ions are required to enable the substrate gated anion conductance. In order to amplify the anion currents, 20 mM thiocyanate has been added to
the perfusion medium since its permeability around 70-fold larger than that of chloride (30,34). Fig. 6A illustrates the I/V plots of the D-aspartate (3mM) net currents in MTSET treated I421C-EAAC-1 transporters at varying sodium concentrations. Replotting these data versus the sodium concentration shows a cooperative behavior at all voltages tested (Fig. 6B). The sodium concentration which gives rise to half-maximal currents are similar (t-test at 0.05 significance level) at -100mV and at +40mV, namely 23.0 +/- 0.4 and 23.3 +/- 0.3 mM (n = 6), respectively. The same is true for the Hill numbers, 3.0 +/- 0.2 and 2.9 +/- 0.1 at -100mV and +40mV, respectively (t-tested at the 0.05 significance level). The data shown in Figs 6A and B are normalised currents. Examination of the absolute net D-aspartate currents reveals a striking result - treatment with MTSET causes a marked stimulation of both inward and outward currents (Fig. 6C), here shown at 96mM sodium. At +40mV and -100mV the increase is 4.43 +/- 0.31 and 2.56 +/- 0.17 fold respectively (n=6). This result indicates that when the anion conductance is the major component of the current, blocking the coupled cycle enables the transporter to reside longer in the conformation gating the anion conductance.
Discussion

Our studies show that in I421C-EAAC-1 transporters, which otherwise behave identical to wild type EAAC-1, sulfhydryl reagents abolish uptake of D-[\(^{3}\text{H}\)]-aspartate (Fig. 1) and modify the substrate induced currents (Figs. 2 and 3). Characterisation of those currents indicates that it is the coupled flux that is affected. While this flux is completely absent, the substrate-gated anion conductance is not affected by the sulfhydryl reagents (Figs. 2–4). We have used the mutant to study the anion conductance in isolation of the coupled current - to obtain novel insights into the similarities and differences between these processes as well as their interrelation.

Possible pathways connecting coupled and uncoupled fluxes are shown in Fig. 7. The sodium-coupled glutamate translocation, with the participating potassium ion and proton omitted for simplicity, is represented by Steps 1–4. After binding of sodium and glutamate on the extracellular side (Step 1), the translocation complex is formed. After translocation (Step 2), the substrates are released on the intracellular side (Step 3). The unloaded transporter binds potassium (not shown) and after translocation to the outside (Step 4), potassium is released and the transporter is ready to start a new cycle. Because glutamate (Glu in the figure for brevity)— as well as the other acidic amino acid substrates — gate the uncoupled anion conductance in a sodium dependent manner, most models (34–37) propose a transition step of the outward facing substrate loaded transporter to an anion-conducting state — out\(^{T*}\)Na\(_{2}\)Glu (Fig. 7, Step A). According to this scenario, the modification of the cysteine introduced at position 421 may be explained by a perturbation of either Steps 2 or 3. HeLa cells expressing mutants locked in the exchange mode - in which Step 4 is impaired selectively - still exhibit normal D-[\(^{3}\text{H}\)]-aspartate uptake (15,26). The complete inhibition of this uptake in I421C-EAAC-1 by MTSET (Fig. 1) renders selective inhibition of Step 4
unlikely. The fact that the apparent affinity for D-aspartate to activate the anion conductance in I421C is almost 10-fold lower than that to activate the total current (Fig. 5C), can be easier accommodated by the alternative possibility depicted in Fig. 7, Step B. According to this proposal the anion conducting state \( \text{out}^{*}\text{Na,Glu} \) can be reached without entering the coupled cycle. An inherent assumption is that the mutation has not somehow altered the apparent affinity of the uncoupled process for D-aspartate. Since the voltage dependence and the D-aspartate concentration dependence of the currents in the untreated mutant is identical to that of the wild type, it is likely that this assumption indeed is correct. Three sodium ions are translocated with glutamate in the coupled cycle (3,16). Our study of the uncoupled flux indicates that this process also may require multiple sodium ions (Figs 6A and B). One possibility is therefore that two or three sodium ions bind to the transporter and subsequently the sodium-bound transporter binds glutamate and enters either the coupled cycle or the anion-conducting state.

The role of the anion conductance has been postulated to counteract the depolarisation caused by excessive glutamate flux (20). At high extracellular glutamate levels, the magnitude of this depolarisation — caused by the coupled process — could be relatively large. Under these conditions, glutamate is likely to saturate the activation site of the anion conductance. Thus at the same time influx of chloride will be activated, and this is expected to limit the depolarization. It is of interest that sulfhydryl modification of I421C also abolishes substrate-sensitive transient currents (Fig. 2). These transients likely represent voltage dependent conformational changes associated with the coupled transport cycle, but not those occurring during gating of the anion conductance.

The idea that the activation of the anion conductance is separate from that of the coupled transport (Fig. 7, Step B), is in harmony with several recent independent lines of evidence. Kinetic experiments indicate that the onset of the uncoupled current is delayed compared with that of the coupled current (35–37) and the two exhibit different pH profiles
Furthermore, it has been found that Zn\textsuperscript{2+} selectively perturbs the two processes; in the EAAT-1 transporter (the human homologue of GLAST-1), Zn\textsuperscript{2+} was found to inhibit the coupled but not the uncoupled current (39) and the reverse is true for EAAT-4 (40).

The scheme shown in Fig. 7 indicates that - regardless of which of the two events in fact takes place - the steps of the coupled flux should be in dynamic equilibrium with the conformation gating the anion-conductance. Selective blockade of the coupled cycle at either Steps 2 or 3 is expected to increase the probability of entering the anion gating conformation. The experiments shown in Figures 2 and 6 support this idea. Upon modification of I421C with MTSET, net outward D-aspartate currents emerge at positive potentials (Fig. 2). Before addition of the sulfhydryl reagent, the current due to the coupled flux, which is inward even at +40mV, is almost balanced by the outward current which reflects the substrate-gated anion conductance (30). Selective elimination of the coupled current by sulfhydryl reagents reveals the outward anion current. In the presence of the highly permeable thiocyanate, the contribution of the anion-conductance to the total current is markedly increased. At positive potentials this conductance becomes the major component of the total current - even before the addition of MTSET (30,34-36). The result of the sulfhydryl modification is a more than four-fold stimulation of the anion-current (Fig. 6C), due to the enhanced probability of entering the conformation gating the anion conductance. At potentials more negative than the reversal potential, the stimulation of the inward current after MTSET treatment is about 2.5 fold. This is because two events now take place: The coupled current, which contributes significantly to the inward current, is inhibited and at the same time the anion current is stimulated.

Even though we have obtained new functional insights into coupled glutamate translocation and substrate-gated anion conductance, the structural basis of their possible interrelations remains unclear. Our observations that modification of a cysteine residue
introduced in the hydrophobic linker region selectively inhibits coupled flux, suggest that this region may be less important for the activation of the anion conductance. It has been suggested that the same translocation pathway is used for both processes (30). An alternative speculation is that the coupled glutamate flux occurs within a glutamate transporter monomer, whereas the anion-conducting pathway is formed in the space between the monomers in a pentameric structure (41). If the latter idea is correct, one way to identify the chloride conducting pathway may be to search for residues that participate in the formation of interfaces between monomers.
References


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Note added to revised manuscript:

While this manuscript was reviewed, two groups have also documented that sulfhydryl modification of cysteine replacement mutants of the related human glutamate transporter EAAT-1, results in the elimination of the coupled flux but not the uncoupled process. In one report the cysteine was introduced at a position corresponding to residue 417 of EAAC-1 (Seal, R.P., Shigeri, Y., Eliasof, S., Leighton, B.H. and Amara, S.G. (2001), *Proc. Natl. Acad. Sci. USA* **98**, 15324-15329) and in the other, the cysteine replacement was at a position corresponding to EAAC-1 residue 420 (Ryan, R.M. and Vandenberg, R.J. (2002), *J. Biol. Chem.*, in press).
Figure legends

Fig. 1 Effect of MTSET on D-[3H]-aspartate uptake in HeLa cells expressing substitution mutants at position 421 of EAAC-1.
Aspartate uptake (10 min at 37 C) in cells incubated with (dark bars) or without (grey bars) 500 µM MTSET was measured as described in Experimental Procedures, using 2 µCi/ml of D-[2,3-3H]-aspartate (18 Ci/mmol). The results are expressed as percent activity of the untreated wild-type EAAC-1 and are mean +/- S.E. of 6-9 determinations.

Fig. 2 Net currents in sodium medium mediated by D-aspartate in I421C-EAAC-1 transporters.
Currents recorded in the absence of D-aspartate during 250ms voltage pulses from -100mV to +40mV subtracted from currents recorded during superfusion of 1mM D-aspartate (net current). The prepulse potential was -25mV. Horizontal line indicates zero current level. The net currents were measured before MTSET application (Control), after MTSET application (MTSET) and after MTSET followed by DTT (MTSET then DTT). MTSET were used at 5mM but similar results were obtained with 1mM on other oocytes. DTT were used at 10mM. A typical experiment is shown (n = 10).
Fig. 3 Voltage dependence of steady-state net currents before and after MTSET treatment.

Voltage dependence of net D-aspartate currents in oocytes expressing wild-type EAAC-1 (A) or I421C-EAAC-1 (B) before MTSET application, after MTSET and after MTSET followed by DTT, as indicated in the figure (n = 10). Net currents at -100mV ranged from -500nA to -1000nA (A) and from -300nA to -620nA (B).

Fig. 4 Net currents in lithium medium mediated by L-aspartate in I421C-EAAC-1 transporters.

Currents recorded in the absence of L-aspartate during 250ms voltage pulses from -100mV to +40mV were subtracted from currents during superfusion of 1mM L-aspartate. The application order of MTSET (1mM) and DTT (10mM) was as described in the legend to Fig 2. A typical experiment is shown (n = 6).

Fig. 5 Concentration dependence of D-aspartate dependent steady-state net currents in I421C-EAAC-1.

Voltage dependence of steady-state net currents at the indicated concentrations of D-aspartate before (A) and after treatment with 2.5mM MTSET (B). The dependence of EC50, determined from (A) and (B), on voltage are displayed in C. Net currents at -100mV ranged from -260nA to -460nA (A) at 1mM D-aspartate and from -330nA to -460nA at 3mM D-aspartate (B). Data are mean +/- S.E. of three oocytes.
Fig. 6 D-aspartate steady-state net currents in I421C-EAAC1 in the presence of thiocyanate.

Voltage dependence of D-aspartate (3mM) steady-state net currents at the indicated sodium concentrations after treatment with 1mM MTSET (A). Sodium dependence of the net D-aspartate currents (B); replot of data from (A). A typical experiment depicting the absolute currents at 96mM sodium are shown before (control) and after MTSET (C). Absolute net currents at 96mM sodium ranged from +370nA to +1156nA and from -380nA to -2002nA before MTSET at +40mV and -100mV respectively, and from +1551nA to +4767nA and from -818nA to -4150nA after MTSET at +40mV and -100mV respectively. Data are mean +/- s.e. of nine oocytes from four different batches.

Fig. 7 Possible interrelationships between coupled and uncoupled pathways.

Depicted is the coupled pathway (Steps 1-4) and the two possible entry routes into the anion conducting state $T^{*}_{\text{Na,Glu}}$. Further details are given in the discussion.
Figure 1

![Graph showing D-[H]Asp uptake (% WT) for WT, I421C, I421A, and I421S. The graph has bars for each protein variant, with WT showing significantly higher uptake compared to the others.]
Figure 2

Control

MTSET

MTSET then DTT
Figure 3

A

WT-EAAC1

Control or MTSET
MTSET → DTT

B

I421C-EAAC1

MTSET
Control or MTSET → DTT
Figure 4

Control

MTSET

MTSET then DTT
Figure 5

A

I421C-EAAC1

[D-Asp] (µM)

Vhold (mV)

I/Imax

B

I421C-EAAC1

[D-Asp] (µM)

Vhold (mV)

I/Imax

C

D-Asp EC50

MTSET

Control

Vhold (mV)
Figure 6

A

[Graph of I421C-EAAC1 showing voltage dependence on sodium concentration]

B

[Graph of I421C-EAAC1 showing sodium concentration dependence on maximum current]

Fig 6C

Control

MTSET

Figure 7
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