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# Arginine 447 Plays a Pivotal Role in Substrate Interactions in a Neuronal Glutamate Transporter*

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**Glutamate transporters from the central nervous system play a crucial role in the clearance of the transmitter from the synaptic cleft. Glutamate is cotransported with sodium ions, and the electrogenic translocation cycle is completed by countertransport of potassium. Mutants that cannot interact with potassium are only capable of catalyzing electroneutral exchange. Here we identify a residue involved in controlling substrate recognition in the neuronal transporter EAAC-1 that transports acidic amino acids as well as cysteine. When arginine 447, a residue conserved in all glutamate transporters, is replaced by cysteine, transport of glutamate or aspartate is abolished, but sodium-dependent cysteine transport is left intact. Analysis of other substitution mutants shows that the replacement of arginine rather than the introduced cysteine is responsible for the observed phenotype. In further contrast to wild type, acidic amino acids are unable to inhibit cysteine transport in R447C-EAAC-1, indicating that the selectivity change is manifested at the binding step. Electrophysiological analysis shows that in the mutant cysteine, transport has become electroneutral, and its interaction with the countertransported potassium is impaired. Thus arginine 447 plays a pivotal role in the sequential interaction of acidic amino acids and potassium with the transporter and, thereby, constitutes one of the molecular determinants of coupling their fluxes.**

Glutamate transporters prevent neurotoxicity by their ability to maintain low synaptic glutamate concentrations despite high intracellular glutamate levels in neurons and glia surrounding the cleft (1–5). Moreover, at some synapses glutamate transporters play an important role in limiting the duration of synaptic excitation (6–9). They achieve their remarkable concentrative power by an electrogenic process (10–12) in which the transmitter is cotransported with three sodium ions and a proton (3) followed by countertransport of a potassium ion (3, 13–15).

One of the five known glutamate transporters, GLT-1, has been purified from rat brain to near homogeneity and reconstituted (16, 17). It has been cloned and expressed (18) and is related to three different glutamate transporters from the central nervous system, GLAST-1 (19), EAAC-1 (20), and EAAT-4 (21), as well as to one from the retina (22). Studies of the highly conserved carboxyl-terminal half of the glutamate transporters indicate a non-conventional topology containing two reentrant loops, two transmembrane domains, 7 and 8, long enough to span the membrane as $\alpha$-helices as well as an outward facing hydrophobic linker (23–25). A very recent study arrives at a somewhat different model including the assignment of transmembrane domain 7 as a reentrant loop (26). Two adjacent amino acid residues of GLT-1 located in transmembrane domain 7, tyrosine 403 and glutamate 404, appear to be involved in potassium binding and are close to one of the sodium binding sites (15, 27). Because of the sequential nature of the transport process (13–15), mutations in these residues cause the transporter to be locked in an obligatory exchange mode (15, 27). Moreover, tyrosine 403 behaves as if it is alternately accessible to either side of the membrane (28). Analysis of GLT-1 mutants where serine 440, located in one of the reentrant loops, has been modified indicates that at least part of this loop is crucial for the coupling of sodium and glutamate fluxes and that it is close to the glutamate binding site (29).

We now report on a residue that controls the binding of the $\gamma$-carboxyl group of glutamate. Conradt and Stoffel (30) already noted that arginine 479 of GLAST-1, located in transmembrane domain 8 (23, 25), is conserved in all dicarboxylic acid transporters of the family but not in the small neutral amino acid transporter ASCT1 (31, 32), whose substrates have only a single carboxyl group. They found that mutation of arginine 479 to threonine, which occupies the same position in ASCT-1, abolishes glutamate uptake, but they noted that there may be other reasons underlying such a defect (30). Nevertheless the idea of a role of the arginine in glutamate binding remained viable, especially after the subsequent cloning and sequencing of the isotransporter ASCT-2 revealed that it contains a cysteine residue at the equivalent position (33, 34). We reason that mutation of this arginine in a glutamate transporter, which also transports a non-dicarboxylic acid substrate, might leave the transport of this substrate intact. It was shown that EAAT-3, the human homologue of EAAC-1 (35), also exhibits considerable transport of cysteine (36). We report here that mutation of the equivalent arginine 447 of EAAC-1 to neutral or negative amino acid residues completely abolishes transport of L-glutamate and D- and L-aspartate without impairing cysteine transport. Surprisingly, this cysteine transport is electroneutral rather than electrogenic. This appears to be due to a defective interaction with potassium. We propose that arginine

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447, by sequentially participating in the binding of glutamate and potassium, is enabling the coupling of their fluxes.

## EXPERIMENTAL PROCEDURES

*Expression of Transporters*—Complementary DNAs encoding the histidine-tagged EAAC-1 and its derived mutants, subcloned in the oocyte expression vector $pOG_2$ (see below), were linearized with *Sac*I, and cRNA was transcribed from each of the cDNA constructs with T7 polymerase and capped with 5′7-methyl guanosine by use of the mMESSAGE mMACHINE (Ambion Inc., Austin, TX). Approximately 50 ng of the various cRNAs was injected into defolliculated stage V-VI *Xenopus laevis* oocytes (36), and expression was assayed 3–5 days later by two-microelectrode voltage clamp recording (15) and/or radiotracer flux experiments (see below).

*Radiotracer Flux Measurements*—Uptake of D-[$^3$H]aspartate (10.5 Ci/mmol) and L-[$^3$H]serine (21.7 Ci/mmol) (both from PerkinElmer Life Sciences) and L-[$^3$H]glutamate (60 Ci/mmol), L-[$^3$H]aspartate (32 Ci/mmol), and L-[$^{35}$S]cysteine (1075 Ci/mmol) (all from American Radiolabeled Chemicals, St. Louis, MO) was performed by placing 5 *X. laevis* oocytes per determination in 500 $\mu$l of frog Ringer's solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.5, supplemented with 1 $\mu$Ci of the radiolabeled amino acids, except for cysteine, where 10 $\mu$Ci was used. The amino acids were used carrier-free unless indicated otherwise. After 20 min at room temperature (uptake was linear with time for at least 30 min), the oocytes were washed by passing through 4 wells filled with 1 ml of Ringer's solution followed by a 15-min incubation in 10% SDS (500 $\mu$l/oocyte). Radioactivity was determined in a Kontron liquid scintillation counter. Each experiment was performed 3–8 times with different batches of oocytes, and representative experiments based on five oocytes per determination are shown.

*Voltage Clamp Recording*—During two-microelectrode voltage clamp recording (15), oocytes were perfused with frog Ringer's solution with and without amino acid substrates. In experiments where the external chloride concentration was replaced or varied, equimolar anion substitution was employed, and recordings were made with the bath grounded via a 3 M KCl/agar bridge connected to a 3 M KCl reservoir containing an Ag/AgCl electrode. In the figures, records from representative oocytes ($n$ = 3–8, at least 2–3 different batches) are presented. The chloride equilibrium potential was determined by measuring the reversal potential of endogenous calcium-dependent chloride channels after activation with A23187 (37).

*Radiotracer Flux during Voltage Clamp*—Current measurements were made during superfusion of 30 $\mu$M L-[$^{35}$S]cysteine ($2.27 \times 10^{14}$ cpm/mol) onto oocytes voltage-clamped at −23 mV for 150 s. After washout of the bath for 1 min, oocytes were transferred to a scintillation tube, lysed, and measured for radioactivity. Currents induced by L-[$^{35}$S]cysteine were recorded using chart software (AD Instruments). The chloride equilibrium potential measured in two different oocytes of the batch used was −21 and −23 mV.

*Subcloning and Site-directed Mutagenesis*—The EAAC-1 cDNA, residing in pBluescript SK$^-$, was generously donated by Dr. Mathias Hediger. A tail of nine histidines was engineered at its carboxyl using site-directed mutagenesis using uracil-containing single strand DNA (38, 39). Briefly, the parent DNA, EAAC-1 or EAAC-1H9 (see below), was used to transform *Escherichia coli* CJ236 (dut$^-$, ung$^-$). From one of the transformants, single-stranded uracil containing DNA was isolated upon growth in a uridine-containing medium according to the standard protocol from Stratagene using helper phage $R_{408}$. This yields the sense strand, and consequently, the mutagenic primers were designed to be antisense. Our original intention was to replace the last residue (phenylalanine) by methionine and to add a deca-histidine tail. However after sequencing throughout the entire coding region from both directions, it appeared that a single error had occurred, causing a frameshift. As a consequence, instead of a tail of 10 histidines, it contained only 9, and the open reading frame was extended by the sequence SLEALASQDCHEGPSMRVIS before reaching the new stop codon TAG (EAAC-1H9). Expression of this transporter in HeLa cells using the recombinant vaccinia/T7 virus (40) gave rise to identical D-[$^3$H]aspartate transport as the original EAAC-1 cDNA. The EAAC-1H9 construct served as the parent construct (wild type) for subsequent site-directed mutagenesis (38, 39), except for the R447C mutant, for which the original EAAC-1 cDNA served as a parent construct.

The histidine-tagged EAAC-1H9, the original EAAC-1, and the R447C-EAAC-1 were subcloned into the oocyte expression vector $pOG_2$, which contained a 5′-untranslated *Xenopus* $\beta$-globin sequence and a 3′-poly(A) signal, using *Xho*I and *Bgl*II. This causes a reduction of the 3′-untranslated region of EAAC-1 from 1621 to 730 base pairs (EAAC-1H9-$pOG_2$). The mutants were identified by diagnostic restriction sites and subcloned into the EAAC-1H9-$pOG_2$ constructs using the restriction enzymes AgeI and *Spe*I. A similar subcloning was also done for the R447C mutation from the EAAC-1 to the EAAC-1H9 constructs. The subcloned cDNAs were then sequenced from each direction between the two restriction sites. In the case of the mutant R447C, which was the first one prepared for this study, initial characterization of its properties was carried out by comparing oocytes injected with cRNA transcribed from R447C-EAAC-1 and EAAC-1 (both without the histidine tail). The features of radiotracer uptake and cysteine currents in these oocytes were the same as those shown in Figs. 1 and 4 for EAAC-1H9 and R447C-EAAC-1H9 (therein labeled as R447C). Thus the R447C mutant does not behave differently because of the presence of the 20 extra amino acids and the 9 histidines present in EAAC1H9.

## RESULTS

*Tracer Flux of Amino Acids in the R447C Mutant—X. laevis* oocytes injected with R447C-EAAC-1H9 cRNA exhibit strikingly different transport properties from their counterparts injected with EAAC-1H9 wild type cRNA (Fig. 1). The mutant transporters are almost totally defective in the transport of D-[$^3$H]aspartate, L-[$^3$H]aspartate, and L-[$^3$H]glutamate. All three acidic amino acids are substrates for the wild type transporter (Fig. 1*A*). Significantly, R447C transporters are capable of taking up L-[$^{35}$S]cysteine, as is the wild type (Fig. 1, *A* and *B*). There is no significant L-[$^3$H]serine uptake in oocytes injected with wild type cRNA as compared with uninjected oocytes (Fig. 1*C*), but L-[$^3$H]serine uptake is observed with the R447C mutant (Fig. 1*C*). Uptake of both L-[$^3$H]serine and L-[$^{35}$S]cysteine is fully sodium-dependent (Fig. 1, *B* and *C*). Not only are the acidic amino acids not taken up by the R447C transporters, but they are unable to bind them. This is exemplified by the inability of unlabeled L-aspartate to compete with L-[$^{35}$S]cysteine uptake in the mutant (Fig. 2). Whereas the wild type L-[$^{35}$S]cysteine uptake is potently inhibited by 100 $\mu$M L-aspartate (4–10-fold the reported $K_m$ for this substrate (20, 35)), L-aspartate does not inhibit L-[$^{35}$S]cysteine uptake by the mutant, even at 1 mM (Fig. 2). Similarly, no inhibition of L-[$^{35}$S]cysteine uptake in the mutant is observed with L-glutamate or D-aspartate (data not shown). L-[$^{35}$S]Cysteine uptake is not inhibited by nonrelated compounds like $\gamma$-aminobutyric acid, neither in the mutant nor in the wild type (Fig. 2). The data presented in Fig. 2 also indicate that the mutant has a higher affinity for cysteine than the wild type, and the same phenomenon is observed with serine (Fig. 2). That this is indeed the case is seen from the apparent $K_m$ values, measured as the ability to induce steady state currents (see "Electrophysiological Characterization of the R447C Mutant" below).

*Tracer Flux in Other Substitution Mutants at Position 447*—The inability of the R447C mutant to interact with acidic amino acids is caused by the removal of the arginine rather than the introduction of a cysteine at this position. Mutants in which arginine 447 has been replaced by glutamate (R447E), lysine (R447K), glycine (R447G), and serine (R447S), retain the ability to take up L-[$^{35}$S]cysteine, although it is reduced in R447K (Fig. 3*A*). In contrast, none of them, with the exception of R447K, is able to transport D-[$^3$H]aspartate (Fig. 3*B*). However, even though in R447K transporters the positive charge at this position is maintained, these mutant transporters exhibit a markedly reduced uptake of this acidic amino acid (Fig. 3*B*). The absence of the positive charge at position 447 is correlated with the ability to exhibit significant L-[$^3$H]serine transport; this is observed with all mutants except R447K (Fig. 3*C*). We have tried to restore D-[$^3$H]aspartate transport to R447C using the positively charged sulfhydryl reagents (2-aminoethyl)-methane thiosulfonate (2.5 mM), which would be expected to introduce a lysine-like structure at this position, and [(2-trimethylammonium)ethyl] methane thiosulfonate (10 mM). No

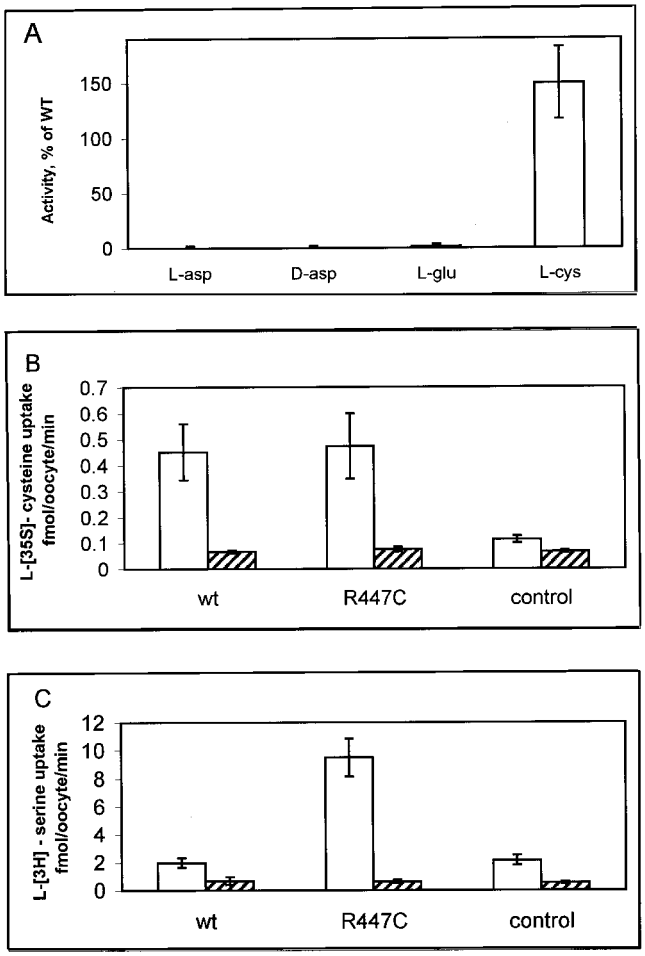


FIG. 1. **Uptake of acidic amino acids, cysteine, and serine by oocytes expressing wild type EAAC-1H9 and mutant R447C.** Uptake was performed using carrier-free D-[$^3$H]aspartate (190.4 nM), L-[$^3$H]aspartate (62.5 nM), L-[$^3$H]aspartate (62.5 nM), L-[$^3$H]glutamate (33.3 nM), L-[$^{35}$S]cysteine (18.6 nM), and L-[$^3$H]serine (92.2 nM) is described under "Experimental Procedures." In the experiment depicted in *A*, values obtained with uninjected oocytes (control) are subtracted, and in those shown in *B* and *C*, unsubtracted values are given. *A*, the transport rates for the R447C mutant are given as the percentage of the rates of the wild type (*wt*). In the experiment depicted in *A*, the wild type rates (mean ± S.E.) were (fmol/oocyte/min): D-[$^3$H]aspartate, 54.1 ± 6.2; L-[$^3$H]aspartate, 6.8 ± 0.7; L-[$^3$H]glutamate, 5.2 ± 0.6; L-[$^{35}$S]cysteine, 1.68 ± 0.41. In *B* and *C*, rates have been determined in the presence (*open bars*) or absence (*hatched bars*) of sodium (choline substitution). The large differences in rates are due to the differences in concentrations and apparent $K_m$ values of the various substrates (20, 35, 36).

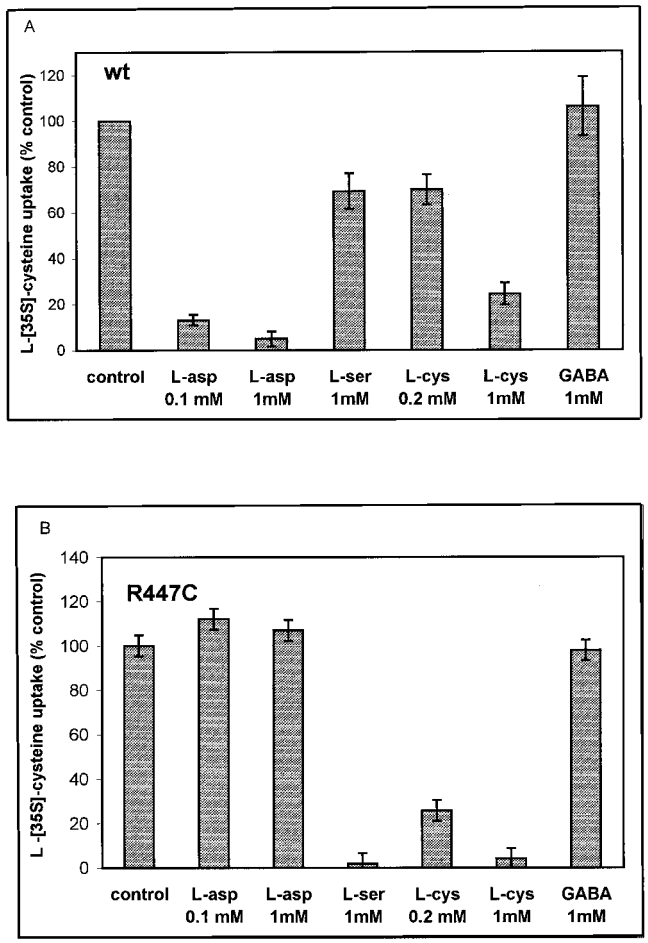


FIG. 2. **Effect of unlabeled amino acids on uptake of L-[$^{35}$S]cysteine in wild type and R447C.** Uptake of L-[$^{35}$S]cysteine (10 $\mu$Ci, 25 $\mu$M final concentration) was measured in the presence of the unlabeled amino acids at the indicated concentrations in oocytes injected with the wild type or R447C transporter cRNAs. Values obtained with uninjected oocytes have been subtracted. Values are given as percent of control (no additions). The control values were 1.11 ± 0.12 and 1.40 ± 0.15 pmol/oocyte/min for wild type and R447C mutant, respectively. *GABA*, γ-aminobutyric acid.

effects on the transport of any of the substrates was observed after 5-min preincubations of oocytes expressing R447C with these reagents or the negatively charged (2-sulfonatoethyl) methane thiosulfonate (10 mM) (data not shown).

*Electrophysiological Characterization of the R447C Mutant*—Uptake of excitatory amino acids by all glutamate transporter clones thus far characterized results in the activation of a current reflecting the sum of the inward current (resulting from cotransport of coupled ions such as sodium) together with a chloride current flowing through a thermodynamically uncoupled conductance pathway (21, 37). The voltage dependence of the currents mediated by wild type EAAC-1H9 and its mutant R447C has been determined by clamping oocytes expressing the transporters at potentials between −100 and +40 mV (increments of 20 mV) in the presence and absence of the substrates D-aspartate and L-cysteine. In the wild type EAAC-1H9, steady state D-aspartate (Fig. 4*A*) and L-cysteine currents (Fig. 4*D*) are observed that reverse at potentials greater than +30 mV (Fig. 4, *C* and *F*). The behavior of R447C is quite different. No significant currents are observed with D-aspartate (Fig. 4*B*) and L-glutamate and L-aspartate (data not shown), consistent with the inability of R447C transporters to take up acidic amino acids (Figs. 1*A* and 3*B*). With L-cysteine, transport currents are observed (Fig. 4*E*), but the L-cysteine currents of R447C exhibit a reversal potential around −25 mV, similar to the chloride equilibrium potential (Fig. 4*F*). The apparent $K_m$ value for cysteine was 27.9 ± 7.9 $\mu$M for the R447C-EAAC-1H9 mutant (measured at +20 mV, $n$ = 3) and 110 ± 24 $\mu$M for the wild type (measured at −80 mV, $n$ = 3). The corresponding values for serine- and alanine-induced currents in the mutant were 13.9 ± 4.1 and 17.9 ± 3.1 $\mu$M, respectively ($n$ = 3). In the wild type, 1 mM each of the latter two amino acids induced very small currents (<10% of those with D-aspartate), consistent with the very low affinities observed with its human EAAT-3 homologue (36).

In addition to the steady state current, a transient cysteine-induced current is observed with R447C (Fig. 4*E*). This transient current appears to be capacitative, because the charge movements, estimated by subtraction of the steady state currents, after following hyperpolarizing or depolarizing pulses are equal to those following the return to the original potential

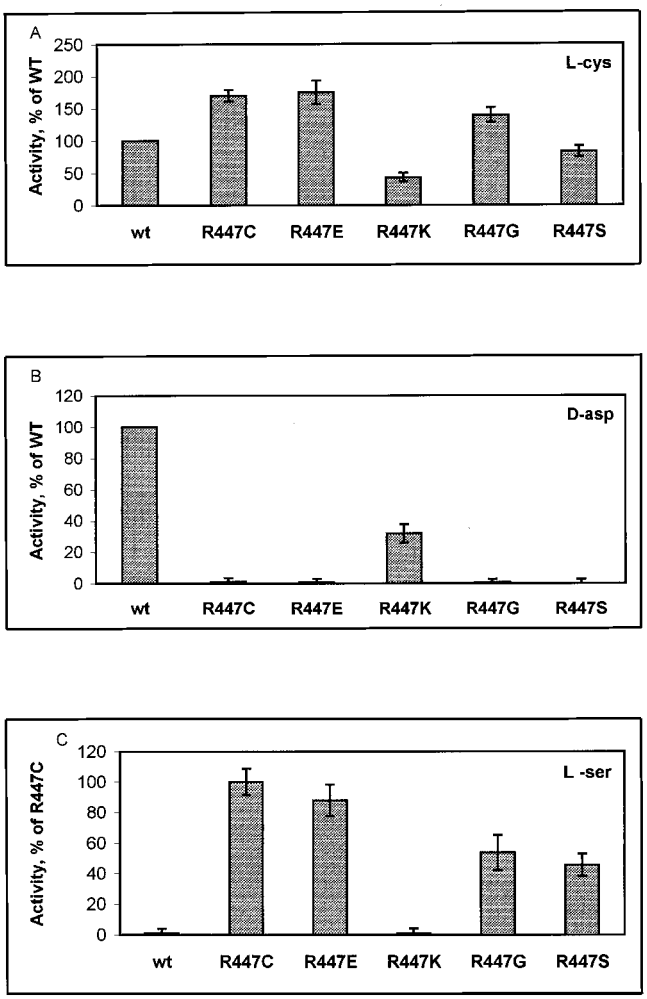


FIG. 3. **Uptake of L-cysteine, D-aspartate, and L-serine by substitution mutants at position 447.** Uptake of L-[$^{35}$S]cysteine, D-[$^{3}$H]aspartate, and L-[$^{3}$H]serine (all carrier-free) was performed as described in Fig. 1. The values are corrected for those obtained with uninjected oocytes and are given as percent of wild type (*wt*, *A* and *B*) or R447C (*C*) uptake. The control uptake rates (fmol/oocyte/min) were: L-cysteine, wild type, 0.67 ± 0.06; D-aspartate, wild type, 52.0 ± 3.9; L-serine, R447C, 22.1 ± 4.9.

(Fig. 5). Neither steady state nor transient cysteine currents were observed in the absence of sodium (lithium or choline substitution) and also not in uninjected oocytes (data not shown). Similar observations have been made on oocytes injected with R447E-EAAC-1H9 cRNA, although the outward currents are smaller than when cysteine occupies this position (data not shown). Varying $[Cl^-]_o$ shifted the reversal potential of R447C by 48 mV/10-fold change in $[Cl^-]_o$ (Fig. 6). Furthermore, in R447C, substitution of $Cl^-$ by gluconate in the Ringer's solution bathing the oocytes abolishes the outward current at positive potentials up to +70 mV (data not shown). These observations suggest that in R447C, $Cl^-$ ions carry the major part of the current activated by L-cysteine superfusion.

The selective loss of the ability of the R447C mutant to catalyze the inwardly rectifying electrogenic transport current (Fig. 4*E*) indicates that the uptake of L-[$^{35}$S]cysteine is electroneutral rather than the electrogenic process observed with the wild type. Evidence supporting this idea is presented in Fig. 7. Oocytes expressing wild type EAAC-1 or R447C were voltage-clamped at −23 mV, near the equilibrium potential of chloride, and superfused with 30 $\mu$M L-[$^{35}$S]cysteine. This non-saturating concentration, around the apparent $K_m$ for cysteine, was used to enable the uptake measurements without having to add excessive amounts of radiolabel. Currents recorded simultaneously during radiolabeled uptake are dramatically different between the two groups of cells (Fig. 7). Although uptake of L-[$^{35}$S]cysteine in the two groups is similar, an electrogenic uptake current is only observed in wild type EAAC-1 (Fig. 7*A*) but not in the mutant (Fig. 7*B*).

The behavior of R447C is reminiscent of that of mutants E404D (15) and Y403F (27) of the glutamate transporter GLT-1. They are locked in the electroneutral exchange mode because they cannot interact with potassium, which is required for the return of the unloaded transporter. We have previously used reconstitution of solubilized membrane proteins from HeLa cells expressing wild type GLT-1, E404D, and Y403F to show that D-[$^{3}$H]aspartate uptake is defective with internal potassium (net flux) but not with unlabeled glutamate or aspartate (exchange) on the inside (15, 27). HeLa cells exhibit a very high endogeneous L-[$^{35}$S]cysteine transport, and transfection with wild type EAAC-1H9 does not elevate it significantly. We therefore tried to purify EAAC-1H9 from the cysteine transporters endogeneous to the HeLa cells on $Ni^{2+}$-nitrilotriacetic acid beads. However, with several detergents we tried, inactivation was so rapid that no reconstitutable transport could be recovered after elution of the beads with imidazole. Therefore, we used an alternative approach to probe the interaction of potassium with wild type and R447C transporters. In oocytes, which have high concentrations of endogenous substrates (35, 41), elevation of external potassium induces reverse transport (13, 15, 42). This also results in the activation of the transporter-mediated anion conductance, readily observed when highly permeant anions, such as nitrate or thiocyanate, are present (15). This anion conductance is not activated by external potassium in the E404D mutant (15). In the presence of 38 mM thiocyanate, potassium induces an outward current at positive potentials in wild type EAAC-1H9 (Fig. 8*A*). Amino acid substrates can also induce the transporter-mediated anion conductance (37). In the thiocyanate supplemented medium, cysteine induces currents of similar size as potassium (Fig. 8*A*). Neither cysteine- nor potassium-dependent anion currents are observed in uninjected oocytes (data not shown). In contrast to the wild type, potassium is not able to induce these currents in the mutant (Fig. 8). This is not due to a defective anion conductance *per se*, because cysteine induces anion currents in R447C that are even larger than those in the wild type (Fig. 8*B*).

## DISCUSSION

Our results indicate that arginine 447 plays a key role in recognition of the γ-carboxyl group of the anionic transporter substrates. Substitution of this arginine by many other residues eliminated the ability to transport the acidic amino acid substrates L-glutamate and D- and L-aspartate (Figs. 1*A* and 3*B*) without effect on cysteine transport (Figs. 1, *A* and *B*, and 3*A*). Furthermore, competition experiments indicate that the acidic amino acids cannot even bind to R447C transporters (Fig. 2), and similar results have been obtained with R447E transporters (data not shown). Only when another positively charged amino acid residue replaces arginine 447 is a low level transport of acidic amino acids retained (Fig. 3*B*). Thus the requirement for just the positive charge at this position is not enough for optimal transport.

The simplest and most straightforward explanation for these observations is that the guanidinium group of arginine 447 directly interacts with the γ-carboxyl group of L-glutamate. In the absence of a crystal structure of any cotransporter, it is impossible to rule out indirect effects. However, a long range conformational change caused by replacing arginine would need to be as subtle as to impede amino acids to bind to a

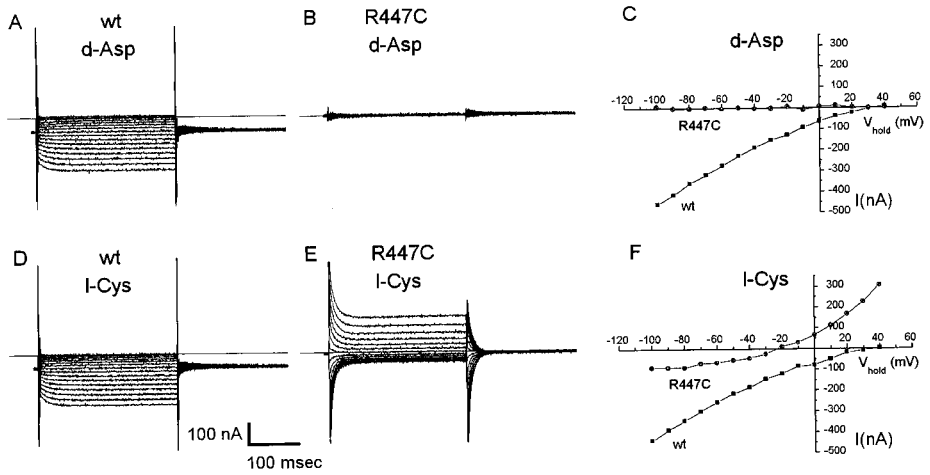


FIG. 4. **Currents mediated by wild type (*wt*) and R447C transporters.** Subtracted records showing currents induced by D-aspartate (*A* and *B*) and L-cysteine (*D* and *E*) of representative oocytes expressing EAAC-1H9 wild type (*A* and *D*) and R447C (*B* and *E*). Currents recorded in the absence of substrate during 250 ms voltage pulses from −100 to +40 mV were subtracted form those during superfusion of 1 mM D-aspartate or L-cysteine. The prepulse potential was −30 mV. Voltage dependence of steady state currents taken at 200 ms induced by D-aspartate (*C*) or L-cysteine (*F*) is shown in wild type (*squares*) and R447C mutant (*circles*).

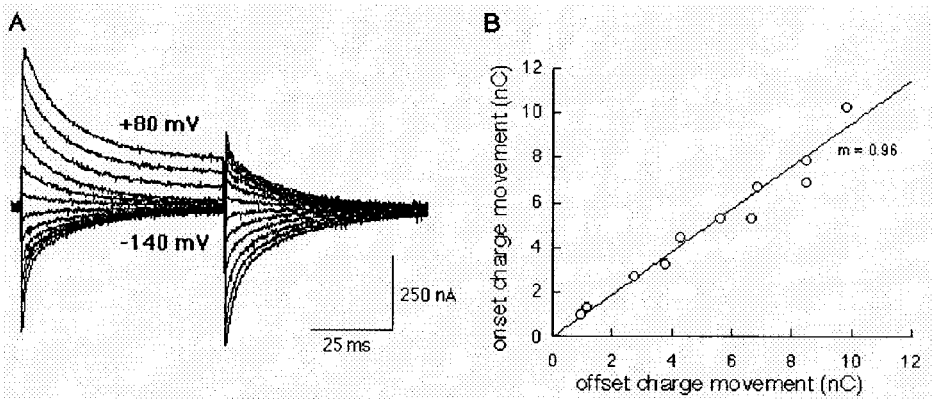


FIG. 5. **Voltage dependence of charge movement by the R447C transporter.** *A*, family of currents induced by application of 30 μM cysteine with 600-ms voltage pulses between +80 and −140 mV in 20-mV increments. The holding potential was −30 mV. *B*, the charge movements were calculated by integrating the currents after subtraction of the steady state component as described in Wadiche *et al.* (12). The on and off charge movements were equal, and similar results were seen in nine other cells.

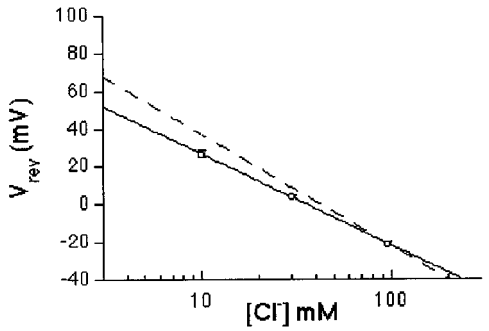


FIG. 6. **Dependence of the reversal potential of steady state cysteine currents by R447C transporters.** Currents induced by 1 mM cysteine with 250-ms voltage pulses between +80 and −120 mV were recorded in oocytes expressing R447C transporters ($n = 3$) using perfusion media of varying chloride concentrations (gluconate substitution). The error bars (S.E.) are smaller than the size of the symbols. The *dashed line* gives the predicted chloride dependence of the reversed potential for the mutant according to the Nernst equation for chloride.

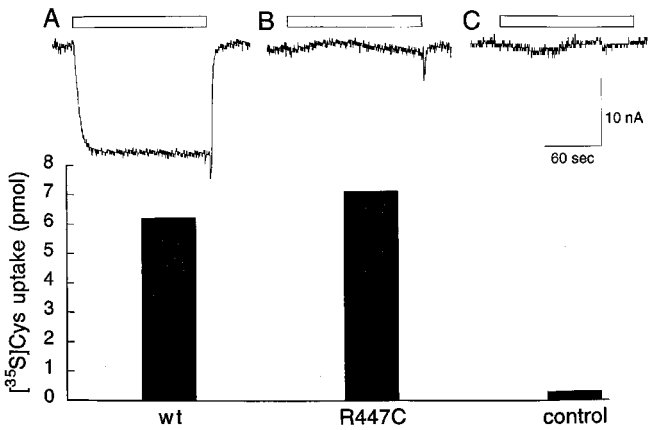


FIG. 7. **Comparison of transport currents and radioactive uptake in wild type (*wt*) and R447C transporters.** Oocytes expressing wild type (*A*), R447C (*B*), and uninjected oocytes were clamped at −23 mV, and currents induced by 30 μM L-[$^{35}$S]cysteine were recorded. Oocytes were washed, and radioactive cysteine taken up was determined by scintillation counting.

distant site, while leaving cysteine binding intact. It is of interest to note that diffraction studies of the crystallized ligand binding domain of the glutamate receptor GluR2 show directly that one of the two carboxyls of the glutamate analogue kainate is liganded by arginine (43). Also in two recent studies on bacterial multidrug-resistant transporters, a critical electrostatic interaction in substrate binding has been inferred (44, 45).

The apparent lack of reactivity of the cysteine, introduced at position 447, with methane thiosulfonate reagents indicates that this residue may not have an unrestricted accessibility to small molecules. This is perhaps not unexpected and may in fact be one of the manifestations of the substrate specificity of the transporter. For instance, binding of some parts of the glutamate molecule such as the α-amino and α-carboxyl groups, could induce a conformational change, exposing arginine 447. Even in the absence of an "induced fit" type of mechanism, it should be kept in mind that the side chain of cysteine is shorter than that of arginine. If the guanidinium group of arginine would be at a water-accessible surface, the sulfhydryl group of the introduced cysteine would be below it.

The presence of an arginine at position 447 is not only important for the interaction of EAAC-1 with acidic amino acids, it also hinders the interaction of the transporter with small neutral amino acids. Very little if any uptake of L-[$^{3}$H]serine is observed in wild type, but highly significant activity is observed

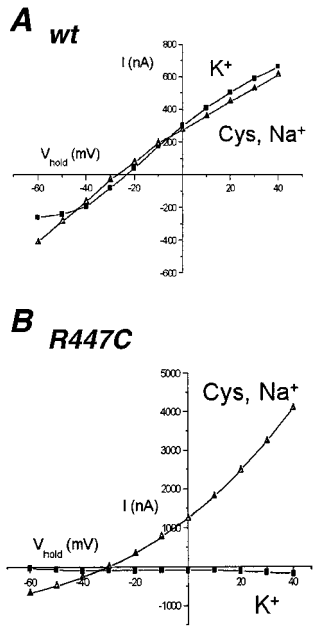


FIG. 8. **Voltage dependence of steady state anion currents of wild type and R447C transporters.** Voltage dependence of subtracted records obtained by subtracting currents recorded in 58 mM NaCl, 38 mM NaSCN,2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.5, from those in a similar solution with 38 mM KSCN instead of NaSCN (*squares*) or currents in the NaSCN containing medium subtracted from those in the same medium supplemented with 1 mM cysteine (*triangles*). The voltage pulses were for 250 ms from −60 to +40 mV, and the prepulse potential was −30 mV. Values recorded after 200 ms were taken as steady state values. *A*, wild type; *B*, R447C.

in many of the substitution mutants at this position (Figs. 1*C* and 3*C*). The same is true for L-[$^3$H]alanine uptake (data not shown). Moreover, in contrast with the wild type, both transient currents and steady state anion currents in R447C are also induced by serine, alanine, threonine, and glutamine (data not shown). Thus, the substrate specificity of R447C-EAAC-1H9 is very reminiscent of that of ASCT-2, where a cysteine residue occupies the corresponding position (33, 34). It is of interest to mention that recently a bacterial family member capable of transporting serine has been cloned (46). Instead of an arginine at this position it contains a glutamate residue, and this is in nice agreement with the strongly enhanced serine transport observed in the R447E-EAAC-1 mutant (Fig. 3*C*).

One of the striking findings of this study is that in oocytes expressing R447C-EAAC-1H9, L-[$^{35}$S]cysteine uptake is unhampered but, unlike the wild type, this uptake is electroneutral (Figs. 4 and 7). This could be due to a change in stoichiometry, allowing net flux to proceed in an electroneutral fashion. Alternatively, L-[$^{35}$S]cysteine uptake in the mutant may represent obligatory exchange. This is certainly feasible since it has been shown (41) that oocytes contain 1.2 mM internal ASCT-1 substrates (alanine, cysteine, threonine, and serine). In the case of GLT-1, where we could use a reconstitution approach, allowing control of the medium composition from both sides of the membrane, we have shown that two mutants, E404D and Y403F, which cannot interact with potassium from either side of the membrane, are locked in an obligatory exchange mode (15, 27). This is because electrogenic glutamate, and probably also cysteine, uptake by their transporters consists of two sequential translocation steps: 1) translocation of sodium and glutamate and 2) translocation of potassium in the opposite direction (13–15). Exchange represents a partial reaction of the full cycle, namely reversible translocation of sodium and the amino acid substrate, which can also take place in the absence of potassium (13, 14) or in mutants with defective potassium interactions (15, 27). In the case of EAAC-1, we can directly monitor only its interaction with external potassium (Fig. 8*A*). In the R447C mutant this interaction is impaired (Fig. 8*B*). It seems therefore likely that R447C-EAAC-1H9, just like GLT-1-E404D (15) and GLT-1-Y403F (27), is locked in an obligatory exchange mode because of its defective interaction with potassium.

In contrast to the wild type, cysteine induces outward currents in R447C-EAAC-1H9 (Fig. 4*E*), which are mainly carried by chloride. The ability of the mutant to mediate gating of the anion conductance mediated by amino acid substrates is consistent with observations that this activity is observed in GLT-1 mutants locked in the exchange mode (15, 27). Electroneutral cysteine transport mediated by the R447C mutant is accompanied by slow transient currents (Figs. 4, *E* and *5*). These may reflect the reversible movement of sodium and substrates across the electric field, which would give rise to capacitative currents and is also consistent with the idea that cysteine transport in R447C is electroneutral because it is operating as an exchanger. The transient currents are not observed in the wild type because it catalyzes net flux, manifested as a resistive current. The substitution mutants of arginine 447 render EAAC-1 similar to ASCT in one more aspect; also, ASCT-1 has been shown to be an obligate exchanger, and induction of this exchange activates an anion conductance (41). R447C appears to display larger outward currents with NaSCN at positive potentials than wild type (Fig. 8). If gating of the anion conductance would be associated with the cysteine-bound form of the transporter rather than the potassium-bound form, the mutant would be expected to exhibit a larger anion conductance than the wild type.

How could mutation of a single arginine residue of EAAC-1 impair both glutamate binding and the interaction of the transporter with potassium? An attractive possibility would be that arginine 447 can form alternate ion pairs with the transported acidic amino acid and an acidic residue of the transporter itself. The intramolecular salt bridge would somehow be important for the interaction with potassium or its translocation. EAAC-1 has several acidic amino acid residues that could be candidate partners, including glutamate 374, which occupies the same position as glutamate 404 of GLT-1. Further work will be required before this possibility can be tested directly. There still is some controversy on the membrane topology of the glutamate transporters (23–26), and no information on the proximity of its transmembrane domains is available now. Approaches, pioneered by Kaback and co-workers (see Ref. 47) to determine proximity relationships in lactose permease, will be important to determine if arginine 447 is close to glutamate 374.

The central finding of this study is that arginine 447 controls substrate binding as well as the interaction of potassium with the transporter. Although it has been shown that in chimeric bacterial cotransporters the selectivity of the ion depends on the nature of the substrate cotransported (48), we have shown here that mutation of a single residue affects separate translocation steps. Regardless of the precise mechanism by which mutation of arginine 447 influences potassium and substrate selectivity, the results suggest the existence of a common binding pocket for countertransported solutes. The results further suggest a plausible countertransport mechanism involving a competitive and sequential binding of substrate and potassium. This may be a widespread principle applying to other countertransporters as well, as has recently been suggested for the multidrug transporter EmrE in which glutamate 14 sequentially binds substrates and protons in a mutually exclusive

fashion, providing a molecular basis of cationic drug/$H^+$ antiport (49).

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# Arginine 447 Plays a Pivotal Role in Substrate Interactions in a Neuronal Glutamate Transporter

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