1997

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 3, Issue of January 17, pp. 1703–1708, 1997
Printed in U.S.A.

Mutation of an Amino Acid Residue Influencing Potassium Coupling in the Glutamate Transporter GLT-1 Induces Obligate Exchange*

(Received for publication, July 23, 1996)

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Glutamate transporters maintain low synaptic concentrations of neurotransmitter by coupling uptake to flux of other ions. After cotransport of glutamic acid with Na^+, the cycle is completed by countertransport of K^+. We have identified an amino acid residue (glutamate 404) influencing ion coupling in a domain of the transporter implicated previously in kainate binding. Mutation of this residue to aspartate (E404D) prevents both forward and reverse transport induced by K^+. Sodium-dependent transmitter exchange and a transporter-mediated chloride conductance are unaffected by the mutation, indicating that this residue selectively influences potassium flux coupling. The results support a kinetic model in which sodium and potassium are translocated in distinct steps and suggest that this highly conserved region of the transporter is intimately associated with the ion permeation pathway.

The role of electrogenic (Na^+ + K^+)-coupled glutamate transporters, located in the plasma membranes of nerve terminals and glial cells is to keep the extracellular concentration of the neurotransmitter below neurotoxic levels (1, 2). Furthermore, together with diffusion, they may help to terminate its action in synaptic transmission (3, 4).

A glutamate transporter (GLT-1) has been purified to near homogeneity and reconstituted (5, 6). It represents around 0.6% of the protein of crude synaptosomal fractions and is one (7) of four glutamate transporters expressed in brain cloned thus far (8–10). The stoichiometry of the electrogenic transport process (11–13) is three sodium ions per glutamate (14), whereas potassium is transported in the opposite direction (11). In addition, glutamate transport is accompanied by alkalinization of the external medium (15), and transport of glutamate can be driven by a pH gradient (16). It is not clear whether a proton is cotransported with glutamate or an hydroxyl is antiported (17), but recent evidence favors cotransport with a proton (14). Mechanistic studies have shown that the transport process is ordered and that sodium and potassium are translocated in distinct steps. The sodium ions bind first, followed by glutamate. After their translocation and release on the inside, potassium binds on the inside and is translocated outwards to complete the translocation cycle (18, 19).

* This work was supported by the Israel Science Foundation, which is administered by the Israel Academy of Sciences and Humanities, the Charles H. Revson Foundation, the Bernard Katz Minerva Center for Cell Biophysics (to B. I. K.), and Grant GM 48709 from the National Institutes of Health (to M. P. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Because all substrates are charged molecules, conserved charged amino acids located in hydrophobic stretches of the transporter proteins are likely to be important for the binding and translocation of these substrates. Using site-directed mutagenesis, we have identified four amino acid residues (GLT-1 numbering) important for the transport process: histidine 326 (20) and the acidic amino acid residues aspartate 398, glutamate 404, and aspartate 470 (21). In this report, we describe the in-depth characterization of a mutant in which glutamate 404 has been changed into aspartate. This mutant, E404D, appears to be able to catalyze the exchange of glutamate and aspartate but unable to carry out net flux of the acidic amino acids. We show that glutamate 404 is important for carrying out the potassium transporting limb of the cycle, suggesting that this residue may represent part of the potassium binding site.

EXPERIMENTAL PROCEDURES

Expression and Electrophysiological Recording—Capped mRNAs transcribed from the cDNAs encoding the rat brain glutamate transporter GLT-1 (7) or its mutant E404D (21) were microinjected into state V-VI *Xenopus* oocytes (50 ng/oocyte), and membrane currents were recorded 3–6 days later (13). Recording solution (Ringer) 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 control 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 <1 megaohms. 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 −100 and +40 mV from corresponding current records in the presence of substrate.

^{3}H-Labeled Amino Acid Flux—Current measurements were made during superfusion of 100 μM [^{3}H]D-aspartate (0.42 Ci/mmol; Amersham Corp.) 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.

Uptake in Reconstituted Systems—HeLa cells were cultured in DMEM supplemented with 10% fetal calf serum (heat-inactivated), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Infection with recombinant vaccinia/T7 virus vTF7–3 and subsequent transfection with plasmid DNA was done as described (22) using pT7-GLT-1 (shortened insert; Ref. 23), pT7-GLT-1 E404D (21), or vector

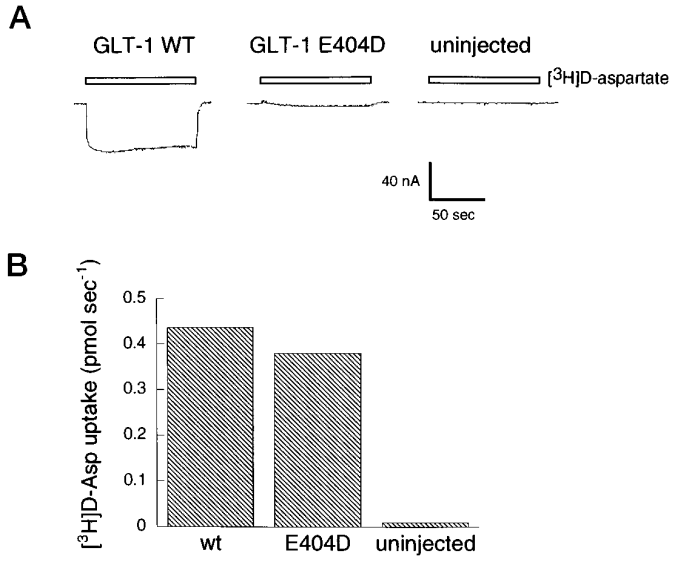

FIG. 1. **Comparison of transport currents and radioactive uptake in wild-type and E404D transporters.** *A,* application of 100 μM [^{3}H]D-aspartate induces an inward current in a representative oocyte expressing wild-type GLT-1, whereas current amplitudes were greatly reduced in oocytes expressing E404D. No current is induced in oocytes not injected with RNA. Oocytes were voltage-clamped at −30 mV. *B,* uptake of [^{3}H]D-aspartate into the corresponding oocytes from *A* demonstrating similar flux of radiolabel in wild-type and E404D in spite of marked differences in current amplitudes. Similar results were obtained with 12 other oocytes.

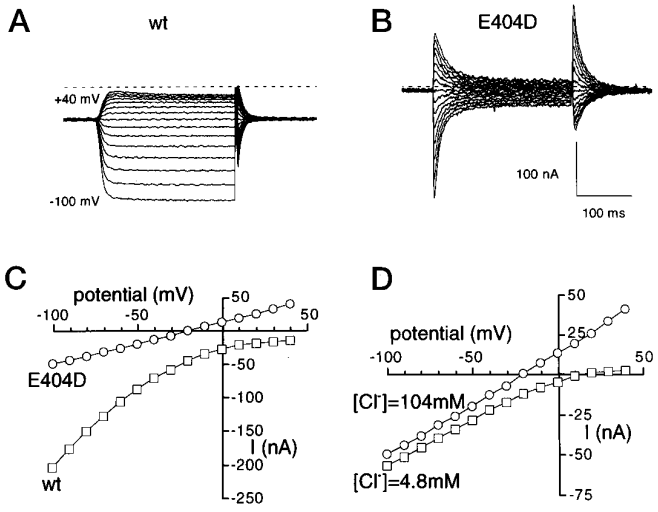

FIG. 2. **Currents mediated by wild-type and E404D transporters: subtracted records showing currents induced by D-aspartate in representative oocytes expressing GLT-1 wild-type (*A*) and E404D (*B*).** Currents recorded in the absence of D-aspartate during 250-ms voltage pulses from −100 mV to +40 mV were subtracted from currents recorded during superfusion of 100 μM D-aspartate. The prepulse potential was −30 mV; the *dashed line* indicates 0 current. Voltage dependence of steady-state currents induced by 100 μM D-aspartate in oocytes expressing wild-type and E404D (*C*). Wild-type transport current shows inward rectification, whereas currents in oocytes expressing the E404D mutant reverse at approximately −20 mV. Chloride dependence of D-aspartate (100 μM) induced steady-state currents mediated by the E404D transporter (*D*). Marked attenuation of outward current was seen following reduction of extracellular chloride from 104 to 4.8 mM.

alone (pBluescript SK$^-$) at 2.8 μg/well (3-cm diameter).

Cells from two wells were washed twice with 1 ml PBS and then taken up in 20 μl of PBS. The cell suspension was mixed with liposomes (4 μmol of asolectin and 0.7 μmol of brain phospholipids) and 0.9% cholate in a final volume of 220 μl of a solution of identical composition as the one used to equilibrate the spin columns, used for the subsequent reconstitution (24). The columns were equilibrated with 5 mM Tris-SO_4, pH 7.4, 1 mM $MgSO_4$, 0.5 mM EDTA, 1% glycerol, and other ions and amino acids corresponding to the desired "in-medium" as detailed in the figure legends. In the case of exchange experiments, acidic amino acid, usually 10 mM L-aspartate, was included in the equilibration buffer of the spin-column. The external L-aspartate was removed by passing these proteoliposomes over a spin column of identical composition but lacking the amino acid. The control liposomes (without entrapped L-aspartate) were also passed through a second spin column. Uptake of [^{3}H]D-aspartate was measured as described (21) using 20 μl of proteoliposomes into 360 μl of 0.15 M NaCl and 1 μCi of [^{3}H]D-aspartate (11.5 Ci/mmol) unless indicated otherwise in the figure legends. Uptake in intact cells was performed as described (22).

RESULTS

The Transport Current of the E404D Mutant—When expressed in HeLa cells using a vaccinia/T7 recombinant virus, uptake of [^{3}H]D-aspartate mediated by the rat brain glutamate transporter GLT-1 is similar to that in cells expressing a mutant GLT-1 transporter containing the conservative substitution E404→D (21). Although similar levels of radiotracer uptake were also seen in voltage-clamped *Xenopus* oocytes injected with RNA transcribed from the corresponding wild-type and mutant cDNAs, membrane currents recorded simultaneously during radiolabel uptake were dramatically different in the two groups of cells (Fig. 1). While uptake of [^{3}H]D-aspartate in the two groups was similar, the magnitude of currents associated with flux mediated by the mutant transporter was less than 5% of that seen in oocytes expressing the wild-type transporter (Fig. 1).

These data show that the mutant E404D transporter mediates uptake of radiolabeled D-aspartate, but they suggest that this uptake is occurring in an essentially electroneutral fashion at −30 mV. Uptake of excitatory amino acids by all glutamate transporter clones thus far characterized results in 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 (10, 25). The voltage dependence of the currents mediated by wild-type GLT-1 and the E404D mutant (GLT-1 E404D) has been examined by clamping oocytes, expressing the transporters, at potentials between +40 and −100 mV in the presence and absence of the transport substrate D-aspartate at 100 μM. In the wild-type GLT-1, inward currents are observed (Fig. 2*A*) that do not reverse (Fig. 2, *A* and *C*), similar to those observed in its human homolog (25). The behavior of E404D is quite different. In addition to the steady-state current, which is greatly diminished as compared with the wild-type, a relatively slow transient current is observed (Fig. 2*B*). Neither of these currents are observed in water-injected oocytes (13). The very small steady-state current of E404D does, however, reverse at potentials more positive than −20 mV (Fig. 2, *B* and *C*). The aspartate-dependent transient currents appear to be capacitative because the charge movements, following hyperpolarizing or depolarizing pulses, are equal to those following the return to the original potential (Fig. 2*B*). Although we will speculate on the nature of these transient currents in the "Discussion," it is important at this point to note that their magnitude indicates that the greatly reduced steady-state currents of E404D are not due to corresponding reduced expression levels of the mutant transporter. This is further supported by the fact that the much faster kainate-sensitive, sodium-dependent transients, which reflect sodium binding to the transporter or a subsequent conformational change (13), are similar for wild-type and mutant transporters (data not shown). The acidic amino acid-dependent outward current observed in glutamate transporters expressed in oocytes is largely due to a sodium- and glutamate-dependent anion conductance not thermodynamically coupled to the

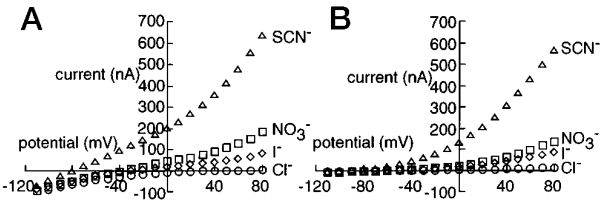

FIG. 3. **Anion selectivity of wild-type and E404D GLT-1 transporters.** Voltage dependence of steady-state currents induced by application of 300 μM D-aspartate to representative oocytes expressing wild-type GLT-1 (*A*) or E404D (*B*) transporters with bath solution containing normal Ringer's solution (Cl^-) and with Cl^- substituted by I^-, NO_3^-, or SCN^-.

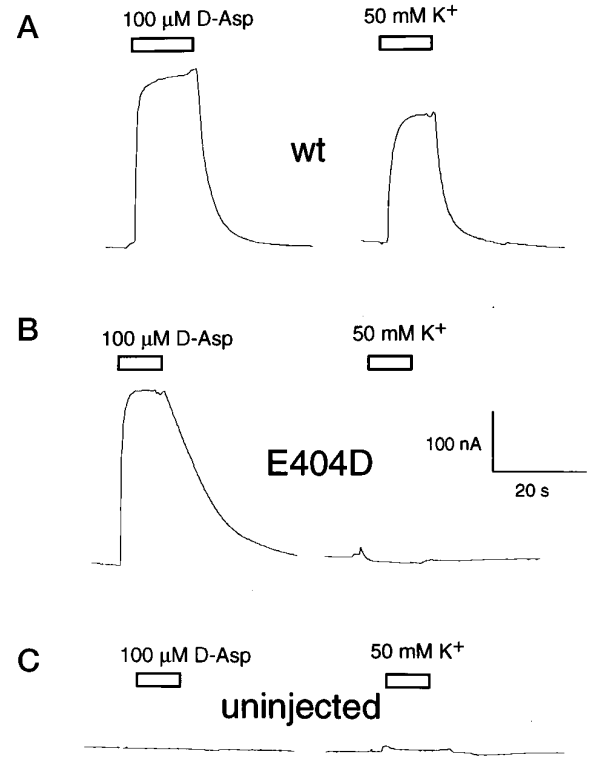

FIG. 4. **Anion conductance in wild-type and E404D transporters.** Anion currents activated by superfusion of 100 μM D-aspartate (influx conditions) or 50 mM K (efflux conditions) in oocytes expressing wild-type (*A*), E404D (*B*), or uninjected oocytes (*C*). Cells were voltage-clamped at 0 mV and perfused with Cl^--free Ringer's solution (NO_3^- substitution). The anion current in E404D was significantly smaller in the presence of Cl^- (5.2 ± 1.2% of magnitude in NO_3^-; $n = 4$).

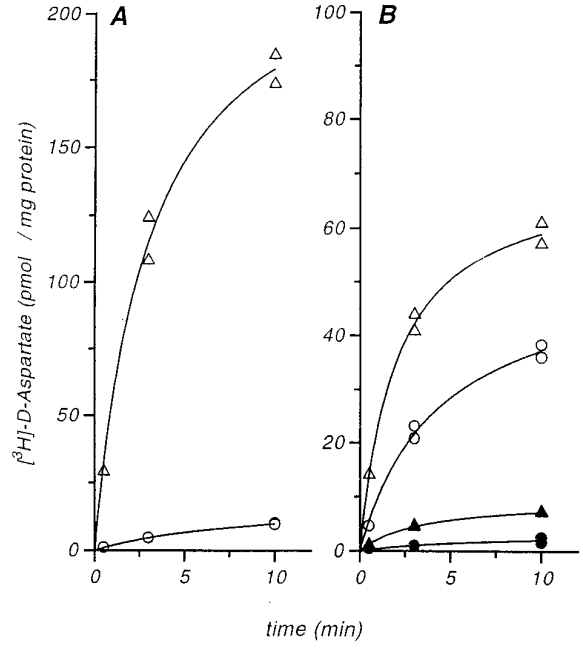

FIG. 5. **Uptake of [^{3}H]D-aspartate in proteoliposomes prepared from HeLa cells expressing wild-type and E404D.** Reconstitution of wild-type (*triangles*) and E404D (*circles*) transporters was performed as described under "Experimental Procedures" using 20 μl of proteoliposomes. The protein concentration in all cases was around 1 mg/ml; data are expressed as pmol/mg protein to normalize for the small variations in protein content resulting from individual reconstitutions. Reactions were stopped at the indicated times. *A,* net uptake, the "in"-medium contained 0.12 M KP_i, pH 6.8; *B,* exchange, the "in"-medium contained 0.12 M Tris P_i, pH 6.8 with (*open symbols*) or without (*closed symbols*) 10 mM L-aspartate; valinomycin (final concentration, 2.5 μM) was also present in the influx solutions. Its presence stimulates the initial rate of net influx 2–3-fold, due to the electrogenicity of the process, but has no effect on exchange.

transport cycle (25). The outward steady-state current in the E404D transporter (Fig. 2*C*) is largely carried by chloride ions moving into the oocyte because it is greatly attenuated when the external chloride concentration is reduced from 104 to 4.8 mM (Fig. 2*D*). Further support for this comes from the observations that much larger outward currents are observed when the external chloride is substituted by the highly permeant NO_3^- ion (Fig. 3).

The anion conductance activated during uptake shows a selectivity sequence favoring chaotropic anions (25, 26), and this selectivity sequence was unchanged in the E404D mutant (Fig. 3). Raising external potassium induces reverse transport (18, 27). This phenomenon is observed in *Xenopus* oocytes, which have approximately 12 mM intracellular substrate concentrations (28). The data presented suggest that the E404D transporter may be impaired in potassium binding. Evidence supporting this is presented in Fig. 4. Oocytes expressing the wild-type and E404D transporter have been clamped at 0 mV in Cl^--free Ringer's solution (substituted with nitrate). In the wild-type, superfusion with 100 μM D-aspartate induces the well characterized anion conductance of the transporter as measured by a large outward current, which is dependent on nitrate (Fig. 4*A*; this current is 20-fold lower in normal Ringer's solution; see also Fig. 2*C*). This anion conductance can also be induced by exposing the wild-type transporters to external potassium, which will cause the transporter to catalyze efflux of internal glutamate and/or aspartate (Fig. 4*A*). In the E404D transporters, this anion conductance can be activated by external aspartate but not by external potassium (Fig. 4*B*), indicating that potassium cannot interact with them.

E404D Function in Reconstituted Systems—The data from the previous sections indicate that uptake of [^{3}H]D-aspartate by the E404D transporters is due to its exchange with intracellular acidic amino acids but not to its net influx. To prove this, we have solubilized HeLa cells transiently expressing either wild-type or mutant transporters and reconstituted those transporters into liposomes. This system allows control of the composition of the external as well as the internal medium. Fig. 5*A* shows that potassium-loaded proteoliposomes inlaid with E404D transporters are virtually unable to accumulate [^{3}H]D-aspartate. The accumulation in the wild-type is completely dependent on the presence of external sodium (data not shown). This accumulation is dependent on internal potassium and is not observed when it is replaced by Tris (Fig. 5*B, closed triangles*). If, however, 10 mM L-aspartate is included in the internal medium together with Tris, significant accumulation of [^{3}H]D-aspartate is observed with wild-type and E404D transporters (Fig. 5*B*) but not with liposomes inlaid with extracts of

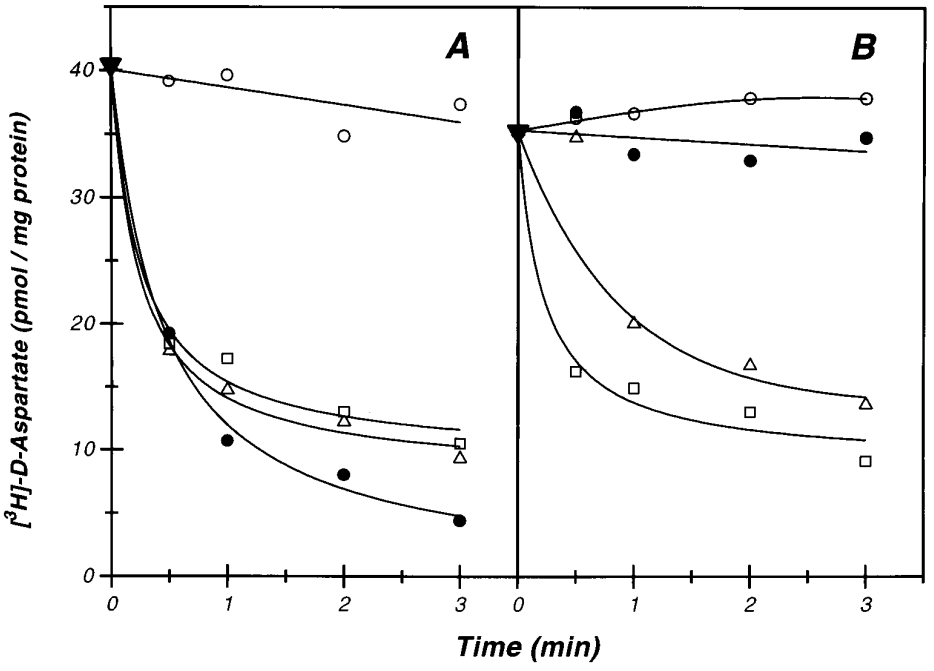

FIG. 6. **Efflux of [^{3}H]D-aspartate from wild-type or E404D proteoliposomes it via exchange.** For each time point 20 μl of proteoliposomes, containing 0.12 M NaP_i and 10 mM L-aspartate, were loaded with [^{3}H]D-aspartate by dilution into 80 μl of 0.15 M NaCl and 1 μCi of label. After 10 min, the mixture was diluted into 1 ml of 0.15 M KCl (●) or 0.15 M NaCl containing the following additions: none (○); 100 μM D-aspartate (□); or 100 μM L-glutamate (△). At the times indicated on the *abscissa,* the reactions were terminated. *A,* wild type; *B,* E404D.

cells transfected with the vector alone (data not shown). Under these conditions, the accumulation is absolutely dependent on external sodium; similar results are also obtained when L-glutamate is present on the inside and also when sodium is used instead of Tris (data not shown). These experiments prove that E404D proteoliposomes catalyze exchange but cannot catalyze net influx, which is dependent on internal potassium. The ability of external potassium to interact with the mutant proteoliposomes has been investigated after their preloading with [^{3}H]D-aspartate by exchange. After a 10-min accumulation of [^{3}H]D-aspartate into the proteoliposomes by exchange, they have been diluted 10-fold into media containing sodium chloride and 100 μM of unlabeled L-glutamate or D-aspartate (Fig. 6). The two substrates induce a rapid efflux of the previously accumulated [^{3}H]D-aspartate in both the wild-type (Fig. 6*A*) and the E404D transporter (Fig. 6*B*). Whereas dilution of the proteoliposomes inlaid with the wild-type transporter into sodium-containing media without anionic amino acids does not result in efflux, a rapid net efflux is obtained into a potassium-containing medium (Fig. 6*A*). However, no such net efflux is observed in the case of the mutant (Fig. 6*B*). Dilution into choline-containing media does not lead to efflux, neither in the wild-type nor in the mutant (data not shown).

To further emphasize the selectivity of the defect of potassium-dependent net flux in the mutant, we have compared another important parameter, *i.e.* affinity for external sodium. Initial rates of sodium-dependent [^{3}H]D-aspartate transport in HeLa cells expressing wild-type and mutant have a very similar dependence on the external sodium concentration (Fig. 7). Although in the experiment D-aspartate has been used at concentrations below its K_m, similar results are obtained when saturating levels (200 μM) are used (data not shown). An identical sodium dependence of [^{3}H]D-aspartate exchange has been observed in proteoliposomes inlaid with wild-type and E404D transporter (data not shown). Thus, E404D is selectively impaired in one or more of the interactions of potassium with the transporter.

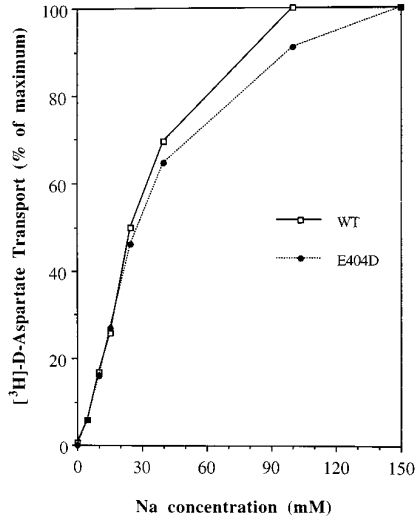

FIG. 7. **Sodium dependence of [^{3}H]D-aspartate uptake in HeLa cells expressing either wild-type or E404D transporters.** Transport was measured for 10 min using influx solutions containing the indicated NaCl concentrations, which were supplemented with Tris-HCl, pH 7.4, to a final concentration of 150 mM. Values are averages of triplicate determinations.

DISCUSSION

The transport cycle of glutamate, as it emerges from mechanistic studies (18, 19), is illustrated in Fig. 8. On the outside, the three sodium ions have to bind to the transporter first (step 1). This is followed by the binding of the acidic amino acid (glutamate in this example; step 2), followed by translocation (step 3). Then the acidic amino acid debinds (step 4), followed by the three sodium ions (step 5). This leaves the binding sites facing inward. To start a new cycle, the transporter has to reorient these sites, and for this purpose, potassium is required. After it binds from the inside (step 6), it is translocated (step 7) and released from the outside (step 8). In the case of net efflux, this cycle is reversed, going clockwise, instead of counterclockwise. Exchange represents a partial reaction of this cycle (steps 1–4 or 5 and back), including the reversible translocation of the acidic amino acid. Net flux is dependent on *cis*-sodium is external sodium is required for influx and internal sodium for efflux. Also exchange requires sodium, but it does not matter on which of the two sides it is present. Although there are some differences in the relative permeabilities

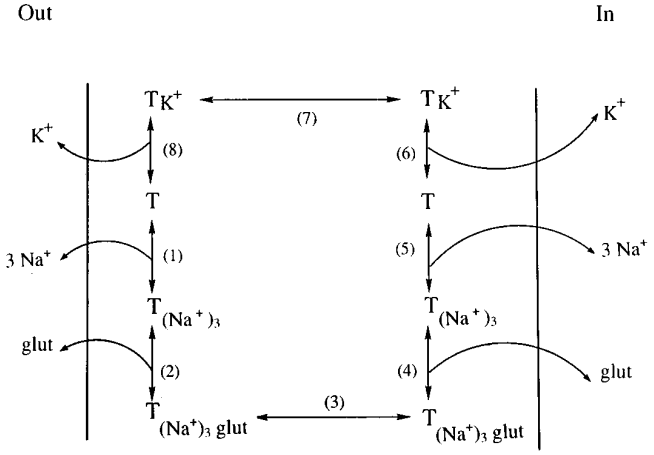

FIG. 8. **A model describing the transport cycle.** Details are given in the text. The place in the cycle where the pH changing species, presumably a proton, binds and debinds is not known and not indicated in this scheme. *glut,* glutamate which may be the anionic or zwitterionic species. Other transport substrates like D- or L-aspartate, bind as L-glutamate.

of glutamate and aspartate between E404D and wild-type (21), the dominant feature is that E404D cannot carry out the whole cycle (Fig. 5*A*) but can exchange (Figs. 5*B* and 6). These data are in harmony with the electrophysiological data, which indicate that E404D is "locked" in the exchange mode (Fig. 1). Besides the ability to translocate sodium and the acidic amino acid, E404D exhibits an unimpaired substrate-dependent anion conductance (Figs. 2–4) and a similar affinity to external sodium as the wild-type, both in intact cells (Fig. 7) as well as in proteoliposomes (data not shown). Thus, we are not dealing here with some structural mutation altering the conformation of the transporter, but a specific step is affected. Two possibilities come to mind: 1) potassium binding and/or translocation is impaired; and 2) the affinity for sodium is increased such that the rate of unbinding is dramatically decreased (step 5 for influx, step 1 for efflux). This latter possibility can be excluded because potassium-dependent efflux in E404D liposomes is defective (Fig. 6), whereas the affinity for external sodium is the same as for the wild-type in this preparation. In addition, no marked differences in affinity for internal sodium between wild-type and mutant proteoliposomes are observed (data not shown). Furthermore, whereas in intact cells the affinity of the mutant transporter for external sodium is identical to that of the wild-type (Fig. 7), the mutant transporter has lost the ability to interact with external potassium (Fig. 4). Therefore, our results indicate that potassium binding and/or translocation is impaired in the E404D mutant from the inside as well as from the outside, and that perhaps glutamate 404 is one of the liganding groups for potassium. It is especially striking that the removal of a single methylene group is sufficient to cause this defect. In view of this extremely high specificity, it is perhaps not surprising that other potassium congeners, *i.e.* rubidium, ammonium, and cesium, which can replace potassium in the wild-type to varying extents, cannot enable net flux in the mutant transporter (data not shown).

Voltage jumps in the presence and absence of external D-aspartate reveal that exchange mediated by the E404D mutant is accompanied by a marked transient current that decays with a time constant between 10 and 20 ms (Fig. 2). This transient current is significantly slower than the previously described sodium-dependent and kainate-sensitive transient current in EAAT2, the human homolog of GLT-1 (t = 3–4 msec; Ref. 13). This latter current is thought to reflect sodium binding and/or state transitions involving charge movements subsequent to sodium binding. Such sodium-dependent conformational changes have recently been monitored experimentally in GLT-1 (29). The slower transient currents associated with the exchange step may reflect reversible movement of sodium and glutamate across the electric field during this partial reaction cycle.

The E404D mutant mediates a sodium- and aspartate-dependent anion conductance (Fig. 2–4). The selectivity of this conductance is unchanged from the wild-type transporter ($SCN > NO_3 > I > Cl$; Fig. 3; see also Refs. 25 and 26). This result indicates that the anion-conducting state is likely to correspond to a sodium- and glutamate-occupied state (25), rather than a state in the potassium-transporting limb of the transport cycle (Fig. 8). A similar conclusion has been reached with independent experiments (17). The current activated by aspartate in E404D reverses close to the reversal potential for Cl, consistent with electroneutral exchange with little or no net electrogenic transport.

The residue E404D is conserved among all cloned mammalian glutamate transporters and is expected to fulfill a similar role in them. Mutation of the corresponding residue in EAAT3 (30), the human homolog of EAAC1 (9), also abolished potassium-dependent efflux.[1] Interestingly, this residue is not conserved in the related neutral amino acid transporter ASCT-1 (31, 32), and recent work shows that this transporter also mediates potassium-independent amino acid exchange (33). Glu_{404} is located in the middle of a structural domain that influences binding of the glutamate analogue kainate (34) and is also near residue Asp_{398}, which is required for transport (21). Amino acid sequences in this region of the glutamate transporters are highly conserved and contain multiple short hydrophobic segments (7–10). Pore-forming domains in both voltage-gated and ligand-gated ion channels tend to be highly conserved in these proteins (35) and often consist of relatively short loop structures (36). A similar role for the domain encompassing Glu_{404} in glutamate transporters is suggested both by its critical role in potassium permeation as well as its highly conserved structure.

Acknowledgments—We thank Dr. Bernard Moss for the provision of recombinant virus vTF-3 and Beryl Levene for expert secretarial help.

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Mutation of an Amino Acid Residue Influencing Potassium Coupling in the Glutamate Transporter GLT-1 Induces Obligate Exchange

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J. Biol. Chem. 1997, 272:1703-1708.
doi: 10.1074/jbc.272.3.1703
