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Molecular determinant of ion selectivity of a (Na^+ + K^+)-coupled rat brain glutamate transporter

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ABSTRACT **Glutamate transporters remove this neurotransmitter from the synaptic cleft by a two-stage electrogenic process, in which glutamate is first cotransported with three sodium ions and a proton. Subsequently, the cycle is completed by translocation of a potassium ion in the opposite direction. Recently, we have identified an amino acid residue of the glutamate transporter GLT-1 (Glu-404) that influences potassium coupling. We have now analyzed the effect of seven other amino acid residues in the highly conserved region surrounding this site. One of these residues, Tyr-403, also proved important for potassium coupling, because mutation to Phe (Y403F) resulted in an electroneutral obligate exchange mode of glutamate transport. This mutation in the transporter also caused an approximately 8-fold increase in the apparent sodium affinity, with no change in the apparent affinity for L-glutamate or D-aspartate. Strikingly, although exchange catalyzed by the wild-type transporter is strictly dependent on sodium, the selectivity of Y403F mutant transporters is altered so that sodium can be replaced by other alkaline metal cations including lithium and cesium. These results indicate the presence of interacting sites in or near the transporter pore that control selectivity for sodium and potassium.**

Glutamate transporters limit receptor activity by maintaining low synaptic concentrations of the transmitter (1–3) and by buffering synaptically released transmitters (4). Transport occurs by an electrogenic process (5–7) where the transmitter is cotransported with three sodium ions and a proton (8), followed by countertransport of a potassium ion (9–11). It has been postulated that potassium binds at a site previously occupied by one of the sodium ions (12). Despite the detailed mechanistic knowledge, little is known about the structural determinants of the translocation pathway.

A glutamate transporter has been purified to near homogeneity and reconstituted (13, 14). It represents around 0.6% of the protein of crude synaptosomal fractions and is one (15) of four glutamate transporters expressed in brain that have been cloned thus far (16–18). Recently, we identified a residue of the cloned glutamate transporter GLT-1 (15) important for potassium coupling (Glu-404) (11). This residue is located in the most conserved region of the glutamate transporter family. It is essential for potassium coupling (11), and the defect is very specific. Thus, transporters where this residue is mutated to Asp (E404D) exhibit a sodium affinity that is identical to that of the wild type (11). The E404D transporters are locked in an obligatory exchange mode. Apparently, they are not able to functionally interact with potassium, which normally interacts with the wild-type transporter after the sodium and acidic amino acid (AAA) have been transported and released (9–11).

In this paper, we describe studies to identify additional residues in the highly conserved stretch surrounding Glu-404 that are involved in potassium coupling. Only one, the neighboring residue, Tyr-403, exhibits this phenotype. However, unlike E404D, transporters in which the tyrosine is replaced by other aromatic amino acid residues (Y403F and Y403W) exhibit a marked increase in sodium affinity and a striking broadening of cation selectivity. These and other observations suggest that sodium- and potassium-binding sites of GLT-1 overlap.

EXPERIMENTAL PROCEDURES

Cell Growth and Expression. HeLa cells were cultured (19), infected with recombinant vaccinia/T7 virus vTF_{7-3} (20), and transfected with plasmid DNA encoding wild-type or mutant GLT-1, as described (19). Solubilization of transporters expressed in the HeLa cells, their reconstitution in proteoliposomes (11, 15), and transport measurements (11, 15) were done as described. Data presented are after subtracting the values obtained from cells transfected with the vector Bluescript SK^- alone. Exchange data are presented as net exchange after subtracting the values obtained on proteoliposomes not containing 10 mM L-aspartate but only 0.12 M Tris phosphate (pH 7.4). These values are negligible (see ref. 11). Protein was determined by Lowry's method (21).

Electrophysiology. Capped mRNAs encoding wild-type or mutant transporters were injected into state V–VI *Xenopus* oocytes (50 ng per oocyte) and currents were recorded by using a two-microelectrode voltage-clamp circuit (11). Recording solution (Ringer's solution) contained 100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM Hepes (pH 7.4). For recordings in the presence of NO_3^-, NaCl was partially substituted by $NaNO_3$ (20 mM).

Site-Directed Mutagenesis. Mutagenesis (22, 23) was done by using uracil-containing single-strand DNA derived from the shortened GLT-1 clone (24). After verification of the mutants by DNA sequencing, the mutations were subcloned into wild type by using either *Hin*dIII/*Asp*700/*Bst*EII (22) or *Bsr*GI/*Bst*EII. Subcloned DNAs were sequenced in both directions between the *Bst*EII site and the *Asp*700 or *Bsr*GI site.

RESULTS

The defect in potassium coupling in E404D-GLT-1 appears to be caused by defective potassium binding from either side of the membrane (11). This implies that other residues of GLT-1, close to E404D in space, also should be involved in binding the potassium. To identify such residues, we have mutated residues of GLT-1 located near Glu-404 in the primary sequence of the transporter. Fig. 1*A* shows the sodium-dependent uptake of the nonmetabolizable substrate D-[^{3}H]aspartate in HeLa cells,

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Abbreviation: AAA, acidic amino acid.
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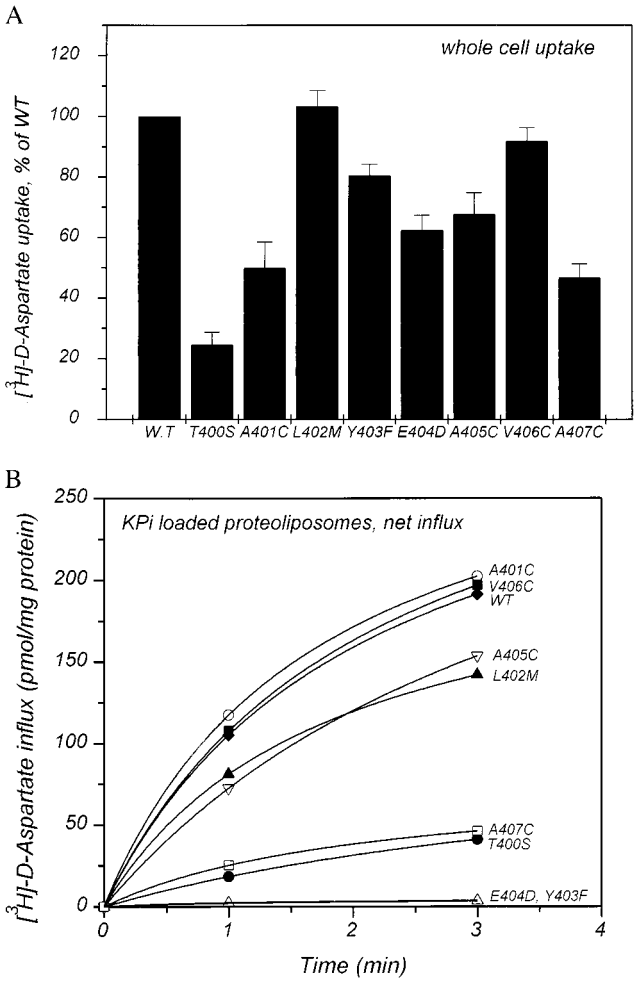

FIG. 1. (*A*) Sodium-dependent D-[^{3}H]aspartate uptake in HeLa cells expressing wild-type and mutant transporters. Values are the average ± SEM of triplicate determinations. (*B*) Sodium-dependent uptake of D-[^{3}H]aspartate in proteoliposomes prepared from HeLa cells expressing wild-type (◆) and mutant transporters. The following mutant GLT-1 transporters were tested: T400S, ●; A401C, ○; L402M, ▲; Y403F or E404D, △ (the same symbol is used for both because the values are superimposable); A405C, ▽; V406C, ■; A407C, □. The medium contained 0.12 M potassium phosphate (pH 7.4). The protein concentration was in all cases around 1 mg/ml; data are expressed as pmol/mg of protein to normalize for the small variations in protein content resulting from individual reconstitutions. Reactions were stopped at the indicated times.

expressing mutant transporters with replacements in the highly conserved residues 400–407. With the exception of T400S, the mutants exhibit uptake levels of at least 50% of that of the wild type (Fig. 1*A*). The Thr at position 400 is important for expression of glutamate transport, because only residual activity is observed when it is replaced by Ser (Fig. 1*A*) and none whatsoever is observed when it is replaced with Ala, Cys, or Asn (data not shown).

E404D-GLT-1 transporters are locked in an obligatory exchange mode (11). To determine whether the uptake in the intact cells of the mutants at positions 400–407 represents exchange with the endogenous AAA or trans-potassium-dependent net flux, the transporters from these cells were solubilized and reconstituted. Similar to the wild type (11), in the majority of the mutant transporters sodium-dependent net flux of the radiolabeled aspartate is observed after their reconstitution into potassium-loaded proteoliposomes (Fig. 1*B*). Only the transporters mutated at position 403, Y403F-GLT-1, are defective in this activity (Fig. 1*B*) just as E404D-GLT-1 (Fig. 1*B*, ref. 11). The same is true for net flux of L-[^{3}H]glutamate (data not shown). It should be noted that with most other mutants, including T400S, the activity is more or less proportional to what is observed in the intact cells (Fig. 1 *A* and *B*). Thus the defect in T400S-GLT-1 is not caused by a defective interaction with potassium. The net flux by A407C-GLT-1 in the reconstituted system is low relative to the uptake in the whole cells, but this case is less extreme than Y403F-GLT-1, which is totally defective in net flux (Fig. 1 *A* and *B*).

In reconstituted proteoliposomes, no sodium-dependent uptake is observed with the wild type when trans-potassium is replaced by other cations such as Tris (11) and also is not observed in Y403F-GLT-1 (data not shown). However, if potassium-free proteoliposomes inlaid with the wild type contain in addition internal L-aspartate (or other transportable AAA) exchange takes place and substantial D-[^{3}H]aspartate is accumulated in a sodium-dependent fashion (11). The Y403F-GLT-1 transporters similarly catalyze this exchange (Fig. 2, zero time and also see Fig. 5). These results indicate that, just like E404D-GLT-1, Y403F-GLT-1 is also locked in the exchange mode and is unable to catalyze net uptake. Thus it does not interact with internal potassium (Fig. 1*B*). It also cannot interact with external potassium. The latter promotes a net efflux of internal D-[^{3}H]aspartate from the wild type but not in the mutant (Fig. 2). In these experiments D-[^{3}H]aspartate was accumulated before the efflux by exchange into proteoliposomes containing sodium ions and unlabeled L-aspartate. Net efflux from mutant liposomes was also not observed by using other cations in the external solution, namely, rubidium, cesium, and lithium (data not shown). The only way to deplete the Y403F-GLT-1 liposomes from the D-[^{3}H]aspartate (previously accumulated by exchange) is by back exchange herein demonstrated with external glutamate (Fig. 2).

Fig. 3 provides electrophysiological evidence that Y403F transporters are locked in the exchange mode. In standard sodium and chloride containing medium, glutamate induces a slow capacitative current and a relatively small steady-state current in voltage-clamped oocytes expressing Y403F transporters (Fig. 3*A*). These currents are reminiscent of those observed in E404D (11), and neither is observed in water-injected oocytes (7). The small steady-state inward current of Y403F becomes outward at potentials more positive than −30

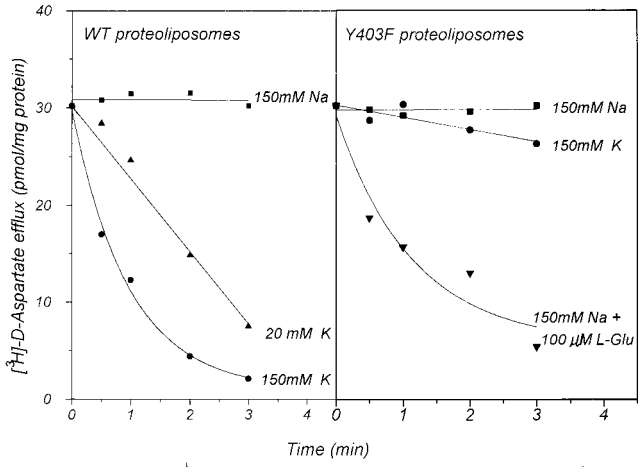

FIG. 2. Efflux of D-[^{3}H]aspartate from wild type (*Left*) or Y403F proteoliposomes (*Right*) in which it was previously accumulated by exchange. For each time, 20 μl of proteoliposomes containing 0.12 M sodium phosphate and 10 mM L-aspartate was loaded with D-[^{3}H]aspartate by dilution into 80 μl of 0.15 M NaCl and 1 μCi of labeled aspartate (1 Ci = 37 GBq). After 10 min, the mixture was diluted into 1 ml of 0.15 M NaCl (■), 20 mM KCl/130 mM NaCl (▲), 150 mM KCl (●), or 0.15 M NaCl/100 M L-glutamate (▼). Reactions were terminated at the times indicated at the abscissa.

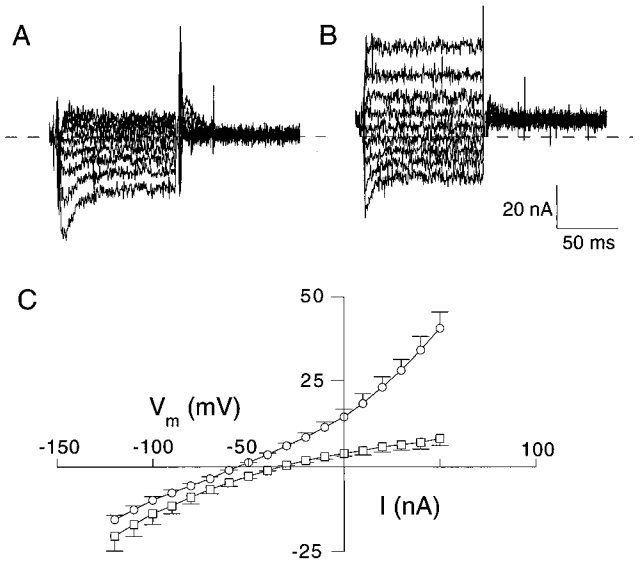

FIG. 3. (*A*) Difference records obtained by subtraction of currents recorded in the absence of glutamate from records in the presence of 100 μM L-glutamate in oocytes expressing the mutant Y403F transporter. Pulses are 100 ms in duration in 20-mV increments to potentials between −120 mV and +40 mV from a holding potential of −30 mV. Dashed line shows zero current. (*B*) Same protocol and oocyte as in *A* showing shift in the reversal potential from −30 mV (E_{Cl}) to approximately −60 mV after substitution of 20 mM Cl with NO_3. (*C*) Summary of voltage dependence of glutamate-induced currents (mean ± SEM, $n = 4$) recorded in 100 mM NaCl (squares) or 80 mM NaCl/20 mM $NaNO_3$.

mV—the electrochemical equilibrium potential of chloride. In contrast, wild-type GLT-1 exhibits much larger steady-state currents that do not reverse up to +40 mV (11). Uptake of excitatory amino acids by all glutamate transporter clones characterized thus far—including wild-type GLT-1—results in activation of a current reflecting the sum of the transport current, which is inward at all potentials (resulting from net sodium-coupled glutamate influx), with a chloride current flowing through a thermodynamically uncoupled pathway (11, 18, 25). At the equilibrium potential of chloride, wild-type GLT-1 still exhibits the transport current (11), but Y403F transporters do not exhibit it (Fig. 3 *A* and *C*). This is to be predicted if Y403F transporters can only carry out exchange but not net flux. That the currents observed with Y403F are caused by anion flux is further illustrated by the fact that substitution of part of the external chloride by the much more permeant nitrate (20 mM) results in a shift of the reversal potential to approximately −60 mV and much larger outward currents are observed (Fig. 3 *B* and *C*). This is to be predicted by the well-characterized selectivity sequence of the anion conductance activated during uptake mediated by cloned transporters favoring chaotropic anions (25, 26).

In striking contrast with E404D (11), Y403F-GLT-1 exhibits a highly increased sodium affinity—8-fold in the experiment shown (Fig. 4). Similar results also are obtained in voltage-clamped oocytes (data not shown). The $K_{0.5}$ for sodium—the concentration giving rise to a 50% activation of transport—is 23.8 ± 3.0 mM (mean ± SEM, $n = 4$) for the wild type and 3.7 ± 0.7 mM ($n = 3$) for Y403F. The dose–response curve for the mutant is characterized by an apparent inhibitory phase after the maximum activation. It is possible that this also could occur in the wild type, but because of its lower affinity, it would require hyperosmotic conditions to become apparent. If we determine $K_{0.5}$ for sodium relative to the value at 150 mM, an even lower value is obtained for Y403F—2.7 ± 0.6 mM ($n = 3$). The cooperativity with regard to sodium is essentially unchanged in the mutant; the Hill coefficients for wild type

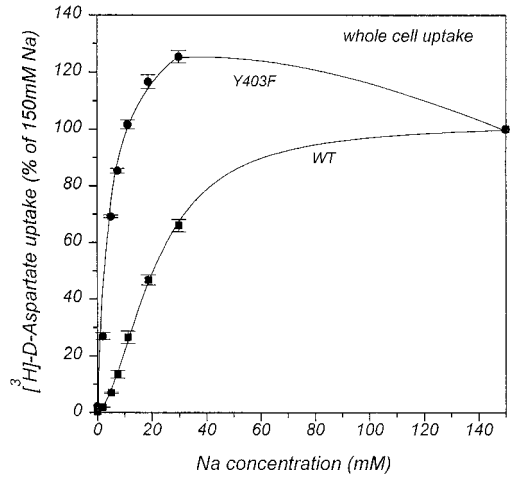

FIG. 4. Sodium dependence of D-[^{3}H]aspartate uptake in HeLa cells expressing wild-type (■) or Y403F-GLT-1 (●) transporters. Transport was measured by using influx solutions containing the indicated NaCl concentrations that were supplemented with choline chloride to a final concentration of 150 mM. Values are the averages ± SEM of triplicate determinations.

and Y_{403}F-GLT-1 are 1.80 ± 0.04 ($n = 4$) and 1.91 ± 0.05 ($n = 3$), respectively. This increased sodium affinity is not only observed in intact HeLa cells expressing the mutant transporters but also in reconstituted proteoliposomes inlaid with them (data not shown).

The impaired potassium binding and increased sodium affinity suggested to us that perhaps the binding sites for sodium and potassium interact closely. This raises the possibility that mutations at Tyr-403 may influence ion selectivity at the sodium binding site. We have tested this idea by monitoring the ion specificity of exchange by Y403F transporters under conditions where this process is completely dependent on external sodium in the wild type. With wild-type glutamate transporters, exchange will proceed only when sodium is present on at least one side of the membrane (9, 10). In the experiment depicted in Fig. 5, exchange of D-[^{3}H]aspartate is measured into liposomes inlaid with either wild type or Y_{403}F-GLT-1 that are loaded with internal Tris and L-aspartate. Indeed, under these conditions the exchange of D-[^{3}H]aspartate into liposomes inlaid with wild-type GLT-1 is completely dependent on external sodium (Figs. 5 *Left*). Strikingly, lithium but not choline supports significant exchange in sodium-free Y403F-GLT-1 liposomes (Fig. 5*A Right*). Its extent amounts to 33.8 ± 3.5% ($n = 4$) of that in sodium. Thus, unlike in the wild type, in the mutant, sodium ions can be replaced by lithium to form the translocation complex (9–11). The lack of exchange in choline indicates that not only this cation but also protons cannot replace the sodium or lithium in the mutant. Consistent with this is the failure to observe exchange at pH 5.8 (by using Bis-Tris medium, data not shown). Of the other cations also tested, cesium is able to replace sodium in the Y403F mutant transporters but not in the wild type (Fig. 5*B Right*). The extent is 60.1 ± 6.4% of that in sodium ($n = 3$). On the other hand ammonium and potassium are not able to do so (Fig. 5*B*).

Although we have noted three functional changes in the Y403F mutant—inability to interact with potassium, increased sodium affinity, and a broadening of the specificity for the sodium site—it is important to note that the overall conformation of the transporter has not changed because of the mutation. Not only does the glutamate-induced anion conductance appear unaltered (Fig. 3) but also the apparent K_m and V_{max} for D-aspartate and L-glutamate are not significantly changed. The apparent K_m values for D-aspartate exchange for wild type and Y403F transporters are 5.1 ± 0.4 and 5.6 ± 0.9

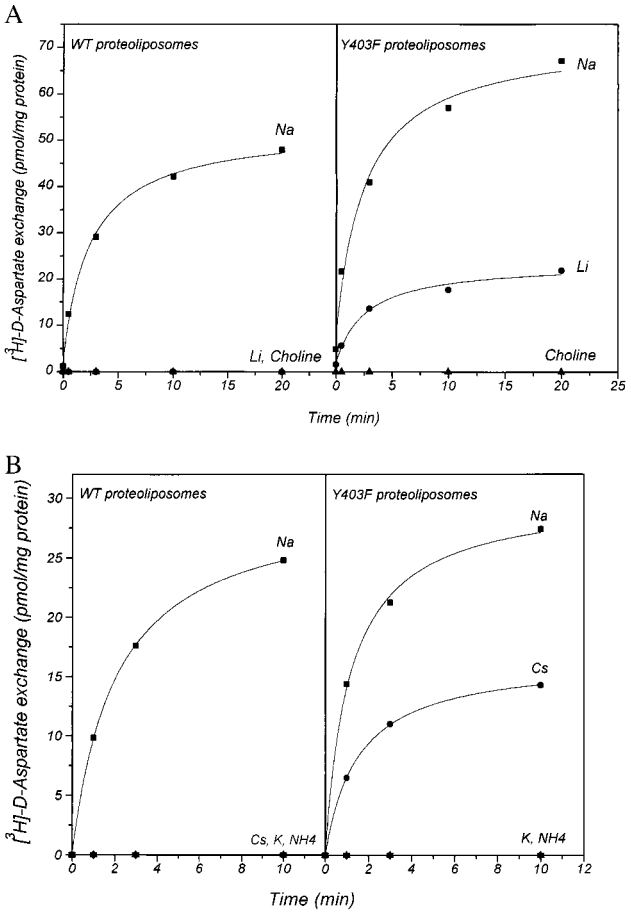

FIG. 5. (*A*) Exchange of D-[^{3}H]aspartate in proteoliposomes prepared from HeLa cells expressing wild-type (WT) (*Left*) or Y403F (*Right*) transporters. The medium contained 120 mM Tris phosphate (pH 7.4) with and without 10 mM L-aspartate. Trans-L-aspartate-dependent exchange is shown in external medium containing 150 mM NaCl, 150 mM LiCl, or 150 mM choline chloride as indicated. (*B*) Ion specificity of exchange of D-[^{3}H]aspartate in wild-type or Y403F transporters. Conditions are as in *A*, where the chloride salts of the indicated cations at 150 mM are added to the external medium.

μM (mean ± SEM, $n = 4$), respectively. For L-glutamate, the corresponding values are 6.4 ± 1.4 and 7.3 ± 1.2 μM ($n = 3$).

To better understand the role of Tyr-403 in the interaction of the transporter with potassium and sodium, we have mutated it to a nonaromatic (Cys, $Y_{403}C$) or to the other aromatic (Trp, $Y_{403}W$) residue. Neither of these mutant transporters is able to carry out net flux of D-[^{3}H]aspartate into reconstituted—potassium-loaded—proteoliposomes (Fig. 6*A*). Nevertheless, intact HeLa cells expressing these mutants are able to take up this AAA (Fig. 6*B*), strongly suggesting that this is caused by exchange with endogenous AAA. This is in fact what happens as shown by using aspartate-loaded proteoliposomes inlaid with the mutant transporters (Fig. 6*C*). Interestingly, the Y403W transporter shows both increased sodium affinity (Fig. 6*B*)—$K_{0.5}$ for sodium 3.8 ± 0.2 mM ($n = 3$) and broadened ion selectivity (Fig. 6*C Left*) just like Y403F (Figs. 4 and 5). The extents in lithium and cesium are 38.6 ± 8.2% ($n = 3$) and 59.5 ± 9.5% ($n = 3$) of that in sodium. On the other hand Y403C transporters neither exhibit the increased sodium affinity—$K_{0.5}$ for sodium 28.3 ± 6.0 mM ($n = 3$)—nor the broadened ion selectivity. Neither of the mutants showed a marked change in the Hill coefficient for sodium, 1.88 ± 0.05 ($n = 3$) and 1.72 ± 0.05 ($n = 3$) for Y403W and Y403C, respectively. These observations strongly suggest that the changes in sodium affinity and selectivity are closely linked.

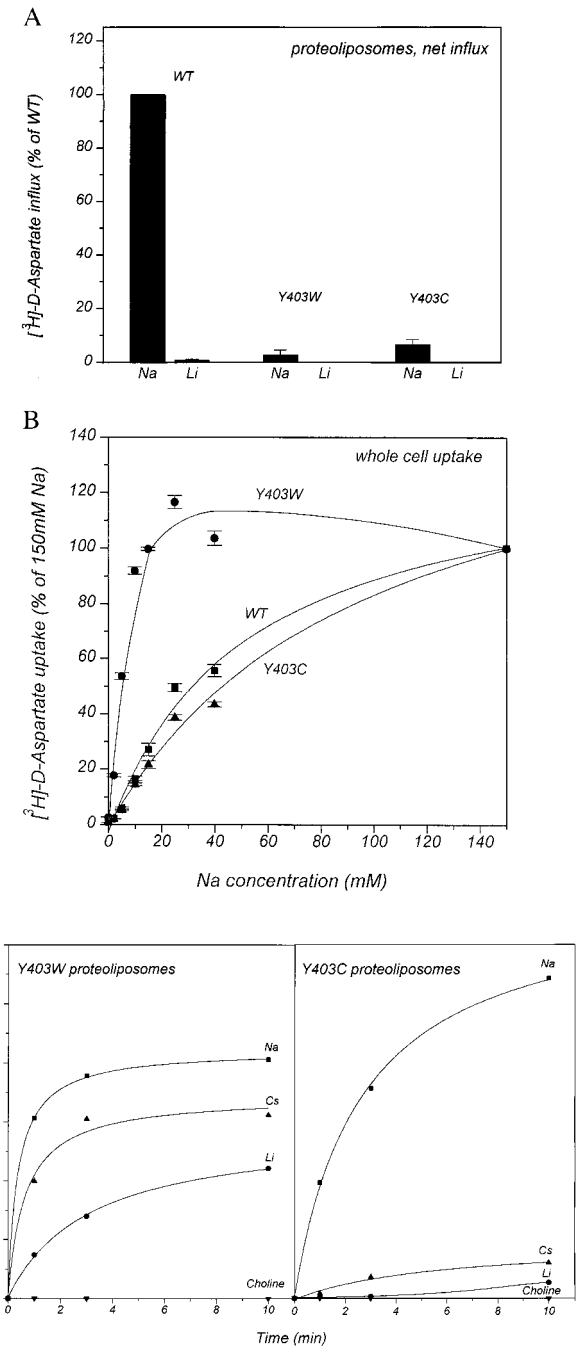

FIG. 6. (*A*) Net sodium-dependent D-[^{3}H]aspartate influx into potassium-loaded proteoliposomes of wild-type, Y403W, and Y403C transporters. The proteoliposomes were loaded with 0.12 M potassium phosphate, and transport values after 10 min (mean ± SEM, $n = 3$ determinations) are given. (*B*) Sodium dependence of D-[^{3}H]aspartate uptake in HeLa cells expressing wild-type (■), Y403C (▲), and Y403W (●) transporters. Conditions were as in Fig. 4. (*C*) Ion specificity of D-[^{3}H]aspartate exchange in Y403W and Y403C transporters. Experimental conditions as in Fig. 5.

DISCUSSION

The fact that all of the mutants at position 403 lose their ability to interact with potassium—regardless of the nature of the side chain inserted—strongly suggests that Tyr-403 is part of the potassium binding site, just like Glu-404 (11). In contrast to the effect on potassium, the nature of the side chain of the substituted amino acid residue at position 403 determines whether both sodium affinity and selectivity are affected. This and the fact that E404D-GLT-1 and the wild type have the

identical sodium affinity (11) indicate that sodium and potassium binding sites are not identical, in contrast to a previous suggestion (12). Our results, however, indicate that the sodium-selective site is located sufficiently close to the potassium site so that it can sense the change at Tyr-403, at least when bulky aromatic residues replace it.

During a single turnover of the glutamate transporter, three sodium ions are translocated (8). Single mutations at position 403 alter the sodium selectivity (Figs. 5 and 6*C*). Does this mean that all three sodium ions interact with the same binding site? This is not necessarily so, as our previous observations suggest the presence of nonidentical multiple sites, some of which may bind lithium (27). It is possible that in the wild type only one out of the three sites is strictly sodium-selective and cannot bind lithium. The aromatic substitutions of Tyr-403 could render this specific sodium site less specific.

It is of interest to note that the analogous tyrosine (Tyr-405) from the related transporter GLAST-1 (16) has been implicated in the binding of the δ-carboxyl group of glutamate (28). This interpretation is hard to reconcile with the lack of effect on AAA affinity in the Y403F mutant that we have observed. The lack of currents observed with Y405F-GLAST-1 (28) is in harmony with our conclusion that the transporter is locked in the exchange mode. The very low radioactive uptake observed in this mutant (28) could be caused by a low expression in the oocytes relative to the expression system used herein. Nevertheless, we have introduced the Y405F mutation in GLAST-1 (provided by W. Stoffel, University of Cologne, Germany) and the behavior of Y405F-GLAST-1 was undistinguishable from that of Y403F-GLT-1 (data not shown).

The close relation of potassium and sodium binding tempts us to speculate on the location of the sodium binding site. This could be close to positions 403 and 404 in the primary structure, but alternatively, it could be located in a completely different stretch of amino acid residues that could be close to the potassium-binding residues in space. The latter is difficult to assess at present as high-resolution structural information is lacking. Several lines of circumstantial evidence indicate that the first possibility may be correct and that part of it may be formed by residues occupying positions 396–400. (*i*) No sodium binding transient currents were observed with transporters mutated at the absolutely essential Asp-398 (22) and the inactive mutant N396Q in which Asn-396 was replaced by Gln (data not shown). (*ii*) Cys scanning mutagenesis indicates that each of the five residues 396–400 when replaced by Cys gives rise to inactive transporters (R.Z., M. Grunewald, M.P.K., and B.I.K., unpublished observations). (*iii*) The T400S and A401G mutants exhibit a greatly impaired sodium affinity (data not shown).

With respect to the potassium site itself, it would appear that there should be other residues that may ligand potassium in addition to Tyr-403 and Asp-404. One residue that at least influences interaction of the transporter with potassium is Ala-407 (Fig. 1). Although the secondary structure of this region is not known, it is of interest to note that if it were α-helical, position 407 would be precisely one turn away from positions 403 and 404 and thus very close to them. In view of the close relationship between sodium and potassium sites suggested by our study, it is possible that some of the residues between positions 396 and 400 may be involved in both sodium and potassium binding. This would not be revealed with the assays currently at our disposal. Alternatively, additional residues forming the potassium site may be located in other parts of the primary structure and may be revealed by subjecting other highly conserved regions to the type of analysis outlined herein.

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