Identification of a Lithium Interaction Site in the γ-Aminobutyric Acid (GABA) Transporter GAT-1*

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Yonggang Zhou, Elia Zomot, and Baruch I. Kanner

From the Department of Biochemistry, Hebrew University Hadassah Medical School, P. O. Box 12272, Jerusalem 91120, Israel

The sodium- and chloride-dependent electrogenic γ-aminobutyric acid (GABA) transporter GAT-1, which transports two sodium ions together with GABA, is essential for synaptic transmission by this neurotransmitter. Although lithium by itself does not support GABA transport, it has been proposed that lithium can replace sodium at one of the binding sites but not at the other. To identify putative lithium selectivity determinants, we have mutated the five GAT-1 residues corresponding to those whose side chains participate in the sodium binding sites Na1 and Na2 of the bacterial leucine-transporting homologue LeuT_Aa. In GAT-1 and in most other neurotransmitter transporter family members, four of these residues are conserved, but aspartate 395 replaces the Na2 residue threonine 354. At varying extracellular sodium, lithium stimulated sodium-dependent transport currents as well as [3H]GABA uptake in wild type GAT-1. The extent of this stimulation was dependent on the GABA concentration. In mutants in which aspartate 395 was replaced by threonine or serine, the stimulation of transport by lithium was abolished. Moreover, these mutants were unable to mediate the lithium leak currents. This phenotype was not observed in mutants at the four other positions, although their transport properties were severely impacted. Thus at saturating GABA, the site corresponding to Na2 behaves as a low affinity sodium binding site where lithium can replace sodium. We propose that GABA participates in the other sodium binding site, just like leucine does in the Na1 site, and that at limiting GABA, this site determines the apparent sodium affinity of GABA transport.

Many neurotransmitters are removed from the synaptic cleft by transporters, which thereby enable efficient synaptic transmission. Many of these transporters are sodium- and chloride-dependent and form a family which, besides the transporters for γ-aminobutyric acid (GABA), also includes those for serotonin, dopamine, norepinephrine, and glycine (for reviews, see Refs. 1 and 2). The GABA transporter GAT-1 (3, 4) is the first identified member of this family, which catalyzes electrogenic sodium:chloride:GABA cotransport with a stoichiometry of 2:1:1 (5–8). The role of chloride in this process is still under debate because it has been suggested that during sodium-coupled GABA transport, obligatory chloride out/chloride in exchange takes place (9). In contrast to sodium, lithium cannot support transport of [3H]GABA (10, 11), but it can stimulate GABA-induced currents at reduced sodium concentrations, suggesting that during transport, one of the two sodium ions can be replaced by lithium (11). In the absence of GABA, leak currents are observed when lithium replaces sodium. These leak currents have a much steeper voltage dependence than the GABA-induced transport currents observed in the presence of sodium (12, 13). These lithium leak currents are inhibited by low concentrations of sodium ions, and this inhibition is not competitive. These observations indicate that when sodium binds to GAT-1, the leak mode is converted into a conformation that enables coupled transport (11, 14).

Recently, a high-resolution crystal structure from a bacterial family member of the Na+/Cl-–dependent neurotransmitter transporters, the leucine transporter LeuT_Aa, was determined (15). The transporter consists of 12 transmembrane domains (TMs), with TMs 1–5 related to TMs 6–10 by a pseudo-2-fold axis in the membrane plane. Two sodium ions, Na1 and Na2, and a leucine molecule are occluded in this structure (15). Although the overall sequence identity between the eukaryotic and procaryotic counterparts is only 20–25%, there are clusters of high sequence conservation. These are distributed throughout the primary structure and include functionally important residues (reviewed in Ref. 15). Therefore, it is possible that the published structure may be a useful model to study the neurotransmitter transporter members of this family.

In this study, we have investigated the possibility that one or more amino acid residues, which correspond to those that coordinate Na1 and Na2 in LeuT_Aa, participate in the interaction of lithium with GAT-1. This would provide an important initial clue toward understanding the structural basis of cation selectivity in neurotransmitter transporters. As a parent construct, we have used GAT-1 with cysteine 74 mutated to alanine (C74A). Transport by this mutant, which has similar properties as wild type GAT-1, is not inhibited by membrane-impermeant sulfhydryl residues (14, 16). Our results provide evidence for the idea that the architecture of the sodium binding sites in GAT-1 is similar, but not identical, to that of LeuT_Aa. The sodium binding site of GAT-1 that corresponds to Na1 is highly specific, whereas the ability of lithium to substitute for sodium at the other sodium binding site and to mediate leak currents by the transporter is controlled by aspartate 395.

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1 To whom correspondence should be addressed. Tel.: 972-2-6758506; Fax: 972-2-6757379; E-mail: kannerb@cc.huji.ac.il.

2 The abbreviations used are: GABA, γ-aminobutyric acid; TM, transmembrane domain; MTS, methanethiosulfonate; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate; MTSES, (2-sulfonatoethyl) [methanethiosulfonate.

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EXPERIMENTAL PROCEDURES

Generation and Subcloning of Mutants—Mutations were made by site-directed mutagenesis of the wild type GAT-1 in the vector pBluescript SK(−) (Stratagene) according to the Kunkel method as described (17, 18). Briefly, the parent DNA was used to transform Escherichia coli CJ236 (dut−, ung−). From one of the transformants, single-stranded uracil-containing DNA was isolated upon growth in uridine-containing medium according to the standard protocol from Stratagene using helper phage R408. This yields the sense strand, and consequently, mutagenic primers were designed to be antisense. Mutants were subcloned into a construct containing C74A-GAT-1 in the pOG1 vector using two unique restriction enzymes. The pOG1 vector is an oocyte expression vector that contains a 5′-untranslated Xenopus β-globin sequence, the T7 RNA promoter, and a 3′-poly(A) signal. The coding and non-coding strands were sequenced between the two restriction sites.

Cell Growth and Expression—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 200 units/ml penicillin, 200 μg/ml streptomycin, and 2 mM glutamine. Infection with recombinant vaccinia/T7 virus vTF7-3 (19) and subsequent transfection with plasmid DNA, as well as [3H]GABA transport, was done as published previously (20). Effects of sulfhydryl reagents on transport were studied as described (16).

cRNA Transcription, Injection, and Oocyte Preparation—Capped run-off cRNA transcripts were made from transporter constructs in pOG1, linearized with SacII, using mMessage mMachine (Ambion). Oocytes were removed from anesthetized Xenopus laevis frogs and treated with collagenase (type 1A; Sigma number C-9891) until capillaries were absent and injected with 50 nl of undiluted cRNA on the same or the next day. Oocytes were maintained at 18 °C in modified Barth’s saline containing the following (in mM): 88 NaCl, 1 KCl, 1 MgSO4, 2.4 NaHCO3, 1 CaCl2, 0.3 Ca(NO3)2, 10 HEPES, pH 7.5, with freshly added 2 mM sodium pyruvic acid and 0.5 mM theophylline, and supplemented with 10,000 units/liter penicillin, 10 mg/liter streptomycin, and 50 mg/liter gentamicin.

Oocyte Electrophysiology—This was done as described (14). Oocytes were placed in the recording chamber, penetrated with two micropipettes (backfilled with 2 M KCl, resistance varied between 0.5 and 2 megaohms), and voltage-clamped using a GeneClamp 500 amplifier (Axon Instruments) and digitized using a Digidata 1200A (Axon Instruments), both controlled with the pClamp6 suite (Axon Instruments). Currents were acquired with Clampex 6.03 at 10 kHz every 0.5 ms and low pass-filtered online. The membrane potential of the oocytes was jumped from −140 mV to +60 mV in 25-mV increments, using −25 mV as holding potential, unless stated otherwise in the figure legends. Each potential was held clamped for 500 ms. The membrane potential was measured relative to an extracellular Ag/AgCl electrode in the recording chamber. Recording solution contained 96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, and 5 mM Hepes, pH 7.4. In substitution experiments, sodium ions were replaced with equimolar choline or lithium (as indicated in the figure legends). Figures were prepared using Origin 6.1 (Microcal). The GABA-induced currents are defined as the currents in the absence of GABA subtracted from those in its presence (at the concentrations indicated in the figure legends). In those experiments, in which the effect of the impermeant sulfhydryl reagents MTSET or MTSES was analyzed, this was done exactly as described (21).

Homology Modeling—Figures were prepared using the DeepView Swiss-PDB viewer downloaded from the ExPasy Proteomics Server. Homology modeling of the Na2 binding site of GAT-1 based on the LeuTa structure (accession code 2A65) was performed using the Tripos Sybyl7.0 computational software. Initially, modeling of the Na2 binding pocket was carried out for amino acid residues located within 6 Å from the sodium ion for greater precision, and afterward, energy minimization of the wild type and D395T were carried out for amino acid residues within a distance of 4 Å from the Na2 ion and compared. Energy computation and minimization were performed using the Powell method with Simplex initial optimization, Tripos force field, Gasteiger-Huckel charges, and a constant dielectric function.

RESULTS

Ion Dependence of GABA Transport by Putative Na2 Site Mutants—The GABA (1 mM)-induced transport currents by oocytes expressing C74A at various sodium concentrations are shown in Fig. 1A. When lithium was the substituting ion, the GABA-induced currents reached saturation at lower Na+ concentrations than when choline replaced sodium. In the presence of lithium, the transport currents at 2 mM sodium were approximately half of those at 96 mM, whereas this value increased to about 18 mM in its absence (Fig. 1A). Although the results shown are for currents recorded at −140 mV, similar results were also obtained at the other potentials analyzed, −40 and −90 mV, and similar results were also obtained with wild type GAT-1 (data not shown). In the LeuTa structure, Na2 is coordinated by three main chain carbonyl oxygens and the hydroxyl oxygens from the TM8 residues threonine 354 and serine 355 (15). The equivalents of these latter two residues in GAT-1 are aspartate 395 and serine 396, respectively. Significant transport currents were observed by the mutants in which aspartate 395 was replaced by serine or threonine, D395S or D395T, respectively. However, with the D395S/C74A and D395T/C74A mutants, no stimulation of the transport currents by lithium was observed (Fig. 1, A–C). Importantly, the sodium concentration dependence of the GABA-induced currents by these mutants in the presence of either choline or lithium was almost the same as that for C74A in the presence of choline (Fig. 1, A–C). Similar results were also observed when these mutants were in the background of wild type GAT-1 (data not shown). In the case of D395C, a very minor stimulation by lithium was observed (Fig. 1, A–C). For this mutant, GABA was used at 10 mM rather than at 1 mM because its Km at −140 mV and 96 mM sodium was ~400 μM, which is ~2.5-fold of that in D395S/C74A and D395T/C74A and ~8-fold that of C74A (Table 1). However, the value of Km is also dependent on the sodium concentration (22). At 20 mM sodium, the Km for GABA in the D395S/C74A mutant was 478 ± 40 μM. Therefore, we also analyzed the cur-
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Currents induced by 10 mM GABA in the D395S/C74A and D395T/C74A mutants, and the results were similar to those depicted in Fig. 1, B and C (data not shown). Transport currents by the D395C/C74A mutant were not affected by preincubation of the oocytes with the membrane-impermeant sulphydryl reagents MTSET or MTSES (data not shown). The D395E mutant was inactive (data not shown), but the D395N/C74A mutant exhibited GABA-induced transport currents, which were also not stimulated by lithium (Fig. 1E).

In contrast to the transport currents induced by 1 mM GABA, when transport of [3H]GABA was measured in HeLa cells expressing C74A (Fig. 2A), the stimulation by lithium was barely visible. This is in agreement with previous studies (10). A major difference between the experiments depicted in Figs. IA and 2A is that, besides the expression system, the actual GABA concentration in the latter is 50 nM, ~ 2 orders of magnitude below the $K_m$ for radioactive uptake at 150 mM sodium (Table 1). Indeed, when the GABA concentration was increased to 20 $\mu$M, a clear stimulation by lithium was observed (Fig. 2B). When transport of [3H]GABA was measured under the same conditions in HeLa cells expressing D395S/C74A or D395T/C74A, the stimulation by lithium was not observed (Fig. 2, C and D). Again, similar results were obtained when wild type GAT-1 was used as a background (data not shown). In contrast to transport currents, uptake of [3H]GABA could not be observed by the D395C/C74A mutant.

GABA-induced currents could be measured with oocytes expressing the S396T/C74A and S396C/C74A mutants (Fig. 3). In the case of S396T/C74A, the voltage dependence of these currents was similar to that of the GABA-induced currents by C74A shown in Fig. 4 (data not shown), indicating coupled transport. On the other hand, the voltage dependence of the GABA-induced currents by S396C/C74A was more similar to that of the lithium leak currents shown in Fig. 4B (data not shown), suggesting that perhaps GABA induces a sodium leak in this mutant rather than sodium-coupled GABA transport.

| $K_m$ | $V_{max}$ |
|------|----------|
| C74A | 56 ± 4   |
| N327A/C74A | 173 ± 13 |
| D395S/C74A | 153 ± 12 |
| D395T/C74A | 407 ± 54 |
| D395N/C74A | 6652 ± 1072 |
| S396T/C74A | 1277 ± 127 |
| S396C/C74A | 3693 ± 448 |
| S295T/C74A | 53 ± 3   |
The S396T/C74A and S396C/C74A mutants did not exhibit any radioactive GABA transport (data not shown), apparently due to the high \( K_m \) values of these mutants (Table 1). Radioactive uptake is measurable in the \( \mu M \) range (at higher concentrations, prohibitive amounts of radiolabel are needed), and due to the high \( K_m \) the transport rate at these concentrations would be an undetectably small fraction of \( V_{max} \).

**Lithium Leak Currents by Asp-395 Mutants**—The voltage dependence of the sodium-dependent GABA currents by the Asp-395 mutants was more linear than in C74A (Fig. 4A), perhaps due to an altered interaction between the two sodium sites. The similar voltage dependence of the GABA-induced currents in the presence of sodium in the Asp-395 mutants indicates that also in D395C/C74A, these currents reflect coupled transport, which is difficult to detect using the radioactive uptake assay due to the increased \( K_m \) for GABA. Not only were these coupled currents by the Asp-395 mutants lacking the stimulation by lithium, but the leak currents were also not observed in the presence of lithium (Fig. 4B). The same result was also observed with D395N (data not shown). On the other hand, the S396T and S396C mutants exhibited lithium leak currents that had similar voltage dependence to those by wild type GAT-1 (data not shown).

**Ion Dependence of GABA Transport by Putative Na1 Mutants**—In the Leu\(_{\alpha}T\alpha\) structure, Na1 is coordinated by two main chain carbonyl oxygens, the carboxyl oxygen of the transported leucine, the side chain carbonyl oxygens of asparagine 27 and asparagine 286, and the hydroxyl oxygen from threonine 254 (15). The equivalents of the latter three residues in GAT-1 are asparagine 66, asparagine 327, and serine 295. As described previously (14, 16), mutation of asparagine 66 of GAT-1 to glutamine, aspartate, or cysteine caused defective transport currents as well as the lack of measurable \(^{[3]H}\)GABA transport. No transport currents could be observed by the asparagine 327 to alanine or cysteine mutants (data not shown). However, \(^{[3]H}\)GABA transport, albeit diminished, could be measured in both mutants. The background in this assay is very low, \( \sim 0.3-0.4\% \) of the signal, so that the sodium dependence of a mutant that has 5\% of the activity of the wild type can still be measured. Analysis of the kinetic parameters of \(^{[3]H}\)GABA transport by these mutants at 150 mM sodium revealed a 20- and 80-fold reduction of \( V_{max} \) in N327A and N327C, respectively (Table 1). This is apparently the reason for the lack of detectable transport currents by these mutants in the oocyte system because in this assay, 5\% of wild type activity can only be detected in the best expressing batches of oocytes. The size of these currents reflects \( V_{max} \). In contrast to the transport currents in which unlabeled GABA is used and its concentration can be increased at will, radioactive uptake is performed at concentrations below \( K_m \) and therefore depends both on \( V_{max} \) and on \( K_m \). The \( K_m \) values for GABA were 3.7 ± 1.0 and 0.6 ± 0.1 \( \mu M \) for N327A/C74A and N327C/C74A, respectively, as compared with 5.5 ± 0.5 \( \mu M \) for C74A (\( n = 3 \)) (Table 1). In both mutants, transport of \(^{[3]H}\)GABA was stimulated by lithium (Fig. 5, A and B). Similar results were also obtained with the more conserved mutants N327D and N327Q (data not shown). Transport by N327C was not inhibited by preincubation of the HeLa cells with MTSET or MTSES (data not shown). Regarding the \( K_m \) values for GABA, it is important to note that they can only be compared at similar sodium concentrations and membrane potentials. Lowering the sodium concentration results in an increased \( K_m \) (22), and at less negative membrane potentials, the values for \( K_m \) decrease (7). These facts explain the relatively low \( K_m \) value of \( \sim 5 \mu M \) for radioactive uptake, measured at 150 mM sodium in HeLa cells expressing wild type GAT-1. The membrane potential in these cells infected with the recombinant Vaccinia virus is presumably not very negative. The \( K_m \) in oocytes, expressing wild type GAT-1, at \( -140 \) mV and 96 mM sodium is \( \sim 55 \mu M \) (Table 1).
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A. GABA

![Graph of GABA-induced currents and lithium leak currents](image)

B. Lithium

![Graph of voltage dependence of GABA-induced currents and lithium leak currents](image)

**FIGURE 4. Voltage dependence of GABA-induced currents and lithium leak currents.** Oocytes expressing C74A (●), D395S/C74A (▲), D395T/C74A (▼), or D395C/C74A (◆) were clamped at −25 mV before the membrane potential was jumped to the indicated values for 500 ms before returning to −25 mV again. A, GABA-induced currents in the presence of 96 mM sodium, normalized to those by oocytes expressing C74A measured at −140 mV. GABA concentration used was either 1 mM (C74A, D395S/C74A and D395T/C74A) or 10 mM (D395C/C74A). B, currents in 96 mM sodium were subtracted from those in 96 mM lithium, normalized to those by oocytes expressing C74A at −140 mV. Data are averages of three oocytes ± S.E.

A. N327A/C74A

![Graph of sodium dependence of [3H]GABA transport by asparagine 327 mutants](image)

B. N327C/C74A

**FIGURE 5. Sodium dependence of [3H]GABA transport by asparagine 327 mutants.** HeLa cells expressing N327A/C74A (●) or N327C/C74A (◆) were assayed for [3H]GABA transport as described under "Experimental Procedures." Transport was carried out for 5 min at the indicated sodium concentrations with either choline (○) or lithium (▲) as the substituting ion. Data shown at the indicated sodium concentrations are normalized to those at 150 mM Na⁺ (no choline or lithium substitution). Values are averages (± S.E.) of at least three experiments each performed in triplicate. [3H]GABA uptake at 150 mM Na⁺ by N327A/C74A and N327C/C74A was 5.61 ± 0.46% and 13.53 ± 0.84%, respectively, of that by C74A. (n = 3).

In the case of serine 295 mutants, transport currents induced by GABA could be measured with S295T/C74A and S295A/C74A (Fig. 6) but not with the cysteine replacement mutant (data not shown). The voltage dependence of the currents induced by GABA by these two mutants was similar to that of C74A shown in Fig. 4A, indicating that, at least in the presence of sodium, these mutants were capable of coupled transport. In control experiments performed in the presence of lithium alone, we observed that in contrast with C74A, the leak currents at −140 mV by S295T/C74A and S295A/C74A were potentially stimulated by GABA (data not shown). However, at −40 mV, the effect of GABA on the leak currents was negligible for S295A/C74A (Fig. 6C). The GABA-induced transport currents, measured at varying sodium concentrations at −40 mV, were stimulated by lithium in the S295A/C74A mutant (Fig. 6C) at least to the same extent as in the case of C74A (Fig. 6A). In the case of S295T/C74A, the stimulation of the lithium leak current by GABA was significant even at −40 mV, and the voltage dependence of these currents was similar to that shown in Fig. 4B for the currents in lithium by C74A (data not shown). Significantly, when S295T/C74A was expressed in HeLa cells, [3H]GABA uptake was observed in the presence of sodium but not in lithium (data not shown), also in agreement with the idea that lithium and GABA are not cotransported by this mutant. When the sodium concentration was increased to 5 or 10 mM in the presence of sodium but not in lithium (data not shown), also in agreement with the idea that lithium and GABA are not cotransported by this mutant. When the sodium concentration was increased to 5 or 10 mM in the presence of sodium but not in lithium (data not shown), also in agreement with the idea that lithium and GABA are not cotransported by this mutant. When the sodium concentration was increased to 5 or 10 mM in the presence of sodium but not in lithium (data not shown), also in agreement with the idea that lithium and GABA are not cotransported by this mutant.

**DISCUSSION**

In this study, we have identified an amino acid residue of GAT-1, which controls the interaction of this transporter with lithium. Our studies provide experimental evidence for the model that GAT-1 has two functionally different sodium binding sites: a site of low apparent affinity where lithium can replace sodium and another site of high apparent affinity that interacts with sodium but not with lithium (11). The fact that mutation of a residue, corresponding to a liganding group which controls the interaction of GAT-1 with lithium, our studies provide experimental evidence for the model that GAT-1 has two functionally different sodium binding sites: a site of low apparent affinity where lithium can replace sodium and another site of high apparent affinity that interacts with sodium but not with lithium (11). The fact that mutation of a residue, corresponding to a liganding group which controls the interaction of GAT-1 with lithium, our studies provide experimental evidence for the model that GAT-1 has two functionally different sodium binding sites: a site of low apparent affinity where lithium can replace sodium and another site of high apparent affinity that interacts with sodium but not with lithium (11). The fact that mutation of a residue, corresponding to a liganding group which controls the interaction of GAT-1 with lithium, our studies provide experimental evidence for the model that GAT-1 has two functionally different sodium binding sites: a site of low apparent affinity where lithium can replace sodium and another site of high apparent affinity that interacts with sodium but not with lithium (11).
those obtained at 96 mM Na.

FIGURE 6. Sodium dependence of GABA-induced currents serine 295 mutants at −40 mV. Currents induced by GABA at −40 mV were measured as described under “Experimental Procedures” at the indicated sodium concentrations. Na	extsuperscript{+}-substituting ions were either choline (○) or lithium (▲). Data are normalized to those obtained at 96 mM Na	extsuperscript{+} (no choline or lithium substitution). The GABA concentrations used were 1 mM for C74A (A) and S295T/C74A (B) and 10 mM for S295A/C74A (C). Values are averages of three oocytes ± S.E. The magnitudes of net GABA-induced currents (in nA) obtained in 96 mM Na	extsuperscript{+} at −40 mV by C74A, S295T/C74A, and S295A/C74A were −112 ± 5.3, −68.6 ± 10.9, and −49.6 ± 4.3, respectively.

FIGURE 7. Schematic model of sodium, lithium, and GABA binding to the outward facing form of the transporter. Indicated on the left are the outward (upward) facing forms of the transporter, with its two sodium binding sites, Na1 (sodium-specific, high apparent affinity) and Na2 (accepts also lithium, relatively low apparent affinity), indicated. In the absence or presence of lithium, but in the absence of sodium, the Na2 site is accessible to both sides and thus could provide a lithium permeation pathway. Already at low concentrations, sodium binds to the Na1 site and induces a conformational change, closing the Na2 site off from the inside. At physiological sodium concentrations (upper level), both sites are filled, and subsequent binding of GABA leads to the translocation step (TS), after which the binding sites are facing inward. At low sodium (middle level), the Na1 site is filled, but the Na2 site is occupied in only a fraction of the transporters. This determines the rate of transport provided that GABA is saturating. In the presence of lithium alone (lower level, left), the transporter is in the leak mode. At low sodium, the Na1 site is filled, and this causes the conversion of the leak mode to the transport mode. At saturating GABA, transport is faster than in the absence of lithium because of a higher occupancy of the Na2 site.

The following model (Fig. 7) can explain our observations. At physiological sodium concentrations (Fig. 7, upper level), the sites of high and low apparent sodium affinity, corresponding to Na1 and Na2, respectively, are both filled with sodium. Subsequent binding of GABA, depicted by analogy to the situation in LeuT	extsubscript{A}, at the Na1 site, yields the outward-facing translocation complex. After the translocation step (Fig. 7, TS), the binding sites face inward, and sodium and GABA can be released to the inside. At low sodium concentrations, when most of the sodium is substituted by choline (Fig. 7, middle level), the rate of transport is determined by the number of transporters that have sodium bound to the low apparent affinity Na2 site provided that GABA is present at saturating levels. At low sodium conditions in the presence of lithium (Fig. 7, lower level), many transporters have their Na2 site filled with lithium, yielding an active GABA translocation complex with one sodium and one lithium ion. At saturating GABA levels, transport levels will be higher in the presence of lithium than in the presence of choline. However, at very low concentrations of GABA, the transport rate will be determined by the fraction of transporters that have bound the neurotransmitter, regardless of whether part of the sodium is replaced by lithium or by choline. Indeed, under such conditions, the stimulation by lithium is not observed (Fig. 2A). If GABA participates in the liganding of Na1, the dependence of GABA transport on sodium is predicted to depend on the GABA concentration. The apparent affinity for sodium would be expected to be higher at saturating than at non-saturating concentrations of GABA, and this is precisely what is observed (Figs. 1A and 2A). The apparent affinity for GABA of D395S and D395T, which cannot interact with lithium, is even lower than in wild type. Since Asp-395 corresponds to a Na2 residue, this effect on $K_m$ is probably indirect. At low sodium, GABA at 20 μM is more limiting in the mutants than in the wild type. Therefore, the apparent affinity for sodium in the mutants is even lower than that of the wild type (Fig. 2, B–D).

The leak currents in GAT-1 are observed in the presence of lithium, and low concentrations (1–2 mM) of sodium potently and non-competitively inhibit these leak currents (11, 14). Thus sodium apparently induces a conformational change so that the leak mode of the transporter gets converted into a mode capable of coupled transport (11, 14). Moreover, when the site equivalent to Na2 is changed by mutation such that lithium also cannot interact with it in the coupled mode (Figs. 1 and 2), the lithium leak currents are also abolished (Fig. 4). Therefore, this site presumably also lines the ion permeation pathway, which opens up in the absence of sodium. The fact that very low concentrations of sodium block the lithium leak currents (11, 14) suggests that it is the binding of sodium to the high affinity site equivalent of Na1 that converts the leak mode of the transporter into the mode capable of coupled transport. These features are schematically shown in Fig. 7, middle and lower levels, left-hand sides. Although it is generally assumed that lithium is carrying the current in the leak mode, more complex scenarios cannot completely be excluded. For instance, in the related serotonin transporter SERT, it has been shown that lithium is capable of inducing a
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conformational change (23). Thus in principle, lithium might induce a conformational change in GAT-1 such that another ion could permeate the transporter. However, even in such a case, lithium would still need to bind to the site equivalent to Na2 to exert its effect.

It is noteworthy that cysteine residues, at positions accessible to sodium ions, were not modified by MTS reagents. However, this is probably due to the fact that sodium is much smaller than MTSET and MTSES. We considered using the smaller MTS reagents methanethiosulfonate ethylammonium and methyl methanethiosulfonate. However, these reagents are membrane-permeable. Therefore, the use of these reagents would require mutations of cysteine residues in the parent construct that face the cytoplasm, and that, upon modification, result in inactivation of transport. An example is cysteine 399 (24).

Introduction of the mutations studied here in the background of C399S or C399A, in addition to C74A, would virtually eliminate transport activity. In any case, the size of the substrate or coupling ion molecule is not the only determinant of accessibility. In the glutamate transporter EAAC-1, when the arginine residue, which apparently interacts with the γ-carboxyl group of the transported glutamate (25, 26), is replaced by cysteine, the transport activity remains insensitive to MTS reagents (25). These reagents have a size roughly similar to glutamate but apparently cannot react with the substituted cysteine. It is possible that binding of substrate, but not a non-substrate molecule, brings the arginine into the binding site by a process of “induced fit.” Recent experiments on lactose permease support the idea that the principle of induced fit also applies to transporters (27).

According to the LeuTAs model, mutations in the residues forming Na1 can influence the apparent affinity of the transported substrate by changing the position of the Na1 ion that serves as an interacting group with the substrate. Additionally, some of the residues involved in the liganding of the sodium ions also directly interact with the bound leucine. One of these is serine 355, the counterpart of serine 396 of GAT-1. The methylene group of serine 355 makes contact with the hydrophobic part of the transported leucine molecule (15). In GAT-1, the $K_m$ for GABA of the transport currents of the S396C mutant is increased from $\sim 55 \mu M$ for the wild type to $\sim 1.3 \text{mM}$, and when the bulkier threonine replaces serine 396, the $K_m$ is $\sim 6.5 \text{mM}$ (Table 1). Thus it is very well possible that, also in GAT-1, this serine residue makes contact with the substrate and that the effect of mutation on the GABA affinity is likely due to a direct effect. The stimulation of the transport currents in the S396T and S396C mutants by lithium is smaller than in the wild type (Figs. 1A and 3). This is possibly due to a lack of saturation at low sodium concentrations, even at 10 mM of GABA.

In the case of putative Na1 residue Asn-327, the removal of the amide group may influence the binding of sodium at the Na1 site directly and that of GABA and the Na2 site indirectly. If, as in LeuTAs, Na1 interacts with the carboxyl group of GABA, this could alter the orientation of GABA in the binding pocket as well as its apparent affinity, which in the case of N327C is actually markedly increased. However, the distortion of the Na1 site apparently hampers the rate of translocation.

Because of the marked drop in $V_{\text{max}}$, it was only possible to measure radioactive GABA transport in these mutants. The stimulation of transport by lithium observed in the Asn-327 mutants is in agreement with the idea that Na2 is the promiscuous sodium binding site. The impact of the other asparagine from the putative Na1 site was more severe; we could not observe any radioactive GABA transport or GABA-induced currents, even in the most conserved substitutions at this position. It is possible that asparagine 66 fulfills a much more important role than asparagine 327 in the binding of sodium to the Na1 site in the absence of GABA.
Serine 295 is the GAT-1 counterpart of the LeuT<sub>AX</sub> residue threonine 254, which directly participates in the Na<sup>1</sup> site via both the side chain and the main chain oxygen atoms. Moreover, its main chain carbonyl group interacts with the amino group of the transported leucine. The <i>K<sub>m</sub></i> for GABA in the S295T mutant was similar to that of wild type, in contrast to the markedly increased value in the S295A mutant. This could suggest a difference in substrate binding between the two transporters. However, it is also possible that the lack of the side chain hydroxyl group in the alanine replacement mutant influences the coordination of Na<sup>1</sup> and thereby the binding of GABA. Whatever the scenario, at saturating GABA, S295A is not affected in its sodium interaction (Fig. 6C), implying that if serine 295 is a Na<sup>1</sup> residue, its side chain oxygen would not participate in the interaction with sodium.

A stimulation of uncoupled currents of GAT-1 mutants by GABA, as seen in S295T/C74A, has been observed previously in mutants at glycine 80 (28). Extrapolating from the LeuT<sub>AX</sub> structure (15), this residue is probably not involved in ion coordination. The flexibility afforded by glycine 80 appears to be essential for the execution of some of the conformational transitions of GAT-1, and GABA is able to release some of the glycine 80 mutants from a “frozen” state, in which the leak mode of the transporter is not operative (28). The stimulation of the lithium leak currents of S295T/C74A, as well as of G80C/C74A and G80A/C74A, by GABA suggests that GABA may bind to the transporter, although the Na<sup>1</sup> site is empty, albeit with a low apparent affinity.

How does aspartate 395 enable the interaction of GAT-1 with lithium? In the absence of a high-resolution crystal structure of wild type and aspartate 395 mutants, it is impossible to give a definite answer to this question. To get some ideas on this issue, we used homology modeling of the putative Na<sup>2</sup> binding site of GAT-1 and the D395T mutant. The Na<sup>2</sup> site of GAT-1 appears similar, but not identical, to that of LeuT<sub>AX</sub> (Fig. 8A). When aspartate 395 is replaced by threonine, the same Na<sup>2</sup> binding site present in LeuT<sub>AX</sub> emerges (Fig. 8B). According to this procedure, the mutation causes a small change in the binding pocket as visualized by the superimposition of the two putative sites (Fig. 8C). When, instead of the sodium ion, a lithium ion is introduced, no change in the binding site of the wild type or the mutant is seen (not shown). The distances of some of the liganding groups to the cation are slightly larger in the wild type than in the mutant and are not optimal for the coordination of a lithium ion, which is smaller than a sodium ion. However, in the wild type, there is one liganding atom more than in the mutant, and the carboxyl group can also potentially contribute a negative charge. These features may be the reason for the ability of the wild type, but not the mutants, to interact with lithium. Our results suggest that the LeuT<sub>AX</sub> model may also be a useful model for the study of other aspects of neurotransmitter transporter function, such as the determinants of neurotransmitter specificity and gating.

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