Transmembrane Domain I of the γ-Aminobutyric Acid Transporter GAT-1 Plays a Crucial Role in the Transition between Cation Leak and Transport Modes*

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The sodium- and chloride-dependent γ-aminobutyric acid (GABA) transporter is essential for synaptic transmission by this neurotransmitter. GAT-1 expressed in Xenopus laevis oocytes exhibits sodium-dependent GABA-induced inward currents reflecting electrogenic sodium-coupled transport. In lithium-containing medium, GAT-1 mediates GABA-independent currents, the relationship of which to the physiological transport process is poorly understood. In this study, mutants are described that appear to be locked in this cation leak mode. When Gly\(^{14}\), located in the middle of the highly conserved transmembrane domain I, was mutated to serine or cysteine, sodium-dependent GABA currents were abolished. Strikingly, these mutants exhibited robust inward currents in lithium- as well as potassium-containing media. Membrane-impermeant sulfhydryl reagents inhibited these currents of the cysteine but not of the serine mutant, indicating that this position was accessible to the external aqueous medium. The cation leak currents mediated by wild-type GAT-1 were inhibited by low millimolar sodium concentrations in a non-competitive manner. Mutations at other positions of transmembrane domain I increased or decreased the apparent sodium affinity, as monitored by the sodium-dependent steady-state GABA currents or transient currents. In parallel, the ability of sodium to inhibit the cation leak currents was increased or decreased, respectively. Thus, transmembrane domain I of GAT-1 contains determinants controlling both sodium-coupled GABA flux and the cation leak pathway as well as the interconversion of these distinct modes. Our observations suggest the possibility that the permeation pathway in both modes shares common structural elements.

The neurotransmitter γ-aminobutyric acid (GABA)\(^3\) is a major inhibitory neurotransmitter in the central nervous system. Its synaptic action, like that of most other neurotransmitters, is terminated by sodium-coupled transport. Many neurotransmitters are removed from the synaptic cleft by sodium- and chloride-dependent neurotransmitter transporters (for a review, see Refs. 1 and 2). These transporters form a large family and include transporters for biogenic amines and amino acids. Besides GAT-1, the first member of the family to be identified, it contains three other GABA transporters. One of the best examples of the importance of these neurotransmitter transporters comes from studies of dopamine transporter knockout mice; the decay of extracellular dopamine in brain slices of such mice is ~100 times longer than normal (3).

GAT-1 was purified to homogeneity in a form active upon reconstitution (4) and subsequently cloned (5). The transporter catalyzes electrogenic sodium/chloride/GABA cotransport with a stoichiometry of 2:1:1 (6–9). It should be noted, however, that there still is some dispute regarding this issue. In a recent report, it has been proposed that, during sodium-coupled GABA transport, obligatory Cl\(^–/\)Cl\(^–\) exchange takes place (10).

GAT-1, as well as the other members of the family, is predicted to have 12 transmembrane domains linked by hydrophilic loops with the amino and carboxyl termini residing inside the cell (5). Studies on the serotonin transporter SERT suggest that the theoretical topological model is correct (11). Mutagenesis studies (in particular on GAT-1 and SERT, but also on other members of the family) suggest the importance of transmembrane domains I and III in the interaction of the transporters with the neurotransmitters as well as with sodium and chloride (12–16).

Using biochemical approaches, it has been shown that GAT-1 undergoes extensive conformational changes upon interaction with its substrates (17, 18). Electrophysiological measurements of GAT-1 have demonstrated sodium-dependent GABA-induced inwardly rectifying currents reflecting the electrogenic sodium- and chloride-coupled translocation of GABA (7–9). Moreover, in the absence of GABA, but in the presence of sodium, GAT-1 exhibits capacitative transient currents (8, 13, 14). These transients are thought to reflect a charge-moving conformational change that takes place after the binding of sodium and that enables subsequent GABA binding (8, 13, 19). Remarkably, in the absence of lithium, inwardly rectifying steady-state currents are observed in the absence of GABA (13, 14). Such leak currents have also been observed in many transporters for neurotransmitters (20–23) as well as in other transporters such as proton-coupled metal transporters (24). In the latter case, it has been suggested that this leak pathway may protect cells from metal ion overload (24). However, the relationship of these leak currents to coupled transport is poorly understood.

In the case of the uncoupled currents observed with the glutamate transporters, it has been shown that the conformation of the transporter mediating this process is distinct from the coupled process (25–28). Recently, co-workers and I have...
obtained some evidence that the same may be true for the leak currents mediated by GAT-1 (29). In this study, I show that the leak mode of GAT-1 represents a unique conformation of the transporter. Mutants at a water-accessible position in the middle of the highly conserved transmembrane domain I that appear to be locked in the leak mode are described. Moreover, other determinants located in this transmembrane domain that control the sodium-dependent transition of the leak to transport mode have been identified here.

EXPERIMENTAL PROCEDURES
Generation and Subcloning of Mutants—Mutations were made by site-directed mutagenesis of wild-type GAT-1 in the vector pBluescript SK(−) (Stratagene) according to the method of Kunkel et al. (30) as described (31). Briefly, the parent DNA was used to transform Escherichia coli CJ236 (dam− 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; consequently, mutagenic primers were designed to be antisense. Restriction sites ClaI and AvrII were used to subclone the mutations into the construct containing wild-type GAT-1 in pOG1, (14). The latter is an oocyte expression vector that contains a 5′-untranslated Xenopus β-globin sequence and a 3′-poly(A) signal. The coding and noncoding strands were assembled between the above two restriction sites.

cRNA Transcription, Infection, and Oocyte Preparation—Capped run-off cRNA transcripts were made from transporter constructs in pOG1, linearized with SacII using mMessage mMachine (Ambion Inc.). Oocytes were removed from anesthetized Xenopus laevis frogs and treated with collagenase (type IA, Sigma C-9891) until capillaries were absent and injected with 50 nl of undiluted cRNA the same or the next day. Oocytes were maintained at 18°C in modified Barth’s saline (88 mM NaCl, 1 mM KCl, 1 mM MgSO4, 2.4 mM NaHCO3, 1 mM CaCl2, 0.3 mM Ca(NO3)2, and 10 mM 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 gentamycin.

Oocyte Electrophysiology—Oocytes were placed in the recording chamber; penetrated with two micropipettes (back-filled with 2 M KCl, with resistance varied between 0.5 and 2 megohms); and voltage-clamped using GeneClamp 500 (Axon Instruments, Inc.) and digitized using Digidata 1200A (Axon Instruments, Inc.), both controlled with the pClamp6 suite (Axon Instruments, Inc.). Currents were acquired with pClamp6.03 and low pass-filtered at 10 kHz every 0.5 ms. Oocytes were stepped from −160 to +40 mV in 25-mV increments, using −55 mV as the holding potential unless stated otherwise in the figure legends. Each potential was clamped for 500 ms. The membrane potential was measured relative to an extracellular Ag/AgCl electrode in the recording chamber. The 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. The results shown in Figs. 2, 3, 6, and 7 are typical and representative of results from four to eight oocytes. In the other figures, the currents were normalized as indicated in the legends to plot results of 3–13 oocytes as means ± S.E. Wherever error bars are not visible, the error was smaller than the size of the symbols. Analysis was performed with Clampfit Version 6.05 and Origin Version 6.1 (Microcal).

Cell-surface Biotinylation—Labeling of wild-type and mutant transporters at the cell surface using sulfo-succinimidyl 3′-biotinamido/ethyl-1,3-dithiopropionate, SDS-PAGE, and Western blot analysis was done as described by Stern-Bach et al. (32). For each determination, five oocytes expressing either the wild type or the indicated mutant were used. The weight of the sample was retained as the source of total protein, and the remainder was treated with streptavidin beads to recover the biotinylated proteins. The GAT-1 protein was detected with an affinity-purified antibody to an epitope located in the carboxyl terminus of GAT-1 (residues 571–586), horseradish peroxidase-conjugated secondary antibody, and ECL as described (33).

[3H]GABA Transport in HeLa Cells—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 (34) and subsequent transfection with plasmid DNA, as well as GABA transport (35), were done as published previously.

RESULTS
Characterization of Currents Mediated by the G63S and G63C Mutants—Of the members of the sodium- and chloride-dependent neurotransmitter transporter family, the biogenic amine transporters possess a conserved aspartate residue in the middle of transmembrane domain I. All other members of the transporter family, including GAT-1, have a glycine at the corresponding position (Gly63 in GAT-1). In the study of the structure/function relationships in transmembrane domain I of GAT-1, I mutated Gly63 to aspartate as well as to serine, cysteine, and alanine. This led to the complete loss of sodium- and chloride-dependent [3H]GABA uptake, as observed upon expression in HeLa cells (Fig. 1). A similar loss of function was observed with the sodium-dependent GABA-induced transport currents in X. laevis oocytes (as shown here for the G63C and G63S mutants (Fig. 2, A, C, and E). Remarkably, however, the two mutants exhibited robust GABA-independent inwardly rectifying leak currents in lithium medium (Fig. 2, D and F). The currents of both of these mutants were similar in magnitude and voltage dependence to that mediated by wild-type GAT-1 (Fig. 2B) and were not influenced by GABA (data not shown). The kinetics (but not the steady-state level) of the currents observed in lithium were dependent on the nature of the substitution at position 63. In the G63C (but not G63C) mutant, the onset of these currents was markedly slowed down (Fig. 2, B, D, and F). Results similar to those obtained with G63C were obtained when Gly63 was replaced by alanine, but no lithium leak currents were observed when aspartate was substituted for Gly63 (data not shown).

Even though position 63 is located in the middle of transmembrane domain I, it is possible that this conserved domain lines an aqueous pathway through the transporter, and a cysteine residue introduced at this position is accessible to hydrophilic membrane-impermeant sulphydryl reagents. Consistent with this idea, the lithium-dependent currents of G63C (but not of G63S) were almost completely abolished by positively charged MTSET as well as by negatively charged (2-sulfonato-ethyl) methanethiosulfonate (Fig. 3) (data not shown). A conserved cysteine (Cys74 in GAT-1) located in the first extracellular loop is the only endogenous cysteine that is externally accessible in GAT-1 and most members of this family (36–38). To exclude the possibility that the sensitivity of the lithium-dependent currents observed with G63C is due to an increased accessibility of Cys74, the G63C and G63S mutants were subcloned into a construct in which Cys74 was replaced by alanine (C74A). The currents of this latter mutant were very similar to those mediated by the wild type (data not shown). The lithium-dependent currents of the G63C/C74A mutant (Fig. 3A) were
almost completely abolished by pretreatment with MTSET (Fig. 3B). On the other hand, no effect of MTSET on the lithium-dependent currents of the G63S/C74A mutant was observed (Fig. 3, C and D). Similarly, 2-sulfonatoethyl methanethiosulfonate also inhibited these currents of G63C/C74A, but not of G63S/C74A (data not shown). It is of interest to note that the difference in the kinetics of the onset of the currents observed with G63C and G63S (Fig. 2, D and F) was also observed in the mutants constructed in the C74A background (Fig. 3, A and C).

**Effects of Sodium on the Lithium Leak Currents Mediated by the Wild Type**—The absence of [³H]GABA transport and GABA-induced currents in the G63S and G63C mutants indicated that they were confined to the leak current mode. Further evidence that the leak currents represent a distinct mode of the transporter is presented in Fig. 4. The effect of sodium on the lithium currents of the wild type was measured at varying lithium levels. The lithium-dependent current did not saturate

**Fig. 2.** GABA-induced and lithium currents mediated by wild-type, G63S, and G63C transporters. A, C, and E, currents recorded in sodium medium in the absence of GABA during 500-ms voltage jumps from −160 to +40 mV (steps of +25 mV) have been subtracted from currents in the same medium containing 1 mM GABA. The prepulse potential was −25 mV. B, D, and F, currents recorded in choline medium have been subtracted from those in lithium medium using the same oocytes as in A, C, and E, respectively, with the same voltage jump protocol. The dotted lines indicate 0 current. Representative oocytes are shown. WT, wild type.

**Fig. 3.** Effect of MTSET on lithium-induced currents mediated by G63C/C74A and G63S/C74A. Lithium leak currents were recorded exactly as described in the legend to Fig. 2 on oocytes expressing G63C/C74A and G63S/C74A before (A and C) and after (B and D) treatment with 0.25 mM MTSET for 2 min on the same oocytes. Immediately before the treatment, the perfusion with sodium medium was stopped, and MTSET was added directly to the bath. After 2 min of incubation and a washout of 1 min, the records shown in B and D were obtained.

**Fig. 4.** Leak currents at varying lithium concentrations. Lithium-dependent steady-state currents (obtained by subtracting the response in sodium) at −160 mV were determined in the presence or absence of 1 mM sodium at 28.8, 57.6, and 86.4 mM lithium. Choline was used to bring the cation concentration to 96 mM. The results were normalized to the net current in 86.4 mM lithium and are averages from four different oocytes. The S.E. is smaller than the size of the symbols.
with increasing lithium concentrations, at least not up to 90 m\(\text{M}\) (Fig. 4). Sodium at a concentration as low as 1 m\(\text{M}\) inhibited the current at all lithium concentrations tested. However, the inhibition by sodium was larger at higher lithium concentrations (Fig. 4). Thus, these effects are not due to a competition between lithium and sodium, but rather sodium appears to act at a distinct site to suppress the leak mode. As sodium is required for coupled transport, an important question is whether the sodium-induced conformational changes of the transport cycle are related to the suppression of the leakage mode. To address this, mutants with decreased and increased apparent sodium affinities were analyzed.

**Effect of Sodium on Currents Mediated by Y60T—Tyr\[^{60}\] of GAT-1**, also located in transmembrane domain I, is unique among the GABA transporters. In the three other GABA transporters (GAT-2–4), a glutamate residue occupies the equivalent position of Tyr\[^{60}\]. Interestingly, when the Y60E mutant was expressed in HeLa cells, no sodium- and chloride-dependent \[^{3}\text{H}\]GABA uptake could be detected, and the same was true for the threonine substitution mutant, but the Y60C mutant exhibited significant activity (Fig. 1). Upon expression of Y60E in *X. laevis* oocytes, no sodium-dependent GABA-induced transport currents could be detected, and the same was true for the lithium leak currents (data not shown). In contrast to Y60E, the Y60T mutant exhibited robust lithium leak currents, ranging from \(-1195\) to \(-2791\) n\(\text{A}\) (\(n = 6\)) at \(-160\) m\(\text{V}\) (Fig. 5A). The same was true for oocytes expressing Y60C; but in contrast to G63C, these currents mediated by Y60C could not be blocked by MTSET (data not shown). The size of the lithium-dependent currents of Y60T was even larger than those of wild-type GAT-1 (Fig. 5B): \(-485\) to \(-2081\) n\(\text{A}\) (\(n = 13\)). Sodium- and chloride-dependent \[^{3}\text{H}\]GABA uptake was typically measured at very low GABA concentrations. However, at a saturating concentration of 1 m\(\text{M}\), Y60T exhibited significant GABA transport currents (Fig. 5A), ranging from \(-31.6\) to \(-90.8\) n\(\text{A}\) at \(-160\) m\(\text{V}\) (\(n = 6\)). In the corresponding oocytes expressing the wild type, the GABA transport currents ranged from \(-342\) to \(-1544\) n\(\text{A}\) (Fig. 5C). The voltage dependence of the currents of the mutant and wild type was similar (Fig. 5, A and B), but it is obvious that the ratio of the lithium leak current to the GABA current differed dramatically between Y60T and the wild type.

Even though the GABA transport currents of the mutant were of small magnitude, their size was large enough to determine the sodium dependence of this process (Fig. 5C). More than 5-fold higher sodium concentrations were needed to half-maximally activate the GABA currents of Y60T compared with those of the wild type (Fig. 5C). It was not possible to determine the exact shift of the apparent sodium affinity in the mutant because, even at 96 m\(\text{M}\) sodium, the GABA-induced current was not yet saturated (Fig. 5C). In parallel with the decreased apparent affinity of Y60T for sodium, 28.8 m\(\text{M}\) sodium inhibited the lithium leak current by 26 ± 2% (\(n = 4\)). In contrast, the IC\text{so} for sodium in the same batch of oocytes expressing the wild type was 1.1 ± 0.1 m\(\text{M}\) (\(n = 3\)) (Fig. 5D).

**Effect of Sodium on Currents Mediated by R69K—Arg\[^{69}\]**, also located in transmembrane domain I of GAT-1, is fully conserved in the entire family of sodium- and chloride-dependent neurotransmitter transporters. Mutation of this residue, even to a conserved one such as lysine (R69K), leads to defective \[^{3}\text{H}\]GABA uptake (12). Indeed, no steady-state GABA currents were observed in this mutant even though transients were present (Fig. 6A). Even at 5 m\(\text{M}\) GABA, no currents could be observed, but the transients were somewhat larger (data not
The transients mediated by R69K were capacitative; the transient currents were of the same magnitude as those observed when jumping back to the holding potential (data not shown). The transients observed with R69K were capacitative; the transient currents were of the same magnitude as those observed when jumping back to the holding potential (data not shown). Furthermore, the mutant exhibited significant lithium-dependent steady-state currents (Fig. 6B).

The observation that transient currents could be isolated by GABA (Fig. 6A) indicated that, even though GABA was not transported, it could still bind to the R69K mutant. This was further substantiated by the fact that, in sodium medium, the non-transportable GABA analog SKF100330A could isolate the sodium-dependent transient currents mediated by R69K (Fig. 7A). In contrast to the corresponding currents mediated by the wild type (Fig. 7B), R69K exhibited transients in the "on" phase only when jumping from a holding potential of −25 mV to more positive potentials of up to +50 mV, and the opposite was true in the "off" phase (Fig. 7A). The sodium-dependent transients were observed only when Arg69 was changed to lysine, but not when the substitution was with glutamine or cysteine (data not shown). The transients observed with R69K were capacitative; the transient currents were of the same magnitude as those observed when jumping back to the holding potential (data not shown), and they increased when the potential was jumped to values more positive than +50 mV (data not shown). An attractive explanation is that, at a holding potential of −25 mV, all the transporters are already in the sodium-bound state, and the sodium is released by jumps to positive potentials, thereby giving rise to the outward transient current seen in Fig. 7A. Indeed, when the external sodium concentration was progressively decreased, an inward transient became apparent (data not shown). In parallel with the increased apparent affinity of R69K for sodium, this cation inhibited the lithium-dependent currents of R69K at lower concentrations (IC_{50} = 0.3 ± 0.1 mM, n = 4) compared with the wild type expressed in the same batch of oocytes (IC_{50} = 1.0 ± 0.1 mM, n = 3) (Fig. 8).

Characterization of Potassium Leak Currents—It has been found that GAT-1 also mediates leak currents in cesium, even though they are much smaller than those in the presence of lithium (13). Interestingly, GAT-1 also mediated potassium leak currents, which, at −160 mV, were 62.8 ± 3.8% (n = 6) of those observed in lithium (Fig. 9A). Such currents were not observed in non-injected oocytes (data not shown). The potassium currents had a similar voltage dependence compared with the currents observed in lithium (data not shown); and as with the lithium currents, they were also inhibited by sodium ions (Fig. 9B). The potassium currents mediated by R69K were more sensitive to sodium than those mediated by the wild type (Fig. 9B). In Y60T, the sensitivity of the potassium currents to sodium was markedly reduced (Fig. 9B), as in the case of the lithium currents (Fig. 5D). Importantly, the ratio of the potassium to lithium currents was different in wild type, R69K, and Y60T (Fig. 9A). The same was also true for the G63S and G63C mutants, in which this ratio was 0.95 ± 0.10 and 0.93 ± 0.11, respectively (n = 3). As for the lithium currents, the potassium currents mediated by G63C/C74A (but not by G63S/C74A) were inhibited by treatment with MTSET (data not shown).

Cell-surface Expression—The transients mediated by R69K were smaller than those mediated by the wild type (Fig. 7). To investigate the possibility that this may be due to decreased cell-surface expression, oocytes expressing wild-type and mutant transporters were treated with the impermeant biotinylation reagent sulfoNHS-biotinamide (2-biotinamido)ethyl-1,3-dithiopropionate. Upon solubilization of the membrane proteins with detergent, the biotinylated proteins were isolated using streptavidin beads. After SDS-PAGE of total (unfractionated) and biotinylated proteins, followed by Western blot analysis using affinity-purified antibody against GAT-1, the transporter...
and GABA to the inside (step 4) yields the inward-facing form of the unloaded transporter (iT); and upon reorientation to the outside (step 5), a new transport cycle can commence. The leak mode of the transporter (iT) can be generated from the unloaded outward- and/or inward-facing (iT) transporter upon hyperpolarization (Fig. 5). Sodium inhibited the lithium currents mediated by the GAT-1 transporter (Fig. 4), and this is in accordance with a study that was published during the preparation of this manuscript (39). Moreover, sodium also inhibited the potassium leak currents mediated by GAT-1 (Fig. 9). The inhibition of the leak currents by sodium was noncompetitive (Fig. 4). This indicates that sodium binds at a distinct site from the permeating ions, bringing it into a different conformation. Because sodium induces a conformational change resulting in the inhibition of the leak current of the unloaded outward-facing (iT) and/or inward-facing (iT) transporter. Binding of extracellular sodium converts the transporter into the sodium-loaded outward-facing form of the transporter (step 1). Subsequent GABA binding (step 2) is followed by translocation (step 3) and release of sodium and GABA to the inside of the cell (step 4). Upon reorientation of the binding sites to the outside and binding of extracellular sodium, the transport cycle is completed (step 5). For simplicity, chloride is omitted from the scheme.

DISCUSSION

Mutation of Gly<sup>103</sup>, located in the conserved transmembrane domain I of GAT-1, to serine or cysteine resulted in defective GABA transport and transport currents (Figs. 1 and 2). The G63S and G63C mutants did not even exhibit sodium-dependent transient currents (data not shown), but exhibited lithium leak currents, which were at least as large as those exhibited by the wild type (Fig. 2). Therefore, these mutants appear to be locked in a conformation that mediates the leak current of GAT-1.

The model depicted in Fig. 11 describes how the leak mode of GAT-1 may relate to its transport cycle. For reasons of simplicity, the role of chloride is not indicated. In step 1, sodium binds to the outward-facing form of the transporter (iT). This enables subsequent GABA binding (step 2), followed by the translocation step (step 3). Release of sodium and GABA to the inside (step 4) yields the inward-facing form of the unloaded transporter (iT); and upon reorientation to the outside (step 5), a new transport cycle can commence. The leak mode of the transporter (iT) can be generated from the unloaded outward- and/or inward-facing (iT) transporter upon hyperpolarization (Fig. 5). Sodium inhibited the lithium currents mediated by the GAT-1 transporter (Fig. 4), and this is in accordance with a study that was published during the preparation of this manuscript (39). Moreover, sodium also inhibited the potassium leak currents mediated by GAT-1 (Fig. 9). The inhibition of the leak currents by sodium was noncompetitive (Fig. 4). This indicates that sodium binds at a distinct site from the permeating ions, bringing it into a different conformation. Because sodium induces the initial steps of the transport cycle (Fig. 11), a simple explanation is that these critical steps represent the conformational change resulting in the inhibition of the leak cur-

FIG. 9. Comparison between lithium and potassium leak currents. The ratio of steady-state potassium- and lithium-induced currents obtained by subtraction of the response in sodium at −160 mV is plotted for the wild-type (WT), R69K, and Y60T (A). The potassium current in the presence of 2 or 10 mM sodium (white and black bars, respectively) was normalized to that in the absence of sodium (gray bar) at −160 mV and is depicted for the wild type and the two mutants (B).

FIG. 10. Cell-surface biotinylation of the wild type and mutants. Oocytes expressing the wild type (WT) and the indicated mutants were labeled and processed as described under "Experimental Procedures." The first lane (M) depicts the positions of the prestained molecular mass standards. The next six lanes show the total samples, followed by six lanes of the corresponding biotinylated samples. The first lanes in both groups are total and biotinylated samples of uninjected oocytes (Uninj.). Two independent groups of oocytes expressing G63C were processed in parallel and are shown next to each other. All samples were separated on the same SDS gel, transferred to nitrocellulose, and detected as described under "Experimental Procedures."

FIG. 11. Conversion of an uncoupled mode of GAT-1 to its coupled form is mediated by sodium ions. The leak pathway (iT) can be opened in the absence of extracellular sodium by hyperpolarization (ΔV) of the unloaded outward-facing (iT) and/or inward-facing (iT) transporter. Binding of extracellular sodium converts the transporter into the sodium-loaded outward-facing form of the transporter (step 1). Subsequent GABA binding (step 2) is followed by translocation (step 3) and release of sodium and GABA to the inside of the cell (step 4). Upon reorientation of the binding sites to the outside and binding of extracellular sodium, the transport cycle is completed (step 5). For simplicity, chloride is omitted from the scheme.
Transport and Leak Modes of GAT-1

Supporting this explanation are observations on mutants at two other positions, also located in transmembrane domain I. A decreased (Fig. 5C) or an increased (Fig. 7A) apparent affinity for sodium was paralleled by the ability of sodium to inhibit the leak currents (Figs. 5D, 8, and 9).

The model depicted in Fig. 11 is also in harmony with the observations that the GABA currents began to saturate at negative potentials, whereas this was not the case for the lithium leak currents (Fig. 5B). The GABA currents reflect electrogenic sodium-coupled GABA translocation involving steps 1–5. Therefore, increasingly negative potentials stimulate the translocation of positive charges that accompany GABA transport. However, at very negative potentials, a voltage-independent step apparently becomes rate-limiting for transport. It has been suggested that this is a voltage-independent interaction between GABA and the transporter (8). The leak currents reflect only the voltage-dependent gating of the unloaded transporter. They do not require steps 1–4, one or perhaps more of which involve voltage-independent interactions.

In choline medium, no inwardly rectifying leak currents were observed. Moreover, the lithium currents did not saturate with increasing concentrations of this cation (Fig. 4). Thus, it appears that only small cations (but not the larger choline) pass through the permeation pathway when this is activated by hyperpolarization.

The role of Gly63 in GABA transport is as yet unclear. In the related serotonin transporter SERT, an aspartate residue occupies the position equivalent to Gly63 of GAT-1. This aspartate residue, which is conserved in all biogenic amine transporters of this family, has been implicated in the binding of the amine group of the substrate and also influences the apparent affinity of SERT for the two cosubstrates, sodium and chloride (16). It is tempting to speculate that, in the amino acid transporter members of the family, the carboxyl group of the transported amino acid fulfills a role played by the carboxyl group of the conserved aspartate in the biogenic amine transporters. If, indeed, Gly63 is part of the binding pocket for the carboxyl group of GABA, it is easy to see that even small changes at this position impair GABA transport. As sodium-dependent transients are also defective in the G63S and G63C mutants, the leak currents mediated by these mutants are their only functional property. The presence of these leak currents indicates that the conformation of the outward- and/or inward-facing unloaded transporter is not affected by the mutations at position 63. Thus, even though the leak currents do not have an obvious physiological function, they can be an important tool for structure/function studies in GAT-1.

The lithium leak currents and the transient currents mediated by R69K are usually smaller than those mediated by the wild type (Figs. 6 and 7). This indicates a lower cell-surface expression of this mutant, and this is, in fact, supported by surface biotinylation experiments (Fig. 10). It is not clear why bands of lower mobility are observed in the biotinylated samples of R69K (and also of G63C) than in those of the wild type. One possibility is that, due to the lower expression levels at the cell surface, during the processing of the samples, these mutant transporters are more prone to proteolysis and subsequent aggregation. Whatever the reason, the important point for this study is that the expression level is high enough to test the hypothesis that an increased apparent affinity of sodium binding to the transporter can be correlated with an increased potency of sodium to inhibit the leak currents (Figs. 7–9). In R69K, GABA has lost the ability to induce a transport current (Fig. 6A) as well as to catalyze [3H]GABA uptake (12). Nevertheless, GABA can bind inefficiently to the R69K transporters, as judged by its ability to partially isolate the transient currents at high concentrations (Fig. 6A). A possible role of Arg64 could be to interact with the carboxyl group of GABA. This would be reminiscent of the role of Arg147 of the glutamate transporter EAAC-1 in binding the γ-carboxyl group of glutamate (40). In the other amino acid transporters, this conserved arginine could play a similar role.

Determinants located in the highly conserved transmembrane domain I influence the affinity of the transporter for sodium (Figs. 5C and 7), the ability of sodium to inhibit the leak currents (Figs. 5D, 8, and 9B), and the potassium/lithium selectivity of the leak currents (Fig. 9A), and the interconversion of the leak to transport mode (Figs. 2 and 3). Consistent with the possibility of a direct involvement of at least one amino acid residue of transmembrane domain I in permeation is the fact that the position of Gly63 located in the middle of this domain appears to be accessible via an aqueous pathway to the extracellular medium (Fig. 3). The simultaneous effects of the mutations on GABA transport and the leak mode suggest that the permeation pathway of these two modes may share common structural elements.

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Transport and Leak Modes of GAT-1

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