Using lithium to probe sequential cation interactions with GAT1

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Meinild AK, Forster IC. Using lithium to probe sequential cation interactions with GAT1, Am J Physiol Cell Physiol 302: C1661–C1675, 2012. First published March 28, 2012; doi:10.1152/ajpcell.00446.2011.—Li\(^+\) interacts with the Na\(^+\)/Cl\(^−\)-dependent GABA transporter, GAT1, under two conditions: in the absence of Na\(^+\) it induces a voltage-dependent leak current; in the presence of Na\(^+\) and GABA, Li\(^+\) stimulates GABA-induced steady-state currents. The amino acids directly involved in the interaction with the Na\(^+\) and Li\(^+\) ions at the so-called “Na\(^+\)” binding site have been identified, but how Li\(^+\) affects the kinetics of GABA cotransport has not been fully explored. We expressed GAT1 in Xenopus oocytes and applied the two-electrode voltage clamp and \(^{22}\)Na uptake assays to determine coupling ratios and steady-state and presteady-state kinetics under experimental conditions in which extracellular Na\(^+\) was partially substituted by Li\(^+\). Three novel findings are: 1) Li\(^+\) reduced the coupling ratio between Na\(^+\) and net charge translocated during GABA cotransport; 2) Li\(^+\) increased the apparent Na\(^+\) affinity without changing its voltage dependence; 3) Li\(^+\) altered the voltage dependence of presteady-state relaxations in the absence of GABA. We propose an ordered binding scheme for cotransport in which either a Na\(^+\) or Li\(^+\) ion can bind at the putative first cation binding site (Na\(^2\)). This is followed by the cooperative binding of the second Na\(^+\) ion at the second cation binding site (Na\(^1\)) and then binding of GABA. With Li\(^+\) bound to Na\(^2\), the second Na\(^+\) ion binds more readily GAT1, and despite a lower apparent GABA affinity, the translocation rate of the fully loaded carrier is not reduced. Numerical simulations using a nonrapid equilibrium model fully recapitulated our experimental findings.

cotransport; GABA transporter; kinetic model

the Na\(^+\)/Cl\(^−\)-dependent GABA transporter 1, GAT1, is one of the benchmark transporters in the family of neurotransmitter transporters belonging to the solute carrier 6 gene family (SLC6) (1, 16). The physiological role of GAT1 is to remove GABA (\(\gamma\)-aminobutyric acid) from synaptic clefts upon presynaptic GABA release, and thereby it is an important component in inhibitory neuroactivity. In common with other secondary-active membrane transport proteins that couple uphill movement of substrates to downhill movement of cations (Na\(^+\) or H\(^+\)), a fixed stoichiometry has been proposed for GAT1 with 2 Na\(^+\), 1 Cl\(^−\), and 1 GABA being cotranslocated for each transport cycle (21, 35). Another common feature that has been proposed for these transporters is the ordered binding of solutes, whereby cations bind sequentially to the transporter before the substrate and then translocation of cations and substrate is accomplished by a reorientation of the fully loaded carrier (5, 30, 31, 41). With respect to GAT1, it still remains unclear where in the sequence of partial reactions Cl\(^−\) and GABA interactions occur (3–5, 8, 17, 24, 28, 47).

The two Na\(^+\)-binding sites of GAT1 have been mapped and the coordinating amino acid residues have been ascribed to the “Na\(^2\)” and the “Na\(^1\)” sites according to the Na\(^+\) binding sites of the bacterial homolog, LeuT\(_{\alpha\alpha}\) (44, 45). Na\(^2\) is most likely the first (low apparent affinity) cation binding site, with which both Na\(^+\) and Li\(^+\) ions interact (see below), and Na\(^1\) most likely corresponds to the second (high apparent affinity) cation binding site with which Na\(^+\) exclusively interacts (17, 27). However, in a more recent study the order of Na\(^+\) interaction at the two sites was proposed to be opposite (45).

The Li\(^+\)-induced leak current attributed to GAT1 (28, 29) is apparently due to Li\(^+\) interactions at Na\(^2\) (19, 27, 45). With Li\(^+\) bound, and at sufficiently hyperpolarizing membrane voltages, the transporters occupy conformations that favor this leak mode (32). However, the GABA-induced steady-state currents are strictly dependent on external Na\(^+\) (28), which implies that Li\(^+\) alone cannot induce the conformation that allows binding and translocation of GABA (32). The Li\(^+\)-induced leak current is blocked noncompetitively by low concentrations of Na\(^+\) (\(K_I\) of 2–3 mM) (19), which suggests that with Li\(^+\) bound at Na\(^2\), the transporter is then only able to bind Na\(^+\) at Na\(^1\). With Na\(^1\) occupied, the Li\(^+\) leak is blocked and the protein is placed in conformations that allow binding and cotransport of GABA (27, 32, 45). This strict dependence on Na\(^+\) to drive cotransport contrasts with the Na\(^±\)/glucose cotransporter (SGLT1), the Na\(^+\)/dicarboxylate transporter and the excitatory amino acid transporter for which Li\(^+\) alone is capable of fulfilling the role as driving cation (6, 18, 33).

Cotransporter-associated presteady-state currents indirectly report events at the molecular level such as conformational changes in response to voltage and cation binding/debinding (15, 28, 29, 34). These currents are observed in electrogenic cotransporters that mediate transport of a wide range of substrates, e.g., GAT1 (30), GAT4 (20), SGLT1 (23), the Na\(^+\)/Pi-cotransporter (9), the Na\(^+\)/dicarboxylate transporter (33), the excitatory amino acid transporter (42), and the Na\(^+\)/P\(_c\)-cotransporters (NaPi-IIa, -b) (11, 12). Although these cotransporters are unrelated in their primary structures, they nevertheless show similar voltage and Na\(^+\)-dependent charge movements in the presence of Na\(^+\) and no other substrates. Moreover, specific blockers of transport function also block the relaxations. In particular, for GAT1, the Na\(^+\) interaction results in very slow charge relaxations (typically >100 ms at ~100 mV, 20°C) that are significantly slower than the capacitive charging transient (typically <1 ms) (29, 30). The interactions of Li\(^±\) with GAT1 are also reflected in presteady-state charge relaxations but appear to be faster (<20 ms) than the relaxations observed for Na\(^+\) (e.g., 14, 32). The Li\(^+\) effect on GAT1 presteady-state kinetics was previously investigated (14); however, only a qualitative interpretation of the data was reported.

In the present study, we provide a new approach to unraveling the kinetics of the two Na\(^+\) binding sites; furthermore we demonstrate that: 1) Li\(^+\) can substitute for the first Na\(^+\) ion that binds and 2) Li\(^+\) is cotransported by GAT1 together with Na\(^+\) and GABA. From a detailed analysis of steady-state and
presteady-state data, we propose a kinetic model that successfully accounts for the experimental findings for GAT1 in the forward transport mode. Taken together, our findings are consistent with strictly ordered binding of two Na\(^{+}\) ions, the first of which binds at Na2. We suggest that this site is a nonselective low-affinity cation binding site where Na\(^{+}\) and Li\(^{+}\) compete for occupancy. Our methodology and findings could be applicable to the investigation of multiple cation interactions in other electrogene solute carriers.

**MATERIALS AND METHODS**

*Ethical approval.* Procedures for animal handling and harvesting *Xenopus laevis* oocytes were approved in writing by the University of Zurich and the Swiss Federal Veterinary Authorities.

*Materials and reagents.* Modified Barth’s solution (88 mm NaCl, 1 mm KCl, 0.41 mm CaCl\(_2\), 0.82 mm MgCl\(_2\), 2.5 mM NaHCO\(_3\), 2 mM Ca(NO\(_3\))\(_2\), and 7.5 mm HEPES, pH 7.4) adjusted with Tris supplemented with 5 mg/l doxycycline was used for incubation of *X. laevis* oocytes. Experimental solutions were based on three standard solutions containing: 2 mM KCl, 1 mM MgCl\(_2\), 10 mM HEPES, Tris pH 7.4, and either 100 mM NaCl (100Na), 100 mM choline Cl (100Ch), or 100 mM LiCl (100Li). In cation substitution experiments NaCl was equimolarly substituted with either choline-Cl or LiCl; the solutions were obtained by mixing 100Na with 100Ch or 100Li in appropriate proportions to maintain the same cationic strength of 100 mM. The solutions with intermediate cation concentrations are referred to as, e.g., 20Na80Ch and 20Na80Li for solutions containing 20 mM NaCl + 80 mM choline-Cl and 20 mM Na + 80 mM LiCl, respectively. In experiments where one cation concentration was kept constant and the other varied, the ionic strength was maintained by addition of choline-Cl. For experiments with low Cl\(^{-}\) concentrations, Na-glucosinate replaced NaCl in the standard solution. GABA was added to solutions on the day of the experiment from a stock solution alone from the steady-state current obtained in the test solution and subtracting the steady-state current obtained in the test solution containing saturating [GABA] (from 500 to 3,000 \(\mu\)M).

For Li\(^{+}\) activation, [Na\(^{+}\)] and [GABA] were kept constant while [Li\(^{+}\)] was varied. Data described above were represented as current-voltage relationships (\(I_{GABA}\) vs. \(V_{m}\)). For the above experiments, steady-state transport kinetics were determined by reploting \(I_{GABA}\) as a function of either [GABA], [Na\(^{+}\)], or [Li\(^{+}\)] for each membrane voltage tested following by fitting with a modified form of the Hill equation:

\[
I_{GABA} = I_{max} \times \left[\frac{[S]}{[S]^H + ([S]^H + (K_{0.5}^H)^H)}\right] + I_{OFFSET}_{GABA}
\]

where \(I_{max}\) is the maximal GABA-induced current, \([S]\) is the concentration of the variable substrate (GABA, Na\(^{+}\), or Li\(^{+}\)), \(n_H\) is the Hill coefficient, and \(K_{0.5}^H\) is the apparent affinity constant for the substrate, \(S\). For GABA activation the Hill coefficient was unity, so the modified Hill equation was reduced to a form of the Michaelis-Menten equation. The offset term, \(I_{OFFSET}_{GABA}\), = 0, except for experiments where there was a residual \(I_{GABA}\) (detected prior to addition of the variable substrate) (e.g., data in Fig. 3).

**Presteady-state currents.** Presteady-state currents were acquired from a pulse protocol where the membrane voltage, \(V_{m}\), initially was held at −40 mV and then stepped to a series of test potentials (from +60 to −160 mV with 20 mV increments) for 400 ms before returning to −40 mV. At each test potential, the transporter-specific presteady-state currents of the “On” relaxations (following a voltage step from the holding potential to the test potentials) were extracted by fitting the relaxation currents to a single or double exponential decay function. In experiments with Li\(^{+}\) present in the absence of Na\(^{+}\), the “Off” relaxations (i.e., in response to voltage steps from the test potentials back to the holding potential) were analyzed because the ON relaxations to potentials below −60 mV were superimposed on the intrinsic Li\(^{+}\)-dependent leak, the activation time course of which is unknown. The fitting procedure was started ~4 ms after the voltage jump onset. At this time point, the capacitive transients arising from the oocyte membrane have decayed. To recover all charge movements arising from the transporter, the fitted curve was extrapolated back to the time at which \(V_{m}\) had reached 80% of its value (~1 ms after onset). From such fits the transporter-specific charge movement, \(Q\), was calculated as the time integral of the transient currents. The charge-voltage (\(Q\) vs. \(V_{m}\)) relationships were fitted with a form of the Boltzmann equation:

\[
Q = Q_{max} + \frac{Q_{max}}{1 + \exp \left[\frac{\epsilon(V_{m0} - V_{m})}{kT}\right]}
\]

where \(Q_{max}\) is the total charge available to move, \(Q_{max}\) is the charge at the hyperpolarizing limit and a function of the holding potential, \(\epsilon\) is the elementary charge, \(k\) is the Boltzmann’s constant, \(T\) is the absolute temperature, \(V_{m0}\) is the membrane potential at which 50% of the movable is displaced and \(z\) is the apparent valence of the movable charge. To visualize the effect of changing [Na\(^{+}\)] or [Li\(^{+}\)] on \(Q_{max}\), the \(Q\) vs. \(V_{m}\) data obtained in each test solution were fit with Eq. 2, offset by \(Q_{max}-Q_{on}\), and then normalized to \(Q_{max}\) predicted from the fit to the 100Na data set.

**Combined radiolabeled \(^{22}\)Na- and \([\text{H}]\)GABA uptake and TEVC.** The TEVC instrumentation comprised an OC-725C oocyte clamp (Warner Instruments) interfaced to a data acquisition system (Digidata 1322A, Molecular Devices) controlled by pClamp8 (Molecular Devices). The oocyte was placed in a superfusion chamber with an effective volume of 24 \(\mu\)l and superfused at a rate of 100 \(\mu\)l/min using a peristaltic pump, with excess superfused solution removed by a suction pump. The oocyte was impaled and the membrane voltage clamped in the range −80 to −50 mV and then superfused with one of three different test solutions (20Na80Ch, 20Na80Li, 5Na95Li) until a stable base line was reached. The oocyte was exposed to the same test solution containing 500 \(\mu\)M GABA and \(^{22}\)Na (final specific
activity 0.3 mCi/mmol, Perkin Elmer) or to the 20Na80Li solution containing 500 μM GABA and 60 nM [3H]GABA (Hartmann Analytic) for 5–10 min; in this period the flow rate was reduced to 5 μl/min to minimize the use of radioisotope; washout of radioactive tracers and GABA was done with the test solution. After the baseline was returned to starting level, the oocyte was removed from the chamber and washed three times in ice-cold 100Ch solution. The oocyte was lysed in a vial containing 250 μl of 4% SDS to which scintillation cocktail was then added. The amount of 22Na or [3H]GABA in individual oocytes were determined by scintillation counting (Tri-Carb 2900TR, Packard) and the molar uptake of Na⁺, 3H[GABA], or GABA, n[GABA], was calculated. The net charge transferred (Qt) was calculated from the current trace by subtracting the endogenous holding current and numerically integrating the area under the baseline-corrected curve. The molar equivalent of Qt was calculated from the Faraday constant. Accordingly, Na⁺ or GABA uptake and Qt were expressed as their molar equivalents.

Data analysis and software. Data analysis was performed with Clampfit version 10.2 (Molecular Devices) and Prism version 4.03 (Graphpad Software). Data points are shown as means ± SE. Errors smaller than the symbol size are not shown. Numerical simulations were performed using Berkeley Madonna V8.0.2a8 software (http://www.berkeleymadonna.com).

RESULTS

Li⁺ changes the apparent GABA affinity. Current-voltage relationships of GABA-induced steady-state currents (IGABA-Vm) obtained upon equimolar substitution of Na⁺ with either choline or Li⁺ revealed different effects on the magnitude and voltage dependence of the IGABA compared with the control condition (100Na, 500 μM GABA). In 20Na80Ch and [GABA] varying in the range 1–1,000 μM, IGABA-Vm showed a monotonic voltage dependence with no evidence of rate-limiting behavior at hyperpolarizing potentials down to −160 mV, unlike the control condition. Moreover, IGABA was reduced at a given Vm, even at saturating GABA concentrations (Fig. 1A). In contrast, with Li⁺ substitution, the shape of IGABA-Vm was preserved and IGABA at saturating [GABA] (1,000 μM) was similar for 20Na80Li and the control condition (Fig. 1B). For superfusion with 5Na95Li and [GABA] varying in the range 1–3,000 μM, the voltage dependence was maintained at all [GABA], however, IGABA at a given Vm was slightly lower compared with control conditions (Fig. 1C). We also determined the [GABA] dependence of the IGABA in 40 mM Na and either 60 mM choline or Li⁺, and this yielded identical IGABA-Vm behavior (data not shown).

From these data, we derived the GABA-dependent activation relationship for different holding potentials (IGABA-[GABA] relationships, not shown) by transposing IGABA-Vm data and then fitting with the Michielis-Menten form of the Hill equation (Eq. 1) to determine the apparent GABA affinity (K0.5GABA), the maximal electrogenic transport rate at infinite [GABA], Imax and their respective voltage dependencies (Fig. 1, D and E). For superfusion with 40Na60Ch and 40Na60Li, we obtained comparable estimates for K0.5GABA as previously reported with 100Na, e.g., Ref. 28 (not shown). However, for superfusion with 20Na80Li, K0.5GABA decreased (varying from 34 μM at −80 mV to 43 μM at −160 mV)

![Fig. 1. Li⁺ decreases the apparent GABA affinity.](http://ajpcell.physiology.org/)
compared with that obtained using 20Na80Ch (varying from 16 μM to 25 μM over the same voltage range) (Fig. 1D). For 5Na95Li, the apparent GABA affinity was even lower, with $K_{GABA}^{Na}$ varying between 170 and 158 μM at −80 and −160 mV, respectively. Furthermore, the normalized $I_{max}$ had the same voltage dependence when Na$^+$ was substituted with Li$^+$, whereas a reduction in the [Na$^+$] alone (choline substitution) changed the voltage dependence of the $I_{max}$ at the voltages tested (Fig. 1E).

Li$^+$ substitution changes the apparent Na$^+$ affinity but not its voltage dependence. We determined the Na$^+$ dependence of the GABA-induced current when the substituting ion was either choline or Li$^+$ (from 100 to 20 mM of Na$^+$) using a GABA concentration of 1,000 μM, since this should be saturating at all tested conditions. For choline substitution, the amplitude of $I_{GABA}$ was strongly dependent on the [Na$^+$] and did not reach saturation at the highest Na$^+$ concentrations, e.g., $I_{GABA}$ was not identical in 80Na20Ch and 100Na (Fig. 2A, left). Furthermore, the voltage dependence of $I_{GABA}$ was affected by the change in [Na$^+$]; for all conditions tested, $I_{GABA}$ increased with hyperpolarization, but saturation was observed only for [Na$^+$] ≥ 40 mM at more negative potentials. In contrast, Li$^+$ substitution resulted in $I_{GABA}$ that was less affected by the reduction in [Na$^+$]: the magnitude of $I_{GABA}$ at a given $V_m$ was higher in 5Na95Li compared with 5Na95Ch (Fig. 2A) and $I_{GABA}$ detected in 20Na80Li, was similar to that observed in the control condition (Fig. 2A, right). Furthermore, $I_{GABA}$ showed saturation with negative membrane potentials in all Li$^+$ substituted solutions.

To compare kinetic parameters from the Na$^+$ activation experiments, we fitted the dose dependence ($-I_{GABA}$-[Na$^+$] relationships) with a form of the Hill equation (Eq. 1). The $-I_{GABA}$-[Na$^+$] relationships and the Hill fits (continuous lines) at different voltages are shown in Fig. 2B. For choline substitution experiments, the fitting procedure worked best with a fixed $I_{max}$ (independent of $V_m$) and a Hill coefficient, $n_H > 1.2$ (Fig. 2B, left) in agreement with published data (28). For Li$^+$ substitution experiments, fitting was best achieved with $I_{max}$ unconstrained and $n_H = 1$ (Fig. 2B, right). The estimates of the apparent Na$^+$ affinity, $K_{GABA}^{Na}$, obtained with both choline and Li$^+$ substitution were strongly voltage dependent with values ranging from 21 ± 2.2 mM (−140 mV) to 365 ± 18.8 mM (−20 mV) ($n = 4$) for choline substitution and from 1.3 ± 0.5 mM (−140 mV) to 16 ± 2.0 (−20 mV) ($n = 4$) for Li$^+$ substitution (Fig. 2C). To quantify the voltage dependence of $K_{GABA}^{Na}$, we followed the procedure of Mager et al. (28) and replotted the data on a semilog scale (Fig. 2C, inset). This manipulation revealed a linear relationship between $lnK_{GABA}^{Na}$ and $V_m$ for data obtained from both substitution conditions over the range of test potentials from −160 to 0 mV. Linear regression fits to the

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**Fig. 2.** Apparent Na$^+$ affinity, but not its voltage dependence, is increased by Li$^+$. A: $I_{GABA}$-$V_m$ relationships obtained at saturating [GABA] (1,000 μM) and varying extracellular cation conditions: Na$^+$ was equimolarly substituted with choline (left) or Li$^+$ (right) as indicated. Data are from 1 representative GAT1 expressing oocyte and points are joined for visualization. B: dose response curves for estimation of apparent affinities. The $-I_{GABA}$-[Na$^+$] relationships are shown different voltages (as indicated) for choline substitution (left) and Li$^+$ substitution (right). Data points were fitted with the Hill equation (lines). Data are from 1 representative GAT1-expressing oocyte. Comparison of voltage dependence of $K_{GABA}^{Na}$ (C) and $I_{max}$ (D) for equimolar substitution of Na$^+$ with choline (Ch subst) or Li$^+$ (Li subst) (means ± SE, $n = 4$). Inset: a replot of $K_{GABA}^{Na}$-$V_m$ relationships on a semilogarithmic scale. The lines are linear regression fits to the data points. The slopes of the 2 lines were identical: 0.023 ± 0.001 ln(mM)/mV, corresponding to an e-fold change in $K_{GABA}^{Na}$ per 43 mV.

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**Caption: AJP-Cell Physiol** • doi:10.1152/ajpcell.00446.2011 • www.ajpcell.org
data points predicted identical slopes: there was an e-fold change in $K_{20Na}^{0.5}$ for a change in membrane potential $\approx 45$ mV, independent of the substituting cation. The vertical shift between the two fitted lines reflects the 20-fold decrease in $K_{20Na}^{0.5}$ in the presence of Li$^+$. For example, at 0 mV $K_{Na}^{0.5}$ (apparent Na$^+$ affinity at 0 mV) decreased from 544 $\pm$ 1.0 mM (choline substitution) to 24.5 $\pm$ 1.1 mM (Li$^+$ substitution) ($n = 4$). $I_{\text{max}}$ predicted from the Hill fits (Fig. 2D) reveals the differences in cotransport behavior for each ion substitution condition. For choline substitution $I_{\text{max}}$ was voltage independent over the voltage range applied: this behavior suggested that the rate-limiting step in the GABA cotransport cycle was voltage independent. In contrast, for Li$^+$ substitution, $I_{\text{max}}$ simply reflected the $I_{\text{GABA}}V_m$ relation at 100Na (0 Li) and saturating GABA (control condition). At hyperpolarizing potentials, the predicted $I_{\text{max}}$ for the two substitution conditions coincide, as expected (Fig. 2D).

Determination of the apparent Li$^+$ affinity during cotransport. To estimate the apparent Li$^+$ affinity in the cotransport mode, we fixed [Na$^+$] = 20 mM and varied [Li$^+$] in the range 5–80 mM. For each condition, we measured the $I_{\text{GABA}}$ at different voltages and saturating [GABA] (data not shown). As expected, the $-I_{\text{GABA}}$-[Li$^+$] relationship exhibited a residual component of cotransport activity for 20Na0Li (Fig. 3A). This offset current was taken into account when fitting the data with the Hill equation (Eq. 1). We obtained reliable fits with $n_H$ constrained to 1. Both “Li-Na-activated” and “Na-Na-activated” (20Na0Li) GABA-currents ($I_{\text{GaBA}}$ and $I_{\text{GABA}}$, respectively) were strongly voltage dependent (Fig. 3B). These data are displayed as the fraction of the maximal current ($I_{\text{max}}$ obtained from the fit) that resulted from $I_{\text{GABA}}$ and $I_{\text{LiNa}}$. At more positive membrane voltages, $I_{\text{LiNa}}$ contributed most to the overall electrogenic response.

From the above experiment and its complement (20 mM Li$^+$ constant, variable [Na$^+$]; data not shown), we obtained estimates of the apparent Li$^+$ affinity in the presence of low concentrations of Na$^+$ and vice versa (Fig. 3C). As above, the voltage dependence of $K_{di}^{Na}$ and $K_{di}^{Li}$ were quantified from the semilog plot of the data ($n_{H}, V_m$ relations) (Fig. 3C, inset), and based on the linear regression fit, the voltage dependence of $K_{di}^{Na}$ showed an e-fold change per 90 mV compared with a 32 mV per e-fold change in $K_{di}^{Li}$. The linear regression fits intersected at $-120$ mV and at 0 mV, the fit predicted $K_{Li}^{0} = 92 \pm 1.1$ mM and $K_{Na}^{0} = 1.039 \pm 1.1$ mM.

Uptake experiments under voltage clamp conditions. The hypothesis that Li$^+$ interacts with the GABA transporter and under some conditions substitutes for a Na$^+$ ion during Na$^+$/GABA cotransport is strongly supported by the above results as well as previous studies (19, 27, 45). To provide a more direct test of this hypothesis, we performed $^{22}$Na uptake experiments under voltage-clamp conditions and correlated the molar uptake of Na$^+$ ions [Na$^{2+}$] to net charge translocated ($Q_t$) during GABA-cotransport. From these experiments we obtained the Na$^+$-to-Qt coupling ratio, $CR_{Q_t}^{Na}$, at different cation conditions. Experiments were conducted under three different superfusion conditions: 20Na80Ch, 20Na80Li, and 5Na95Li; experiments with 5Na95Ch were excluded as both $Q_t$ and $^{22}$Na uptake were considered to be too close to, or below detection limits. For individual oocytes, the time of exposure was chosen in the range 5–10 min and constant holding potential, to obtain a broad range of $CR_{Q_t}^{Na}$ to allow linear regression analysis. A

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1 To our knowledge a direct measurement to test this hypothesis has not been reported; it would require radioactive Li$^+$-uptake experiments and correlation with e.g. [H]$GABA$ uptake or net charge translocation. However, a half-time of 838 ms for $^8$Li, the most stable radioisotope of Li, excluded this approach.
representative current trace obtained during $^{22}\text{Na}$ superfusion is shown in Fig. 4A. The $\text{Na}^+ - Q$, relationships for individual oocytes were combined for each condition and linear regression was applied to obtain $CR_{Q Na}^N$: With choline substitution ($20\text{Na}80\text{Ch}$) related data points gave a $CR_{Q Na}^N = 1.1 \pm 0.072$ (slope ± SE of linear regression line, $n = 6$), in agreement with previously published data (24). $\text{Li}^+$ substitution resulted in a $CR_{Q Na}^N < 1$, which varied with the relative activities of $\text{Li}^+$ and $\text{Na}^+$ in the external medium: For $20\text{Na}80\text{Li}$ the slope $= 0.71 \pm 0.02$ and for $5\text{Na}95\text{Li}$ the slope $= 0.53 \pm 0.04$ (slope ± SE of linear regression, $n \approx 7$; Fig. 4B). These data indicated that $\text{Li}^+$ reduced $CR_{Q Na}^N$ and thereby strongly support our hypothesis that $\text{Li}^+$ is cotransported with $\text{Na}^+$ and GABA.

We also tested if the coupling ratio between GABA and $Q$, $CR_{Q GABA}^{\text{GABA}}$, was affected by substitution of $\text{Na}^+$ with $\text{Li}^+$. This series of experiments was conducted as described above, but with $[^3\text{H}]\text{GABA}$ instead of $^{22}\text{Na}$ as the tracer in the uptake solution. As the charge-GABA ratio for $\text{Na}^+$-only conditions was previously established to be two charges per GABA, independent of membrane voltage, e.g., Refs. 10, 26), we performed uptake experiments in $20\text{Na}80\text{Li}$ and 500 $\mu\text{M}$ GABA and analysis gave $CR_{Q GABA}^{\text{GABA}} = 0.45 \pm 0.035$ (slope ± SE of linear regression, $n = 7$) (data not shown). This result confirmed that two charges are translocated together with GABA, even if most of the $\text{Na}^+$ were substituted with $\text{Li}^+$. $\text{Li}^+$ interactions alter the kinetics of the voltage-dependent partial reactions. To gain further insight into how $\text{Li}^+$ and $\text{Na}^+$ determine the voltage dependence of the transport cycle, we analyzed the presteady-state currents induced by step changes in the oocyte membrane voltage (see MATERIALS AND METHODS). These capacitive transient currents result from 1) charging the oocyte membrane; 2) voltage-dependent conformational changes and cation binding/debinding within the expressed proteins (15, 28, 29, 34) and are not seen in noninjected oocytes (Fig. 5). Recordings obtained from the same representative GAT1-expressing oocyte under five different superfusion conditions (100CH, 100Na, 100Li, 100Na90Ch, or 10Na90Li) indicated that the GAT1-dependent relaxations varied significantly according to the cation substitution, with four features being readily discernible from the original recordings (Fig. 5). First, in the absence of external $\text{Na}^+$ or $\text{Li}^+$ (100CH), we observed small relaxations that suggested the presence of charge movements associated with the GAT1 empty carrier alone. These currents were easily distinguishable from those recorded from noninjected oocytes from the same donor frog and in the same solution (100CH) (Fig. 5A). Second, substitution of 100CH with 100Na, resulted in the typical GAT1-associated relaxations (29, 30) (Fig. 5B, left). For the voltage step protocol used (Fig. 5A, inset), these relaxations were approximately symmetrically distributed around the holding potential. Third, substitution of 100Na with 100Li resulted in very fast and small GAT1 associated relaxations that showed significant asymmetry (Fig. 5B) (14, 32). Note that the ON relaxations were superimposed on the $\text{Li}^+$-induced leak current at hyperpolarizing potentials, but their features (asymmetry and time course) were readily visible in the OFF currents at the holding potential ($\approx \pm 60$ mV) at which the $\text{Li}^+$ leak was negligible (Fig. 5B, right). The OFF time constant ($\tau_{\text{OFF}}$) for the relaxations in 100Li to the holding potential of $\approx \pm 60$ mV was typically 6.6 ± 3.0 ms over the range of test potentials (from $\approx \pm 80$ mV). Fourth, with 10 mM $\text{Na}^+$ in the superfuse, the relaxations became strongly asymmetrically distributed compared with 100Na, as previously reported (7, 29, 30) (Fig. 5C, left). Moreover, with 90 mM $\text{Li}^+$ instead of choline, the asymmetry was slightly shifted, and the relaxations were faster, in agreement with (14) (Fig. 5C, right). This behavior suggested that $\text{Li}^+$ ions were able to interact with GAT1 in a voltage-dependent manner.

To quantitate these features, we fitted the current traces of the ON or OFF relaxations with exponential decay functions. The fits were integrated (see MATERIALS AND METHODS) to obtain the charge movement at each membrane potential. For the GAT1-injected oocytes superfused with 100Na, 100Li, and Na-choline solutions, the relaxations were well described by fitting with a single exponential to the currents after the endogenous charging of the oocyte capacitance was completed. However, when $\text{Li}^+$ was equimolarly substituted for $\text{Na}^+$, for $[\text{Li}^+] > 20$ mM, the relaxations were best fit using a double exponential (data not shown).

The normalized charge-voltage relationships ($Q-V_m$) obtained for 100Na, 100Li, and 100CH (Fig. 6A, left) show how the cation composition strongly affected the charge distribution: in particular, in 100Li, the $Q-V_m$ distribution was strongly shifted to hyperpolarizing potentials and saturation of the charge movement was not reached within the range of test.
voltages. For 100Ch, the total charge movement amounted to \(\sim10\%\) of that in 100Na.

The \(Q-V_m\) obtained in the different superfusates containing various [Na\(^+]\) and equimolar substitutions with Ch of Li\(^+\) (Fig. 6A, middle and right) were fitted with a form of the Boltzmann equation (Eq. 2), and the effect of Li\(^+\) substitution could be appreciated by comparing the three fit parameters \(Q_{\text{max}}\), \(z\) and \(V_{0.5}\) (Fig. 6B). To reveal the voltage-dependent shifts in the charge distribution, the \(Q-V_m\) data were normalized to \(Q_{\text{max}}\) in 100Na for equimolar substitution with choline or Li\(^+\) (Fig. 6A, middle and right). We obtained the most reliable fits (Fig. 6A, continuous lines), especially at lower [Na\(^+]\), by constraining the \(Q_{\text{max}}\equiv1\), whereas the slope factor, \(z\), was unconstrained. This gave fits for which the normalized \(Q_{\text{max}}\) was always close to unity except at the lowest [Na\(^+]\) (Fig. 6B, left); and \(z\) lay between 0.9 and 1 for all conditions, except with 10 and 5 mM Na\(^+\), where \(z\) was 0.75 \(\pm\) 0.1 and 0.59 \(\pm\) 0.1, respectively for choline substitution and 0.86 \(\pm\) 0.04 and 0.73 \(\pm\) 0.08, respectively for Li\(^+\) substitution (\(n=5\)) (Fig. 6B, middle). The most profound effect of the substitution of Na\(^+\) with choline or Li\(^+\) was observed for \(V_{0.5}\): for choline substitution from 100Na to 40Na60Ch there was a hyperpolarizing shift \(\sim50\) mV compared with a shift of \(\sim30\) mV from 100Na to 40Na60Li (Fig. 6A, middle and right). To quantify the dependence of \(V_{0.5}\) on [Na\(^+\)] with each substitution, we plotted the data on a semilog scale (\(V_{0.5}-\log[^{\text{[Na}\,^+]\text{]}\text{ relationships}}\), which revealed a linear relationship for both substitutions (Fig. 6B, right). Linear regression fits to these data yielded significantly different slopes: 120 \(\pm\) 7 mV/10 fold change in [Na\(^+]\) with choline substitution and 63 \(\pm\) 6.5 mV/10-fold change in [Na\(^+]\) for Li\(^+\) substitution. These slopes suggested that for choline substitution, two Na\(^+\) ions interacted with GAT1, whereas for Li\(^+\) substitution only one Na\(^+\) ion interacted (see DISCUSSION).

Presteady-state relaxations with mixed cation substitutions. We repeated the presteady-state assays by varying the concentra-
Li⁺ interaction with GAT1 shifts the midpoint voltage, \( V_m \), of the charge movement. \( A \): normalized \( Q-V_m \) relationships obtained by integration of the presteady-state currents at 100Na, 100Li, and 100Ch (left) and at different \([Na^+]\) for substitution with choline (middle) or \( Li^+ \) (right). Cation concentrations were varied as indicated in the figure. Data were normalized to \( Q_{max} \) predicted from the fit of 100Na for each individual oocyte (see MATERIALS AND METHODS). Left: means ± SE (\( n = 4 \)); middle and right: data from 1 representative GAT1-expressing oocyte. Continuous lines are fits using the Boltzmann equation (Eq. 2). \( B \): Boltzmann fit parameters: \( Q_{max}, V_m \) (left), \( z \cdot V_m \) (middle), and \( V_0.5-\log[Na^+] \) (right) relationships for substitution with choline (Na/Ch) or \( Li^+ \) (Na/Li). Data points are means ± SE, \( n = 6 \). The \( V_0.5-\log[Na^+] \) data were fitted with a linear regression line and the slopes ± se in mV/10 fold change in \([Na^+]\) are indicated. The 2 slopes were statistically different from each other (Student’s t-test, \( P = 0.001 \)).

The determination of the effective valence under these conditions was more prone to uncertainty due to a hyperpolarizing shift to the \( Q-V_m \).

2 The determination of the effective valence under these conditions was more prone to uncertainty due to a hyperpolarizing shift to the \( Q-V_m \).
constant at 20 mM or 5 mM and variable [Li⁺] the predicted slopes were 42 ± 6.8 and 40 ± 4.2 mV/decade, respectively.

DISCUSSION

The transport mechanism of GAT1 has been studied extensively throughout the last three decades, starting before its cloning with conventional radio tracer flux experiments by Kanner and coworkers (e.g., 21, 35) and later by means of steady-state and presteady-state voltage clamp assays applied to Xenopus oocytes heterologously expressing wild-type GAT1 (4, 7, 8, 10, 13, 28–30, 39) and in a cell culture system (5). Furthermore, a number of mutational studies have been conducted to elucidate both the functional characteristics as well as structure-function relationships of GAT1 (reviewed in

Fig. 7. Li⁺ interaction decreases the relaxation time constants. A–C: summary of presteady-state data [τ-V⁺m relationships (left) and Q-V⁺m relationships (right)] in GAT1-expressing oocytes obtained under 3 different conditions: A: constant 20 mM Li⁺, varying [Na⁺]. For the 20Li0Na, the arrow in τ-V⁺m indicates τ₀FF = 14.5 ± 2.7 ms at −40 mV for the voltage range −60 to −160 mV. B: constant 20 mM Na⁺, varying [Li⁺]. C: constant 5 mM Na⁺, varying [Li⁺]. Concentrations of the variable cations are given in the figure. Included in all data sets are 100Na serving as control. Data points are means ± SE, n ≥ 3. Q was normalized to the Qmax value obtained for 100Na, as described in MATERIALS AND METHODS. D: cation concentration dependencies of the z value obtained from the Boltzmann fitting (left) and V₀.5-log[Na⁺] relationships (right) obtained from right panels of A–C: 20 mM Li⁺, varying [Na⁺] (20Li var. Na), 20 mM Na⁺, varying [Li⁺] (20Na var. Li), and 5 mM Na⁺, varying [Li⁺] (5Na var. Li). Data sets were fitted with a linear regression line and the values of the slopes ± SE in mV/10 fold change in [Na⁺] are indicated. The 2 slopes obtained for the data sets with varying Li⁺ were statistically different from the slope obtained for the data set of varying Na⁺ (Student’s t-test, \( p < 0.005 \)).
Ref. 1). Recently, further insight into the location of the substrate binding sites and the spatial relationships of the coordinating amino acids (also reviewed in Ref. 1) were predicted from the three-dimensional structure of GAT1’s bacterial homolog LeuT<sub>aa</sub> (37, 38, 44, 46). To gain mechanistic insight into the transport process, kinetic models have been developed, some of which allow simulations to be performed that successfully predict the experimentally observed electrogenic activity (steady state and presteady state) (e.g., 5, 8, 10, 17, 30, 39). Other models are simply hypothesized kinetic schemes that lack an analytical basis (e.g., 4, 14, 19, 27, 32, 45, 47). Despite this plethora of information, several issues concerning GAT1 function remain unclear, two of which we address in this report, namely: the fate of Li<sup>+</sup> as a cosubstrate and the cation binding order during the forward cotransport cycle.

**Evidence that Li<sup>+</sup> can substitute for one Na<sup>+</sup> to drive GABA cotransport.** Experiments performed under voltage clamp conditions revealed that Li<sup>+</sup> reduced the effective coupling ratio, $CR_{Q_i}^{Na}$, during GABA cotransport (Fig. 4), whereas the presence of Li<sup>+</sup> did not change the $r^{GABA/Q_i}$ coupling ratio, $CR_{Q_i}^{GABA}$. These results offer compelling evidence that Li<sup>+</sup> ions can substitute for Na<sup>+</sup> to act as a driving cation, and furthermore they rule out the hypothesis that Li<sup>+</sup> may exit the transporter in a thermodynamically uncoupled manner. It has previously been established for GAT1 expressed in oocytes that under voltage clamp, $I_{GABA}$ is directly proportional to Na<sup>-</sup>-influx with $CR_{Q_i}^{Na} = 1$ (24). We confirmed this result in the present study with Na<sup>-</sup> alone and, moreover, established that $CR_{Q_i}^{Na}$ was independent of [Na<sup>-</sup>], as expected. We hypothesized that for all active GAT1, if one of the two cotransported Na<sup>+</sup> ions were substituted by a Li<sup>+</sup> ion this would define a lower limit for $CR_{Q_i}^{Na} = 0.5$. Moreover, intermediate values for $CR_{Q_i}^{Na}$ would be expected for different Li<sup>+</sup> and Na<sup>-</sup> activities in the external superfuse because of the mixed population of transporters in each mode as we observed for 20Na80Li (Fig. 4B).

To estimate the proportion of GAT1 operating in the Na-Na-GABA modes from the measured $CR_{Q_i}^{Na}$, the respective turnover rates must be known (see APPENDIX). To estimate the turnover rate with Na<sup>+</sup> as the sole active cation (no Li<sup>+</sup>), we need to know $I_{GABA}$ at the test V<sub>m</sub> and the number of active transporters; the latter can be found from the $Q_{max/ez}$ ratio, see (13). However, when Li<sup>+</sup> is also present, $I_{GABA}$ contains contributions from both modes and a more complex relationship is obtained, which depends on the ratio of the turnover rates in each mode (Eq. A4, APPENDIX). For superfusion in 5Na95Li, $CR_{Q_i}^{Na}$ was found to be $=0.5$, which, according to our hypothesis, indicates that $=100%$ of GAT1 operate in the Li-Na-GABA mode (Fig. 9). For 20Na80Li, $CR_{Q_i}^{Na} \approx 0.7$ at V<sub>m</sub> $=-80$ mV. There, we can use the ratio of $I_{GABA}$ in 20Na80Li to $I_{GABA}$, in 20Na80Ch (Fig. 2A) to estimate the ratio of turnover rates for each mode to be $\approx 2$, which would correspond to having at the most 40% of GAT1 operating in the Li-Na-GABA mode (Fig. 9).

It is important to note that the above considerations take no account of Cl<sup>-</sup> as a thermodynamically coupled substrate. Whereas there is considerable evidence to support the role of Cl<sup>-</sup> as a modulator of GAT1 activity (4, 7, 29), whether there is a net Cl<sup>-</sup> influx with a strict thermodynamically defined GABA/Cl<sup>-</sup> ratio of 1:1 as proposed in several studies (5, 7, 17, 21, 35), or Cl<sup>-</sup> is simply exchanged with no net flux as proposed by Loo et al. (24), remains unresolved. Recently, the Cl<sup>-</sup> interactions with GAT1 were further analyzed in mutational studies (3, 47), and it was proposed that the role of Cl<sup>-</sup> in the transport process was to compensate for the positive charges during substrate translocation and thereby facilitate transporter gating. Indeed our simultaneous 22Na flux and TEVC data, which confirm $CR_{Q_i}^{Na} = 1$, are not consistent with the existence of a thermodynamically coupled net Cl<sup>-</sup> influx, which would have given $CR_{Q_i}^{Na} = 0.5$. Our data rather support the Cl<sup>-</sup> exchange model (24), in which external chloride ions modulate GAT1-mediated cotransport. Although the original experiments (21, 35) using preparations from rat brain certainly provided compelling evidence that GABA transport was thermodynamically coupled to Na<sup>+</sup> and Cl<sup>-</sup> influxes, with an overall coupling ratio of 2Na<sup>+</sup>/1Cl<sup>-</sup>/1GABA, discrepancies may possibly arise from differences in experimental procedures and the respective expression systems used. Experiments to fully resolve this issue are beyond the scope of the present study.

**Revisiting apparent Na<sup>+</sup> affinities and identifying sequential cation binding.** Our steady-state TEVC assays have allowed us to separate the cation interactions into sequential steps, whereby Na<sup>+</sup> is occupied first followed by Na<sup>+</sup>. We obtained estimates of the apparent Na<sup>+</sup> affinities for each site based on the difference in apparent affinities for Na<sup>+</sup> and Li<sup>+</sup>. As apparent affinities are a function of all partial reactions in the transport cycle, it was essential in these experiments, to maintain a saturating [GABA], particularly as we found that Li<sup>+</sup> substitution significantly decreased $K_{0.5}^{Na}$ compared with control conditions (Fig. 1D). This precaution has not always been consistently addressed in previous studies (e.g., 19, 27) and could lead to potential misinterpretations of the cation interaction kinetics. The $I_{GABA}/V_m$ relationships from Na<sup>+</sup> activation experiments were very different for choline and Li<sup>+</sup> substitutions and provided clear evidence that Li<sup>+</sup> interacted with GAT1 to modulate its transport properties (Fig. 2A). That the $I_{GABA}/V_m$ relationships had the same voltage dependence as control conditions indicated that the presence of Li<sup>+</sup> did not alter the intrinsic voltage dependence of Na<sup>+</sup> interactions, but simply resulted in a 20-fold scaling of $K_{0.5}^{Na}$ (Fig. 2C). Indeed our data suggest that $K_{0.5}^{Na}$ found in the Li<sup>+</sup> substitution experiments represents the apparent Na<sup>+</sup> affinity for Na<sup>+</sup> and it follows that the $K_{0.5}^{Na}$ obtained from the choline substitution experiments largely reflects the interaction of Na<sup>+</sup> ions at Na<sup>2</sup>.

Moreover, the dose dependencies (Fig. 2B) show a strong dependence on the substituting cation. With Li<sup>+</sup> substitution, the maximal $I_{GABA}$ was reached already between 20 and 40 mM Na<sup>+</sup>, resulting in a higher $K_{0.5}^{Na}$ and $n_H = 1$, which would be consistent with Li<sup>+</sup> replacing one of the two Na<sup>+</sup> ions.

**Presteady-state charge movements confirm the interaction of Li<sup>+</sup> ions with the first cation binding site.** Further support for the sequential cation interaction scenario came from interpretation of presteady-state relaxations. Our data show that the voltage dependence of the cotransport cycle is profoundly influenced by the Li-Na-GABA transport cycle. This can be directly attributed to altered kinetics of partial reactions that precede GABA binding and those carrying charge are manifest as presteady-state relaxations. In addition to the easily discernible Na<sup>+</sup>-dependent relaxations (29, 30), we could also detect relaxations in 100Ch and 100Li. These were smaller in magnitude and generally faster than those observed when Na<sup>+</sup> was present and their unambiguous detection was only possible by
taking precautions with the voltage clamp tuning and micro-electrodes. That we observed relaxations associated with heterologously expressed GAT1 in 100Ch, indicated that the empty carrier underwent conformational changes in response to changes in membrane potential, analogous to those hypothesized for other cotransporters e.g., SGLT1 (15, 23), as well as NaPi-IIa (11) and NaPi-IIb (12).

Of particular significance was the detection of relaxations in 100Li (Fig. 5B). This further supports our hypothesis that Li$^+$ ions alone interact with GAT1 by binding at the Na2 site. The Q-Vm data for Li$^+$ in the absence of Na$^{+}$ (Figs. 6A, 7A) revealed a charge distribution, the equilibrium of which was strongly shifted toward hyperpolarizing potentials compared with those when Na$^{+}$ was also present. The absence of saturation in this membrane potential range meant that we were unable to quantify the maximum charge displacement with confidence; however, for the 100Li case, the trend suggested that $Q_{\text{max}}$ in 100Li was ≤ $Q_{\text{max}}$ in 100Na (Fig. 6A). The interaction of Li$^+$ ions with GAT1 is also consistent with the findings of a previous study on the GAT1 Li$^+$/H11004 activation experiment. This is also reflected in the findings of a previous study on the GAT1 Li$^+$/H1001 substitution experiment. Li$^+$ substitution compared with choline substitution: Li$^+$ ions alone interact in a voltage-dependent manner at the first cation binding site (Na2).

Li$^+$ and Na$^{+}$ compete for Na2 occupancy. When both Li$^+$ and Na$^{+}$ were present, the voltage dependence of $I_{\text{GABA}}$-Vm-v in both GABA-activation (Fig. 1) and Na$^{+}$-activation experiments (Fig. 2) was changed with Li$^+$ substitution compared with choline substitution experiment. This is also reflected in the presteady-state charge movements: The presence of Li$^+$ significantly reduced the relaxation time constants, indicating that the Li$^+$ interactions are different from those of Na$^{+}$ (Fig. 7, A and B, left). In addition, the shift in the Q-Vm relation obtained by lowering [Na$^{+}$] was affected differently with Li$^+$ substitution compared with choline substitution: Li$^+$ appeared to partially compensate for the effect of lowering [Na$^{+}$] by reducing the shift in V0.5. Mechanistically, this means that for a hyperpolarizing voltage step from very positive potentials to a defined Vm, more charge is displaced with Li$^+$ substitution (Fig. 6). If Li$^+$ had substituted perfectly for Na$^{+}$ we would not expect any shift in V0.5 compare to the control condition (100Na). Moreover, the slope of the V0.5-log[Na$^{+}$] relationship ≈ 120 mV/10-fold [Na$^{+}$] with choline substitution, would support a model in which two Na$^{+}$ ions interact with GAT1, whereas the ≈ 60 mV/10-fold [Na$^{+}$] slope obtained with Li$^+$ substitution is consistent with the notion of only one Na$^{+}$ ion interacting with GAT1 after Li$^+$ occupies the Na2 site. Moreover, when Na$^{+}$ was fixed (5 and 20 mM) and Li$^+$ was the variable substrate (Fig. 7, B and C), the small depolarizing shift in Q-Vm with increasing [Li$^+$] correlated with the corresponding shift in the peak of $\tau$-Vm. This behavior is consistent with Li$^+$ ions contributing to the overall voltage dependence of the transport cycle by interacting at Na2.

We reasoned that we could estimate the apparent Li$^+$ affinity in the cotransport process by performing Li$^+$ activation experiments with a constant and relatively low [Na$^{+}$] (20 mM), which would nevertheless be enough to saturate Na1, as discussed above. From these experiments it became evident that the two cotransport pathways have different voltage dependences (Fig. 3B) and that Li$^+$ most likely interacts at Na2 with a higher apparent affinity than Na$^{+}$ (Fig. 3C), at least at the voltage range tested here.

Kinetic scheme to account for cation interactions and GABA cotransport. The cation interactions with GAT1 can be considered in terms of their effect on the presteady-state kinetics (in the absence of GABA) (shaded area of kinetic scheme, Fig. 8A) and the steady-state cotransport data, involving the complete cotransport cycle (Fig. 8A, complete scheme). By appropriate choice of forward and backward rate constants and valency factors associated with the voltage-dependent partial reactions, the numerical solutions to the associated set of differential equations were found to match the experimental findings very closely. The cotransport model is similar to models incorporating ordered substrate binding that have been used in numerical simulations of cation driven cotransport for GAT1 (5, 10, 17) and other Na$^{+}$-coupled cotransporters, e.g., SGLT1 (25), NaPi-IIb (2), and the Na$^{+}$-dependent serotonin transporter (36). In contrast to the previously published GAT1 models (5, 10, 17), chloride interactions have not been explicitly included in this scheme.

We first investigated the model under presteady-state conditions (Fig. 8A, shaded area) that limit the number of possible states to either 1) four states incorporating the empty carrier (C6$^+$+C1) and sequential binding of the two Na$^{+}$ ions (C1++C2++C3) or 2) six states incorporating the empty carrier (C6$^+$+C1) and sequential binding of either 2 Na$^{+}$ ions (C1++C2++C3) or one Li$^+$ ion and one Na$^{+}$ ion (C1++C2++C3$^+$). In this reduced model, the forward and backward rate constants, $k_{f}$ and $k_{b}$, and associated effective valency factors ($z_{ij}$) were chosen to simulate presteady-state relaxations such that the best visual match to the data over the range of experimental conditions used was obtained. Thus, the characteristic slow ON relaxations observed for superfusion in 100Na (Fig. 5B, left) arise from the slow rates of $k_{d}^{012} = 65\,s^{-1}$ and $k_{b}^{21} = 50\,s^{-1}$ and the significantly higher ratio of $k_{d}^{23}/k_{b}^{23}$ (40/M), which would result in the cooperative cation interaction to reach state C3. If the rates were exchanged to give reduced or negative cooperativity (e.g., by setting $k_{d}^{012} = 3,000\cdot M^{-1}\cdot s^{-1}$ and $k_{b}^{21} = 75\,s^{-1}$ and $k_{b}^{23} = 65\cdot M^{-1}\cdot s^{-1}$, $k_{d}^{32} = 50\,s^{-1}$), the simulated data matched poorly to the experimental data. This finding supports the hypothesis that the high-affinity Na$^{+}$-binding site (Na1) is occupied last but contrasts with the conclusions from another study based on steady-state data in which it was proposed that at low [Na$^{+}$], Na1 would be occupied before Na2 (45). The parameter set also allowed us to satisfactorily recapitulate the experimentally obtained linear dependence of V0.5 on log[Na$^{+}$] with a 120 mV/10-fold change in [Na] for equimolar choline substitution and a 60 mV/10-fold change in [Na] for equimolar Li$^+$ substitution (Fig. 8B, middle). Although we found it necessary to assign charge movement to all three partial reactions, the first Na$^{+}$ interaction (C1++C2), with an apparent valence of 0.7, contributed the bulk of charge movement. The valence factors obtained by fitting the simulated Q-Vm data (Fig. 8B, left) with a Boltzmann function lay close to 0.9 for [Na$^{+}$] in the range 5–100 mM in excellent agreement with the experimental findings (Fig. 6B).

With these parameters for the Na-Na pathway, the model was extended to include the Li interaction. To account for the shift in presteady-state charge distribution and faster ON re-
laxations observed when superfusion in e.g., 10Na90Li (Fig. 5C, right), both forward and backward rates of the Li (C1+C2) and Na (C2+C3) interactions were changed compared with the corresponding partial reactions in the Na-Na pathway. Specifically, a low apparent Li⁺ affinity in the absence of Na⁺ (27) and a higher affinity for the second Na⁺ to bind in the Na1 site in presence of Li⁺, were obtained by increasing $k_{2-1}$ (200 s⁻¹) in combination with an increased $k_{2-3'}$ (5,000 s⁻¹) and a decreased $k_{3-2'}$ (30 s⁻¹). These parameters also predicted the voltage dependence of the $V_{0.5}$, with the slope of 60 mV/10-fold change in [Na] for Li⁺ substitutions (Fig. 8B, middle). For both pathways, the dissociation constant for Na2 ($K_{d1} = k_{31}/k_{12}$) was much greater than the dissociation constant for Na1 ($K_{d2} = k_{32}/k_{23}$), which suggested that state C2 never accumulates and positive cooperativity exists between the two cation binding sites (e.g., 43).
To extend the model to account for the cotransport mode, three additional partial reactions were added to each of the presteady-state pathways, namely GABA binding (C3C4C6 and C3C5C4'), translocation (C4C5S and C4'C5S'), and cytosolic release of substrates (C5C6C6 and C5'C6C6) (Fig. 8A, complete scheme). In the cotransport scheme, the cation interaction steps were identical to those in the presteady-state scheme. We found it necessary to assign a slight voltage dependence (k23 = k34' = -0.015) to the GABA binding partial reaction to account for the voltage dependence of the apparent GABA affinity (Fig. 1D). The decrease in KGABAg caused by the presence of Li+ at higher concentrations was also accounted for by decreasing the backward rate 10-fold (from 1,000 for k23 to 100 for k42').

The translocation partial reactions, determined by k56, k57, and k47, which were all the same (5 s⁻¹ at 20°C), established the rate-limiting behavior and is in good agreement with previously published estimates (15 ± 2 s⁻¹, at -50 mV, 21°C) (13). Due to the uncertainty about the partial reactions for "inward facing" conformations of GAT1, we lumped the substrate binding and release transitions into one partial reaction (5+6). The forward rates (k56 and k87) were defined in terms of the other rate constants under conditions of zero driving force to satisfy the detailed balance (22, 34).

An important distinction between the presteady-state and cotransport models relates to the empty carrier transitions (C1 ↔ C6) (Fig. 8A, dark shaded rate constants). If the same rate constants were applied for the cotransport mode as for the presteady-state mode, I_{GABA} and Q showed the same voltage dependence. However, according to previously published data (e.g., Ref. 28, compare Figs. 3B and 7B therein, and Ref. 24, compare Figs. 3A and 4A therein) and confirmatory experiments performed in the present study (Fig. 8C), we observed that there was a hyperpolarizing shift of the I_{GABA}/V_m relation of ~70 mV compared with the Q-V_m relation. So despite the fact that the simulations successfully accounted for the steady-state findings with varying substrates and replicated the essential features observed for GABA, Na⁺, and Li⁺ activation, the voltage dependencies deviated significantly. In our simulations, we could readily account for this discrepancy by altering the rate constants associated with the empty carrier such that for the cotransport cycle, the return rate of the empty carrier to the outward facing conformation after substrate release (transition 6→1) was now slower than for the presteady-state model (i.e., absence of external GABA) (Fig. 8A). To test if this finding simply reflected the underdetermined nature of the model, we included an additional voltage-dependent transition in the cotransport cycle (e.g., C4C5S), but this gave unsatisfactory results. However, exchanging the forward and backward rate constants at zero voltage for the empty carrier, we obtained good predictions for I_{GABA}/V_m in the steady-state mode. Dependence of fraction of GAT1 in the Li-Na-GABA mode on different hypothetical turnover rate ratios (e = 5 vs. 20). Symbols representing different e are given in the figure. Points were joined for visualization. Dotted lines are shown for CRGABA= 0.5 and 0.7 to allow the fraction of transporters in the Li-Na-GABA mode to be predicted, based on our experimental estimates for CRGABA.

Fig. 8. A kinetic model for the GAT1 cotransport cycle to account for cation effects on presteady and steady-state kinetics. A: kinetic scheme to account for presteady- and steady-state data (shaded area); the presteady-state scheme comprises a sequence of 3 partial reactions represented by 4 putative conformational states: C6 (inward facing empty carrier), C1 (outward facing empty carrier), C2 or C2' (outward facing carrier with 2 Na⁺ or 1 Li⁺ and 1 Na⁺ bound, respectively). For the steady-state scheme, each cotransport cycle comprises a sequence of partial reactions between 6 putative conformational states. When the protein is in C1, 1 Na⁺ or 1 Li⁺ ion, and the cycle proceeds sequentially through the respective conformational states, indicated by nonprime and prime labeling, respectively. After the reaching C3 or C3' state, GABA binds to form the C4 or C4' (outward facing fully loaded carrier), leading to translocation of the substrates C5 or C5' into the cytosol (C5'C6 and C5'C6). Lumped together into transitions for simplicity. The voltage-dependent rate constants were defined by assuming a symmetrical barrier model based on Eyring transition state theory and at 20°C. All common rate constants for the 2 models are identical, except the rates of the empty carrier transition, k61 and k69 (dark shaded), which were exchanged (see DISCUSSION): k61 = 600 exp(-V/150) and k69 = 400 exp(-V/150) for the presteady-state model, and k61 = 400 exp(-V/200) and k69 = 600 exp(-V/250) for the cotransport model (these rate constants are indicated in boldface). For the Na-Na-GABA pathway, the rate constants were: k12 = 655 [Na⁺]₁ exp(-V/120), k23 = 500 exp(-V/210), k32 = 3,000 [Na⁺]₁ exp(-V/210), k34 = 75 exp(-V/250), k43 = 10,000 [GABA]₁ exp(-V/250), k42 = 100 exp(-V/350), k56 = 845, k57 = 10 s⁻¹. For the Li-Na-GABA pathway, the rate constants were: k12 = 655 [Na⁺]₁ exp(-V/120), k23 = 500 exp(-V/210), k32 = 3,000 [Na⁺]₁ exp(-V/210), k34 = 30 exp(-V/250), k43 = 10,000 [GABA]₁ exp(-V/250), k42 = 1,000 exp(-V/360), k56 = 845, k57 = 10 s⁻¹. All substrate concentrations are given in moles (M), and all rates are given in s⁻¹ or M⁻¹ s⁻¹. The forward rates at the cytosolic site (k56 and k57) were defined in terms of the other rate constants under conditions of zero driving force to satisfy the detailed balance. The effective valences for the charge displacing transitions were: k12 = 34 = 2.67, k12 = 32 = 0.17, and k34 = 3 = -0.015. For each give transport cycle the sum of the charge displacement equals 1. B: left: simulations of presteady-state charge distribution; Q-V_n of Na⁺ substituted with Li⁺ (top) or choline (bottom) as indicated; middle, top, V_n vs log [Na⁺] relationships obtained by equimolar substitution with choline (Na/Ch) or Li⁺ (Na/Li), right: simulated steady-state parameters: I_{GABA}[Na+] relationships with Li⁺ substitution (top) or choline substitution (bottom) at membrane voltages as indicated; middle, bottom, voltage dependence of K_{0.5} for choline and Li⁺ substitutions (Ch subst. and Li subst, respectively) obtained by fitting the simulated data with Eq. 1. C: comparison of the voltage dependence of normalized Q-V_n relationships obtained in 100 mM NaCl (Q NaCl) and in 100 mM NaGlu (Q NaGlu) with normalized I_{GABA}V_n relationship in 100 mM NaCl, 500 μM GABA (I_{GABA}). Arrow indicates a ~70 mV hyperpolarizing shift of I_{GABA}V_n in 100 mM NaCl relative to Q-V_n in 100 mM NaCl. Data were obtained as described in (24). Briefly, for each GAT1 expressing oocyte the Q-V_n in 100 mM NaCl and 100 mM NaGlu were obtained and normalized as previously described (MATERIALS AND METHODS). The GABA-induced steady-state current was normalized to I_{GABA} at V_m = -140 mV. Each data point is mean ± SE, n = 4. Data points were joined for visualization.
[e.g., Na\(^+\) activation with Li\(^+\) or choline substitution (Fig. 8B, right)] and the voltage dependence of, e.g., \(K_{CR}\) (Fig. 8B, middle). The model also explains how the overall cotransport activity results from contributions from both cycles in a population of transporters; this depends on the probability of which cation binds first at each GAT1.

We speculate that chloride might be intimately associated with empty carrier reorientation and that this interaction would depend on whether or not GABA was previously bound. In this way, chloride interactions with the empty carrier could account for the alterations in the respective rate constants for each model. This notion was strongly suggested from a comparison of experiments done in the absence and presence of chloride: for example, we compared the IgABA-Vm relation obtained in 100Na and the Q-Vm relation obtained in 100NaGlu (Fig. 8C, closed and open circles) and after appropriate scaling of the ordinate axes, there was an obvious coincidence of the two data sets.

Conclusions

Our findings support a sequential cation binding mechanism, whereby Na\(^+\) and Li\(^+\) compete for the occupancy of the first of the two cation binding sites; and occupancy of the second site (NaI) is conditional on occupancy of the first site (Na2). When GABA is also present, Li\(^+\) can assume the role of a driving ion like Na\(^+\) and, as a consequence, Li\(^+\) is cotransported together with Na\(^+\) and GABA. We have incorporated our experimental findings into a kinetic model that successfully predicts the experimental data over a wide range of experimentally definable parameters and suggest that chloride may play an important role in defining the empty carrier reorientation, similar to that proposed by Bicho and Grewer (5). Our findings with respect to the role of Li\(^+\) ions in the GAT1 cotransport cycle prompt speculation that via a ubiquitously expressed neuron transporter, there is a Li\(^+\) transport pathway, the existence of which may have implications for the fate of Li\(^+\) ions in the treatment of bipolar disease. Finally, we note that this approach of determining the steady-state and presteady-state kinetics using data obtained under well-defined experimental conditions could be applied to the elucidation of multiple driving cation interactions in other solute carriers.

APPENDIX

The Na/Qt coupling ratio and GAT1 cotransport mode. We assume that in the steady-state with a given external [Na\(^+\)] and [Li\(^+\)], the net charge translocated (Q) arises from two populations of transporters: those operating in the Na-Na-GABA mode for which Na/Qt \(\approx 1\) and those operating in the Li-Na-GABA mode, for which Na/Qt \(\approx 0.5\). The overall coupling ratio (CRQt) will then lie in the range 0.5 \(\leq CRQt \leq 1\). The relationship between the experimentally determined CRQt and the fraction of GAT1 in the Li-Na-GABA mode is derived as follows:

In general, the GABA-induced current IgABA in the steady-state can be expressed as:

\[
I_{GABA} = eN(1 - 0.5) \frac{R_{LNG}^{NNG} + f R_{LNG}^{NNG}}{R_{LNG}^{NNG} + f R_{LNG}^{NNG}}
\]

where the superscripts NNG and LNG refer to the respective modes, \(N\) is the number of active GAT1, \(e\) is the electronic charge, \(m\) are the number of charges translocated each cycle, \(f\) is the fraction of GAT1 in respective mode, and \(R\) is turnover rate in the respective mode. For saturating GABA, the turnover rates are a function of the driving cation concentrations, external [Cl\(^-\)] and membrane potential. Note that under the experimental conditions in this study (e.g., Fig. 4), \(R_{LNG}^{NNG}\) and \(R_{LNG}^{NNG}\) are not the maximum possible turnover rates e.g., (13). Assuming \(m^{NNG} = m^{LNG} = 2\), the effective coupling ratio is then given by:

\[
CR_{Qt} = \frac{2R_{LNG}^{NNG} + f R_{LNG}^{NNG}}{R_{LNG}^{NNG} + f R_{LNG}^{NNG}}
\]

Using \(R_{LNG}^{NNG} + f R_{LNG}^{NNG} = 1\) and defining \(\epsilon = R_{LNG}^{NNG}R_{LNG}^{NNG}\), this simplifies to:

\[
CR_{Qt} = 0.5 \left[ \frac{2 + f R_{LNG}^{NNG}(\epsilon - 2)}{1 + f R_{LNG}^{NNG}(\epsilon - 1)} \right]
\]

Rearranging, the fraction of GAT1 operating in the Li-Na-GABA mode is given by:

\[
f = \left( \frac{CR_{Qt} - 1}{CR_{Qt}(1 - \epsilon) + 0.5\epsilon - 1} \right)
\]

This relationship is shown graphically in Fig. 9 for a range of \(\epsilon\). For a given \(\epsilon\), each data set shows how the relative fraction of GAT1 in each mode varies with CRQt.

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AUTHOR CONTRIBUTIONS


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