Role of Cl\(^-\) in Electrogenic Na\(^+\)-coupled Cotransporters GAT1 and SGLT1*

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We have investigated the functional role of Cl\(^-\) in the human Na\(^+\)/Cl\(^-\)/\(\gamma\)-aminobutyric acid (GABA) and Na\(^+\)/glucose cotransporters (GAT1 and SGLT1, respectively) expressed in Xenopus laevis oocytes. Substrate-evoked steady-state inward currents were examined in the presence and absence of external Cl\(^-\). Replacement of Cl\(^-\) by gluconate or 2-(N-morpholino)ethanesulfonic acid decreased the apparent affinity of GAT1 and SGLT1 for Na\(^+\) and the organic substrate. In the absence of substrate, GAT1 and SGLT1 exhibited charge movements that manifested as pre-steady-state current transients. Removal of Cl\(^-\) shifted the voltage dependence of charge movements to more negative potentials, with apparent affinity constants \((K_o)\) for Cl\(^-\) of 21 and 115 mM for SGLT1 and GAT1, respectively. The maximum charge moved and the apparent valence were not altered. GAT1 stoichiometry was determined by measuring GABA-evoked currents and the unidirectional influx of \(^{36}\)Cl\(^-\), Na\(^+\), or \(^{3}H\)GABA. Uptake of each GABA molecule was accompanied by inward movement of 2 positive charges, which was entirely accounted for by the influx of Na\(^+\) and Cl\(^-\) in the presence or absence of Cl\(^-\). Thus, the GAT1 stoichiometry was 2Na\(^+\):1GABA. However, Cl\(^-\) was transported by GAT1 because the inward movement of 2 positive charges was accomplished by the influx of one Cl\(^-\) ion, suggesting unidirectional influx of 2Na\(^+\):1Cl\(^-\):1GABA per transport cycle. Activation of forward Na\(^+\)/Cl\(^-\)/GABA transport evoked \(^{38}\)Cl\(^-\) efflux and was blocked by the inhibitor SKF 89976A. These data suggest a Cl\(^-\)/Cl\(^-\) exchange mechanism during the GAT1 transport cycle. In contrast, Cl\(^-\) was not transported by SGLT1. Thus, in both GAT1 and SGLT1, Cl\(^-\) modulates the kinetics of cotransport by altering Na\(^+\) affinity, but does not contribute to net charge transported per transport cycle. We conclude that Cl\(^-\) dependence per se is not a useful criterion to classify Na\(^+\) cotransporters.

Electrogenic sodium cotransporters utilize the movement of Na\(^+\) down its electrochemical potential gradient to drive the secondary active transport of ions, solutes, nutrients, and neurotransmitters into cells (1). This broad group of Na\(^+\)-driven cotransporters contains several distinct gene families (for reviews, see Refs. 2–5). However, there are differences in the ionic requirement of the specific transport systems (1, 2, 6–8). Whereas some require only Na\(^+\) to drive transport (e.g. the Na\(^+\)/glucose cotransporter SGLT1), others, such as the Na\(^+\)-dependent neurotransmitter transporters (e.g. the Na\(^+\)/Cl\(^-\) /GABA\(^1\) transporter GAT1), show an additional dependence on Cl\(^-\).

The role played by Cl\(^-\) in the transport cycle of the Na\(^+\)/Cl\(^-\) -dependent transporters is not well understood. The Cl\(^-\) equilibrium potential in most cells is close to the resting membrane potential, and little free energy would be provided by the Cl\(^-\) electrochemical potential gradient to drive uphill transport of substrate. Therefore, from a thermodynamic viewpoint, additional coupling of Cl\(^-\) to the transport of Na\(^+\) and substrate does not seem to confer a significant energetic advantage. In an effort to better understand the functional role played by Cl\(^-\) in GAT1, we have investigated the coupling between Na\(^+\), Cl\(^-\), and GABA.

Although there is no amino acid sequence homology between GAT1 and members of the Na\(^+\)-driven family of transporters (SGLT family) (5), they share a number of functional similarities. The best studied members of the SGLT and GAT families are the intestinal Na\(^+\)/glucose cotransporter SGLT1 and the brain Na\(^+\)/Cl\(^-\)/GABA cotransporter GAT1. In both transporters, (i) Na\(^+\)-coupled substrate transport is electrogenic and voltage-dependent (9, 10); (ii) both exhibit capacitive transients in response to step changes in membrane voltage (10–12); (iii) the steady-state and transient kinetics of Na\(^+\) and substrate transport are similar (9, 10, 13); (iv) transport is tightly coupled with no or small leakage currents (14, 15); (v) both behave as low conductance water channels and water transporters (16); and finally, (vi) both can be described by similar ordered, alternating access models (17, 18). This close functional resemblance between GAT1 and SGLT1 prompted us to investigate the effect of Cl\(^-\) on their kinetics and coupling stoichiometry.

Our results show that Cl\(^-\) is not absolutely required for either Na\(^+\)/glucose or Na\(^+\)/GABA transport. However, Cl\(^-\) interacts with both transporters to modulate the binding of external Na\(^+\). Although GAT1 transports Cl\(^-\) across the membrane in a 2Na\(^+\):1Cl\(^-\):1GABA stoichiometry, the transported Cl\(^-\) does not contribute to the net charge translocated across the membrane, and so we propose a Cl\(^-\)/Cl\(^-\) exchange mechanism during the transport cycle. SGLT1 does not translocate Cl\(^-\) across the membrane. Based on the results presented here and those in the literature, we suggest that Cl\(^-\) dependence per se is not a useful criterion to classify Na\(^+\) cotransporters.

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¶ The abbreviations used are: GABA, \(\gamma\)-aminobutyric acid; MES, 2-(N-morpholino)ethanesulfonic acid; \(\alpha\)-MDG, \(\alpha\)-methyl-D-glucopyranoside.
EXPERIMENTAL PROCEDURES

Expression in Xenopus Oocytes and Experimental Solutions—Stage V–VI Xenopus laevis oocytes were injected with 25–50 nl (1 µg/µl) of cRNA encoding human GAT1 (19) or human SGLT1 (20) or with 50 nl of water (control oocytes) and were maintained in Barth’s medium (88 mM NaCl, 1 mM KCl, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 2.4 mM NaHCO₃, and 10 mM HEPES, pH 7.4) for 3–4 days (11, 16). GAT1 used in this study was polymerase chain reaction-amplified from a human brain library, and sequencing confirmed that it is identical to that previously reported (19). In the recording chamber, oocytes were normally bathed in Na⁺ buffer (100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 10 mM HEPES, pH 7.5). Na⁺- and Cl⁻-free solutions were prepared by replacing Na⁺ with choline and Cl⁻ with gluconate (i.e., gluconate salts of K⁺, Mg²⁺, and Ca²⁺). Solutions that were both Na⁺- and Cl⁻-free contained choline/gluconate and the gluconate salts of K⁺, Mg²⁺, and Ca²⁺. Chloride replacement was also achieved with MES, with no observed difference in transporter function between gluconate and MES replacement. All experiments were performed at 21 ± 1 °C.

Electrophysiological Measurements—Transporter kinetics were studied using the 2-electrode voltage-clamp technique (11). In experiments in which the Cl⁻ concentration was varied, the reference electrode was connected to the experimental oocyte chamber via an agar bridge (3% agar in 3 mM KCl). To obtain current-voltage relations, the pulse protocol (pCLAMP, Axon Instruments, Inc., Foster City, CA) consisted of 100-ms (SGLT1) or 400-ms (GAT1) voltage steps from a holding potential of −50 mV to a series of test voltages (V_m) from +50 to −50 mV in 20-mV steps. Currents were low-pass-filtered at 500 Hz and sampled at 10 kHz (SGLT1) or 2.5 kHz (GAT1). Substrate-induced steady-state cotransporter currents were obtained from the difference between steady-state currents in the absence and presence of substrates (GABA or α-methyl-D-glucopyranoside (α-MDG)). The effects of external substrate concentration ([GABA], [α-MDG]ι), [Na⁺]ι, and [Cl⁻]ι on the steady-state kinetics were determined by nonlinear curve fitting (using SigmaPlot, SPSS Inc., Chicago, IL) of the substrate-induced currents (I) at each membrane potential (V_m) to Equation 1,

\[ I = \frac{I_{\text{max}}}{1 + \left(\frac{[S]}{K_i}\right)^n} + I_a \]

(Eq. 1)

where S is the substrate ([GABA], [α-MDG]ι), [Na⁺]ι, or [Cl⁻]ι, I_{max} is the maximal substrate-induced current, K_i is the half-maximal substrate concentration, and n is the Hill coefficient.

To obtain the transporter pre-steady-state currents, at every V_m, the total current (I_t) was fitted to Equation 2,

\[ I(t) = I_e^{\text{off}} + I_e^{\text{on}} + I_a \]

(Eq. 2)

where I_e^{\text{on}} is the initial capacitive current with time constant τ_e, t is time, I_e^{\text{off}} is the initial transporter (GAT1 or SGLT1) transient current with time constant τ_p, and I_a is the steady-state current (11). At each V_m, the total transporter-mediated charge (Q) was obtained by integrating the transporter transient currents. The charge-voltage (Q-V) relations obtained were then fitted to a single Boltzmann equation (Equation 3),

\[ \frac{Q - Q_{\text{off}}}{Q_{\text{on}} - Q_{\text{off}}} = \frac{1}{1 + e^{\left(\frac{V_m - V_{\text{1/2}}}{\sigma}\right)}} \]

(Eq. 3)

where Q_{max} = Q_{on} - Q_{off}, Q_{on} and Q_{off} are Q at depolarizing and hyperpolarizing limits, respectively, \( \sigma \) is the effective (or apparent) valence of the movable charge, V_{1/2} is the membrane potential at 50% charge transfer, R is Faraday’s constant, T is the gas constant, and T is the absolute temperature.

Coupling Stoichiometry—The ion/substrate stoichiometry of GAT1 was determined by simultaneous measurement of GABA-induced inward currents and influx of [3H]Cl⁻, [2Na⁺], or [3H]GABA under voltage-clamp conditions. The membrane potential was clamped, and after an initial base-line period in Na⁺ buffer, oocytes that were placed in a chamber (volume of 50 µl) were superfused (160 µl/min) for 0.5–10 min with a buffer containing GABA and [3H]Cl⁻, and [2Na⁺], or [3H]GABA. The experimental conditions were selected to optimize tracer counts, taking into account the specific activity of the reagents and the kinetics of the transporter (see Fig. 2). At the end of the period, GABA and the isotope were removed from the external medium, and the current was monitored until it returned to the base line. The oocyte was removed from the chamber, washed in ice-cold choline buffer, and solubilized for liquid scintillation counting (21–23). The total substrate-evoked inward charge (see Fig. 5) was obtained from the time integral of the substrate-evoked current and was correlated with tracer fluxes in the same cells.

Chloride Efflux Assays—Unidirectional chloride efflux was measured either in voltage-clamped oocytes to quantify the ratio of chloride efflux to inward charge or in groups of 10–20 oocytes to examine the time course of efflux (see Fig. 7). The procedure for Cl⁻ efflux assays was modified from that described in the literature (24). Oocytes were incubated in a modified Barth’s medium in which the Cl⁻-specific activity was 40 µCi/ml, i.e., 40 mCi of the total NaCl was provided from the NaCl stock solution. Oocytes were preincubated in a modified Barth’s medium for 24–72 h to maximize the cytoplasmic [36Cl⁻] specific activity. Before each experiment, oocytes were removed from the modified Barth’s medium, rinsed, and mounted in the experimental chamber. The chamber was perfused with the NaCl buffer, and GABA (500 µM) with or without SKF 89976A (500 µM) was added to the buffer. For the non-voltage-clamped oocytes (see Fig. 7, A and B), 10–20 GAT1-expressing oocytes were placed in a linear perfusion chamber. The chamber was perfused at 1 ml/min, and the superfusate was collected at 1- or 2-min intervals. After an initial control period of [36Cl⁻] efflux, GABA (500 µM) was added to the superfusate, followed by, in some experiments, SKF 89976A (500 µM). Cl⁻ efflux was assessed by scintillation counting of the superfusate fractions from non-voltage-clamped oocytes (Fig. 7C). After establishment of the base-line current, GABA was added to the bathing medium for 10 min, and the GABA-induced inward current was recorded. During this period, the entire superfusate was collected and counted as a single sample. At the end of the experiment, oocytes were rinsed and solubilized to determine the total oocyte tracer content. Cl⁻ efflux is reported as a percentage of total [36Cl⁻] count present in the oocyte at the beginning of the experiment. Total counts at the start of the experiment were determined by adding the efflux counts to the oocyte count at the end of the experiment. The magnitude of Cl⁻ efflux was estimated by assuming an oocyte cytoplasmic volume of 500 nl and a cytoplasmic [Cl⁻] of 33 mM (25). Control oocytes were obtained from the same batch and were treated in exactly the same manner as the GAT1-expressing oocytes.

All experiments were repeated with a minimum of three oocytes from three different donor frogs. Unless otherwise noted, all statistics are expressed as S.E. of the fit.

RESULTS

Steady-state Kinetics of Human GAT1—We first characterized the kinetics of human GAT1, as they have not been previously reported. The current records from an oocyte expressing human GAT1 in response to step jumps in membrane voltage (V_m) from a holding potential of −50 mV to a series of test potentials are shown in Fig. 1. In the absence of GABA, after the initial capacitive transient (with time constant τ = 1 ms), there was a slower decay to the steady state (τ = 40–150 ms) (Fig. 1A). These pre-steady-state, or capacitive, transients were not observed in control oocytes and were blocked by the GAT1-specific inhibitor SKF 899776A (data not shown) (10). Addition of GABA (500 µM) to the bathing medium induced an inward steady-state current and abolished the pre-steady-state current (Fig. 1B). The GABA-evoked current, the difference in steady-state current in the presence and absence of GABA (Fig. 1C), increased with hyperpolarizing membrane voltages and did not saturate at the most negative V_m tested (−150 mV). In the depolarizing direction, the GABA-evoked current tended to asymptote toward zero, but current reversal was not observed even at the most positive V_m tested (+50 mV).

The GABA-evoked inward current depended on the external Na⁺, Cl⁻, and GABA concentrations and on the membrane voltage. The dependences of the half-maximal concentrations (K_m) for GABA (K_m[GABA]), Na⁺ (K_m[Na⁺]), and Cl⁻ (K_m[Cl⁻]) are shown in Fig. 2 (A–F, white bars). The maximal currents (I_{max} for GABA (I_{max}[GABA]), Na⁺ (I_{max}[Na⁺]), and Cl⁻ (I_{max}[Cl⁻]) are also shown (Fig. 2, A–F, black bars). The data were obtained at V_m = −110 mV, and the reported values are from the fits of the data to Equation 1. To compare data from oocytes exhibiting different levels of GAT1 expression, the maximal currents at −110 mV have
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From 5 to 500 μM (Fig. 2C), \(I_{\text{Cl}}^{\text{max}}\) also increased (Fig. 2C), and this was the case at more negative potentials (data not shown). At 500 μM GABA\(_e\), external Na\(^+\) (20–100 mM) had little effect on \(K_{\text{Cl}}^{\text{Na},0.5}\) (Fig. 2D). \(I_{\text{Cl}}^{\text{max}}\) increased from 0.45 ± 0.1 to 0.76 ± 0.1 as [Na\(^+\)]\(_o\) was varied from 20 to 100 mM. However, as the membrane potential approached ~150 mV, the relative \(I_{\text{max}}\) values approached the same value (data not shown), suggesting that the absolute \(I_{\text{Cl}}^{\text{max}}\) was independent of [Na\(^+\)]\(_o\).

GABA exerted a small effect on the apparent affinity for Na\(^+\); \(K_{\text{Na}}^{\text{GABA},0.5}\) increased marginally from 14 ± 1 to 20 ± 5 mM as [GABA]\(_e\) increased 100-fold from 5 to 500 μM (Fig. 2E). Cl\(^-\) increased the apparent affinity of GAT1 for Na\(^+\); \(K_{\text{Na}}^{\text{GABA},0.5}\) decreased from 18 ± 2 to 14 ± 1 m\(\text{M}\) when [Cl\(^-\)]\(_o\) was increased from 20 to 100 mM (Fig. 2F). \(K_{\text{Cl}}^{\text{Na},0.5}\) increased with increasing [GABA] but was independent of [Cl\(^-\)]\(_o\), and was made more negative, the GABA-coupled current increased.

In summary, Na\(^+\) and Cl\(^-\) increased the apparent affinity of GAT1 for GABA. GABA decreased the apparent affinity for Cl\(^-\), whereas Na\(^+\) had relatively little effect on the apparent affinity of GAT1 for Cl\(^-\). GABA had no effect on the apparent affinity for Na\(^+\), but Cl\(^-\) increased the apparent affinity for Na\(^+\). The absolute \(I_{\text{max}}\) values (i.e. \(I_{\text{max}}\) at hyperpolarizing potentials) were apparently independent of the Na\(^+\) and Cl\(^-\) concentrations and depended on the GABA concentration. All in all, the steady-state and pre-steady-state (see below) kinetics of human GAT1 reported here are similar to those of rat GAT1 reported previously (10, 13, 15, 26, 27). The data are consistent with ordered reaction schemes in which Na\(^+\) and Cl\(^-\) bind to the transporter before GABA (2, 18), as is the case for Na\(^+\) and Cl\(^-\) in SGLT1 (17, 28).

**Effect of Cl\(^-\) on Steady-state Kinetics of GAT1 and SGLT1**—

The dependence of steady-state GABA and glucose transport kinetics on external Cl\(^-\) is shown in Fig. 3 (A–F). In 106 mM Cl\(^-\)\(_o\) the steady-state current-voltage curve of the current induced by GABA (500 μM) (Fig. 3A) did not show saturation, whereas the current induced by α-MDG (500 μM) (Fig. 3B) tended toward saturation. Both currents were reduced upon removal of external Cl\(^-\) (gluconate or MES substitution). In GAT1, in the absence of Cl\(^-\), there was virtually no GABA-coupled current at \(V_m\) more positive than ~50 mV, but as \(V_m\) was made more negative, the GABA-coupled current increased. At ~150 mV, the current was ~50% of that in the presence of 106 mM Cl\(^-\). In contrast, in SGLT1, the reduction in α-MDG-induced current was small and relatively constant at every voltage tested. Cl\(^-\) had no effect on the maximal transport rates (\(I_{\text{max}}\)) for GAT1 and SGLT1; \(I_{\text{max}}\) evaluated at the most negative membrane potentials was the same in the presence and absence of external Cl\(^-\) (see Fig. 2A for GAT1; data not shown for SGLT1).

Kinetic analyses of GABA- or glucose-evoked currents indicated that there was an increase in the half-maximal concentration (\(K_{\text{GABA}}\)) for Na\(^+\) and/or the organic substrate with decreasing [Cl\(^-\)]\(_o\) (Figs. 2F and 3, C–F). For example, decreasing [Cl\(^-\)]\(_o\) from 106 to 20 mM increased \(K_{\text{GABA}}^{\text{Na},0.5}\) for GAT1 from 14 ± 2 to 20 ± 2 m\(\text{M}\) (at ~110 mV) (Fig. 3C) and that for SGLT1 from 41 ± 1 to 62 ± 2 m\(\text{M}\) (at ~50 mV) (Fig. 3D). There was a large effect of Cl\(^-\) on the affinity of GABA for GAT1, whereas the effect on the apparent affinity of SGLT1 for α-MDG was small. At ~50 mV, \(K_{\text{GABA}}^{\text{Na},0.5}\) increased from 11 ± 2 to 104 ± 13 μM whereas in SGLT1, \(K_{\text{GABA}}^{\text{Na},0.5}\) increased from 760 ± 20 to 860 ± 30 μM (Fig. 3, E and F). For both transporters, there was no change in the observed Hill coefficient for Na\(^+\) (~2) or the organic substrate (~1) in the presence or absence of Cl\(^-\) (data not shown). In GAT1, the Hill coefficient for Cl\(^-\) was ~1.

**Effect of Cl\(^-\) on Pre-steady-state Kinetics of GAT1 and SGLT1**—

For both GAT1 (as illustrated in Fig. 1A) and SGLT1, in the absence of the organic substrate, step jumps in mem-
brane voltage elicited pre-steady-state capacitive transients or charge movements (10, 11). Fig. 4 (A and B) shows the charge-voltage (Q-V) relations in the presence and absence of external Cl\textsuperscript{−}. For both transporters, removal of Cl\textsuperscript{−} from the external medium shifted the V\textsubscript{0.5} (voltage at 50% Q\textsubscript{max}) toward more negative potentials (Fig. 4, A and B). The shift in V\textsubscript{0.5} (ΔV\textsubscript{0.5}) was −18 ± 3 mV for SGLT1 and −76 ± 4 mV for GAT1. The shift in V\textsubscript{0.5} can be used to estimate an apparent affinity of the transporter for external Cl\textsuperscript{−} (Fig. 4, C and D). The apparent affinity for Cl\textsuperscript{−} was 5-fold greater in SGLT1 than in GAT1 (K\textsubscript{0.5} for Cl\textsuperscript{−} was 21 ± 1 mM versus 115 ± 18 mM). Chloride removal had no effect on Q\textsubscript{max} (Fig. 4, E and F) or the apparent valence of the transporter (z ≈ 1 for both transporters; data not shown).

**GAT1 Stoichiometry**—To determine the ion/substrate stoichiometry of GAT1, influxes of 22Na\textsuperscript{+}, 36Cl\textsuperscript{−}, and [3H]GABA were measured under voltage-clamp conditions (V\textsubscript{m} = −100 mV) (Fig. 5A). At the end of the recording period, the inward charge (Q) and the amount of [3H]GABA, 22Na\textsuperscript{+}, and 36Cl\textsuperscript{−} influx were determined. The slope of the inward charge versus 36Cl\textsuperscript{−} influx was 2.0 ± 0.1 charges/Cl\textsuperscript{−} ion (Fig. 5B). Thus, Cl\textsuperscript{−} is transported by GAT1, with the net inward movement of 2 positive charges coupled to the influx of each Cl\textsuperscript{−} ion. The inward charge per [3H]GABA molecule was 2:1 (slope was 1.9 ± 0.1 charges/GABA molecule) and was the same in the presence and absence of Cl\textsuperscript{−} in the external medium (Fig. 5C). The one-to-one correlation between the inward charge and 22Na\textsuperscript{+} uptake indicated that each inward charge was accompanied by the influx of one 22Na\textsuperscript{+} ion (slope was 0.9 ± 0.1 charge/Na\textsuperscript{+} ion) and independent of the presence or absence of external Cl\textsuperscript{−} (Fig. 5D). Experiments performed with the membrane potential held at −10, −30, −50, and −70 mV yielded similar results (data not shown).

To determine whether Cl\textsuperscript{−} was transported by SGLT1, Na\textsuperscript{+}/α-MDG currents were evoked for 10 min in the presence of 36Cl\textsuperscript{−} (Fig. 6A). There was no difference in 36Cl\textsuperscript{−} uptake (4.3 pmol/min) between control oocytes and SGLT1-expressing oocytes (p > 0.05) (Fig. 6B), indicating that Cl\textsuperscript{−} is not transported by SGLT1. The inward current in these experiments (Fig. 6A) ranged from 540 to 950 nA, and if one Cl\textsuperscript{−} ion was transported per inward charge, the Cl\textsuperscript{−} influx would be expected to be 340–590 pmol/min, well within the resolution of our experiments.

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**FIG. 2. Steady-state kinetics of human GAT1.** Half-maximal concentrations (K\textsubscript{0.5}) and maximal current levels (I\textsubscript{max}) were determined by measuring the GABA-induced inward currents at various concentrations of GABA, Na\textsuperscript{+}, and Cl\textsuperscript{−}. K\textsubscript{0.5} values are shown by the black bars. Each graph (A–F) was collected on a single oocyte, and to correct for different expression levels in different experiments, data are shown by the white bars. A, dependence of K\textsubscript{0.5} and I\textsubscript{max} on [GABA]. B, dependence of K\textsubscript{0.5} and I\textsubscript{max} on [Na\textsuperscript{+}]. C, dependence of K\textsubscript{0.5} and I\textsubscript{max} on [Cl\textsuperscript{−}]. D, dependence of K\textsubscript{0.5} and I\textsubscript{max} on [Na\textsuperscript{+}], [Cl\textsuperscript{−}]. E, dependence of K\textsubscript{0.5} and I\textsubscript{max} on [GABA]. F, dependence of K\textsubscript{0.5} and I\textsubscript{max} on [Cl\textsuperscript{−}]. Each graph was collected on a single oocyte, and to correct for different expression levels in different experiments, data are shown by the white bars. A, dependence of K\textsubscript{0.5} and I\textsubscript{max} on [GABA]. B, dependence of K\textsubscript{0.5} and I\textsubscript{max} on [Na\textsuperscript{+}]. C, dependence of K\textsubscript{0.5} and I\textsubscript{max} on [Cl\textsuperscript{−}]. D, dependence of K\textsubscript{0.5} and I\textsubscript{max} on [Na\textsuperscript{+}], [Cl\textsuperscript{−}]. E, dependence of K\textsubscript{0.5} and I\textsubscript{max} on [GABA]. F, dependence of K\textsubscript{0.5} and I\textsubscript{max} on [Cl\textsuperscript{−}]. Each graph was collected on a single oocyte, and to correct for different expression levels in different experiments, data are shown by the white bars. A, dependence of K\textsubscript{0.5} and I\textsubscript{max} on [GABA]. B, dependence of K\textsubscript{0.5} and I\textsubscript{max} on [Na\textsuperscript{+}]. C, dependence of K\textsubscript{0.5} and I\textsubscript{max} on [Cl\textsuperscript{−}]. D, dependence of K\textsubscript{0.5} and I\textsubscript{max} on [Na\textsuperscript{+}], [Cl\textsuperscript{−}]. E, dependence of K\textsubscript{0.5} and I\textsubscript{max} on [GABA]. F, dependence of K\textsubscript{0.5} and I\textsubscript{max} on [Cl\textsuperscript{−}]. Each graph was collected on a single oocyte, and to correct for different expression levels in different experiments, data are shown by the white bars.
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**GAT1 Chloride Efflux**—Since the net inward charge is totally accounted for by the influx of Na\(^+\), Cl\(^-\) influx appears not to contribute to the GABA-evoked inward current. One explanation is that there is an exchange of an internal anion for Cl\(^-\). To investigate this possibility, we monitored the efflux of \(^{36}\)Cl\(^-\) in oocytes preloaded with \(^{36}\)Cl\(^-\). In the absence of GABA, Cl\(^-\) efflux from GAT1-expressing oocytes was similar to that from control cells. In GAT1-expressing oocytes, activation of forward Na\(^+\)/Cl\(^-\)/GABA transport (addition of 500 \(\mu\)M GABA to the NaCl buffer) increased the rate of \(^{36}\)Cl\(^-\) efflux from 0.2 to 2%/min (Fig. 7A). The GABA-stimulated Cl\(^-\) efflux was blocked by the GAT1 inhibitor SKF 89976A (500 \(\mu\)M), as addition of this compound to the external solution reduced the rate of \(^{36}\)Cl\(^-\) efflux to control values (Fig. 7B).

In an effort to gain a more quantitative measure of Cl\(^-\) efflux, we monitored Cl\(^-\) efflux in individual voltage-clamped oocytes. In these experiments, oocytes that had been preloaded with \(^{36}\)Cl\(^-\) were voltage-clamped; forward Na\(^+\)/Cl\(^-\)/GABA transport was activated for 10 min as was done in the experiments of Fig. 5A; and the inward current was monitored. As
was the case in the non-voltage-clamped GAT1-expressing oocytes (Fig. 7, A and B), there was a significant increase in Cl\(^{-}\) efflux as forward Na\(^+\)/Cl\(^{-}\)/GABA transport was activated (p < 0.05) (Fig. 7C). Assuming that the internal Cl\(^{-}\) concentration is 33 mM and the cytoplasmic volume of the oocyte is 500 nl (25), the ratio of GABA-evoked inward charge to Cl\(^{-}\) efflux was 2.2 ± 1.0 charges/exported Cl\(^{-}\) ion.

**DISCUSSION**

Extracellular Cl\(^{-}\) alters the activity of the brain GABA transporter (GAT1) and the intestinal glucose transporter (SGLT1). A reduction in [Cl\(^{-}\)]\(_o\) decreases the steady-state substrate (GABA and glucose)-evoked inward currents of these transporters by reducing their affinity for external Na\(^+\) and the organic substrate. However, Cl\(^{-}\) is not absolutely required for Na\(^+\)-dependent substrate transport by either transporter. For Na\(^+\)-dependent glucose transport (SGLT1), it has been generally believed that transport is insensitive to external Cl\(^{-}\). For GAT1, the sensitivity to external Cl\(^{-}\) is widely accepted (2, 29). Our findings that Cl\(^{-}\) is not absolutely required and that negative membrane potentials could substitute for Cl\(^{-}\) agree with uptake studies in membrane vesicles (30) and electrophysiological experiments on GAT1-expressed Xenopus oocytes (10, 15). Here, we add the novel observation that in GAT1, the transport stoichiometry of 2Na\(^+\):1GABA is independent of the extracellular Cl\(^{-}\) concentration. Surprisingly, whether external Cl\(^{-}\) is present or absent, 2 net positive charges enter the cell during the GAT1 transport cycle. This is in contrast to the widely held view that the GAT transport cycle introduces only 1 net positive charge into the cell. In addition, we show that forward Na\(^+\)/Cl\(^{-}\)/GABA transport is accompanied by simultaneous Cl\(^{-}\) efflux.

**Effect of Cl\(^{-}\) on Steady-state and Pre-steady-state Kinetics**—The effect of Cl\(^{-}\) on SGLT1 and GAT1 is to increase the apparent affinity of the transporters for Na\(^+\) and the substrate.
Role of Cl⁻ in GAT1 and SGLT1

Fig. 7. Forward Na⁺/Cl⁻/GABA transport induces simultaneous Cl⁻ efflux through GAT1. A, shown is the time course of 36Cl⁻ efflux through GAT1. The ordinate is the Cl⁻ efflux rate, expressed as a percentage of the total 36Cl⁻ counts present in the oocyte in the beginning of the experiment. Control (○) and GAT1-expressing (■) oocytes were preloaded with 36Cl⁻ by incubation for 24–72 h in Barth’s medium containing 36Cl⁻. Oocytes (20 cells) were rinsed, and 36Cl⁻ efflux was monitored at 1-min intervals. At the time indicated by the bar, 500 μM GABA was added to the external medium to activate forward Na⁺/Cl⁻/GABA transport. In the absence of GABA, Cl⁻ efflux was similar in both control and GAT1-expressing oocytes (0.2%/min). GABA stimulated a large increase in Cl⁻ efflux in the GAT1-expressing cells blocked by the GAT1-specific inhibitor SKF 89976A (500 μM). Note that in both A and B, 36Cl⁻ efflux was instantaneous with the addition of GABA, C, a similar experiment was performed on control (n = 4) and GAT1-expressing (n = 7) oocytes under voltage-clamp conditions (V_m = −50 mV). The GABA-evoked inward current was monitored and correlated with Cl⁻ efflux in the same cells. Control oocytes showed no GABA-evoked inward current (data not shown). 36Cl⁻ efflux values in control oocytes and GAT1-expressing cells in the absence of GABA were similar. In GAT1-expressing oocytes, activation of Na⁺/Cl⁻/GABA transport led to a significant increase in 36Cl⁻ efflux. The coupling ratio was estimated to be 2 ± 1 inward charges for every exported Cl⁻ ion (similar to the coupling ratio obtained for Cl⁻ influx). Thus, there appears to be no net Cl⁻ flux during the transport cycle of GAT1. This is indicative of Cl⁻ exchange during the transport cycle of GAT1.

(glucose and GABA), whereas the maximum transport velocity is relatively unaffected by Cl⁻. Because the affinities for the organic substrates are dependent on the affinity for Na⁺, the reduction in the glucose and GABA affinities is, at least in part, secondary to the decrease in Na⁺ affinity.

We examined GAT1 and SGLT1 pre-steady-state currents to gain a better understanding of the nature of Cl⁻ interaction with these proteins in the absence of GABA and glucose. Pre-steady-state currents have been investigated in a variety of Na⁺-coupled transporters, including the Na⁺/glucose (11, 12), Na⁺/Cl⁻/GABA (10, 13), Na⁺/sodium (21), Na⁺/phosphate (31, 32), and Na⁺/K⁺/glutamate (33) cotransporters. They are thought to represent a redistribution of protein conformations in response to rapid changes in membrane voltage and/or ligand concentration (9–11, 28, 34, 35). When voltage perturbations are used, transporter pre-steady-state currents can be represented in the form of charge-voltage (Q-V) relations (10, 11).

Q-V curves are characterized by three Boltzmann factors: Q_max (maximal charge movement), zδ (product of the effective valence of the moveable charge and the fraction of the electric field through which the charge moves), and V_0.5 (membrane voltage at 50% Q_max). These curves represent the distribution of a charged species (with valence z), which translocates across a given fraction (δ) of the membrane electric field, thereby distributing between two predominant conformational states depending on the membrane voltage (11, 12). Q_max is directly related to the total number of functional transporter molecules in the plasma membrane (36, 37). V_0.5 represents the voltage at which approximately half of the moveable charge is in either conformational state. In SGLT1, the Q-V relation has been shown to provide a measure of the accessibility of the ligand-binding sites, as there is a direct correspondence between the Q-V curve and the accessibility of alkylation reagents to a cysteine residue (Q457C) placed in the putative sugar-binding/translocation domain of SGLT1: increasing [Na⁺]o, shifted V_0.5 to more negative membrane potentials and increased the accessibility of the sugar-binding site from the external medium (11, 28). In both SGLT1 and GAT1, a 10-fold reduction in external Na⁺ concentration results in a similar change in occupancy probability: the shift in V_0.5 was −100 mV for both transporters (10–12).

Since varying [Cl⁻]o had no effect on Q_max or zδ in either GAT1 or SGLT1, the reduction in the rate of substrate transport upon Cl⁻ removal is not due to a decrease in the number of transporters at the cell surface or to a decrease in the effective valence of each transporter. The shift of V_0.5 toward more negative membrane potentials as [Cl⁻]o was reduced is similar to that seen with a reduction in [Na⁺]o. Thus, in GAT1 and SGLT1, Cl⁻ appears to alter the occupancy probability in a similar way to Na⁺. From Fig. 4 (A and B), we found that the probability of being in the substrate-binding conformation is decreased from 0.6 to 0.4 for SGLT1 and from 0.8 to 0.3 for GAT1 upon removal of Cl⁻ from the external medium (at −50 mV). The larger shift in V_0.5 seen for GAT1 compared with that seen for SGLT1 is responsible for the greater effect of Cl⁻ on the steady-state transport rates of GAT1 (Fig. 3, A and B). Although the effect of Cl⁻ on the rate of steady-state substrate transport by GAT1 was much larger than that seen for SGLT1, it should be noted that weaker effects of chloride have been noted for other members of the GABA transporter family even though they exhibit 40–50% identity to GAT1 (39).

GAT1 Stoichiometry—Previous studies on the stoichiometry of Na⁺/Cl⁻/GABA cotransport have been based on three methods: 1) kinetic analysis of the dependence of cotransport rates on the substrates in oocytes expressing GAT1 (10, 26), 2) thermodynamic analysis of the dependence of the reversal potentials of cotransport in membrane vesicles (40, 41) and in cells expressing GAT1 (15, 27), and 3) comparison of the ratios of substrate fluxes in membrane vesicles (42) and comparison of the inward charge and substrate fluxes in oocytes expressing GAT1 (26). With the exception of the study of Pustuszko et al. (40), which determined the transport of two Na⁺ ions with one zwitterionic GABA molecule (independent of Cl⁻), all other studies determined a 2Na⁺:1Cl⁻:1GABA stoichiometry. The stoichiometry for GABA influx obtained in this study is consistent with this ratio (2Na⁺:1Cl⁻:1GABA). However, our surprising finding is that in the presence or absence of external Cl⁻, there were 2 inward charges transported with each GABA...
Cl that the Na cell. Several observations suggest that this is an unlikely scenario, operating in the reverse mode due to GABA accumulated in the extracellular solution, this implies that Cl may not play a role in the thermodynamics of the cotransport process (see below). 

**GAT1 Cl** /**Cl** Exchange—GABA stimulated an efflux of Cl through GAT1 (Fig. 7). We estimate that the Cl efflux is comparable to the Cl influx, leading to stoichiometric Cl /Cl exchange during the transport cycle. Therefore, there is no net Cl flux through GAT1. Based on this observation and the finding that the Na/GABA coupling ratio is independent of Cl, it may be inferred that Cl plays no role in the thermodynamics of the cotransport process. However, it is difficult to reconcile this direct result with several other reports suggesting a thermodynamic component to Cl transport (15, 27, 41, 42). 

GAT1 has been shown to be able to operate in the reverse mode (15, 27, 43). This raises the possibility that the observed GABA-stimulated Cl efflux is the result of the cotransporter operating in the reverse mode due to GABA accumulated in the cell. Several observations suggest that this is an unlikely scenario. (i) The GABA-stimulated Cl efflux was concomitant (1-min sampling resolution) with activation of inward Na /GABA cotransport (Fig. 7, A and B), and in view of the unfavorable Na and voltage gradients, transport in the reverse mode seems unlikely. (ii) An accumulation of GABA should alter the direction of Na /GABA cotransport. However, after exposing GAT1-expressing oocytes to 500 μM GABA for up to 10 min, we did not observe outward currents generated by GAT1. (iii) Assuming that the efflux is caused by accumulation of GABA in the cell, it is expected that it should continue (or even be stimulated) after removal of external GABA. This is not the case, however, as Cl efflux ceases immediately after removal of external GABA or addition of the inhibitor SKF 89976A (Fig. 7C). Thus, the observed Cl efflux is unlikely to be caused by accumulation of intracellular GABA leading to GAT1 operating in the reverse mode. Despite overall similarities in external Cl sensitivity, there was a major difference between GAT1 and SGLT1. In SGLT1, despite strong interaction with the transporter (as revealed by the dependence of the Q-V relation on Cl), there is no Cl flux through the protein. However, in GAT1, for every Cl ion transported into the cell, there appears to be an efflux of one Cl ion from the cytoplasm mediated by the transporter (Fig. 8).

**Conclusion**—In summary, our studies show that Cl modulates the function of both the Na -dependent cotransporter SGLT1 and the Na /Cl-dependent cotransporter GAT1. Cl-dependence is not a good criterion to classify transporter families. This is also emphasized by the recent report on the cloning and characterization of the high affinity choline transporter from rat spinal cord (38). This Na- and Cl-dependent neurotransmitter transporter is a member of the SGLT1 gene family. In both SGLT1 and GAT1, extracellular Cl appears to alter the conformational state of the transporter such that it poises the transporter to bind external Na. The effect is much more pronounced for GAT1. GAT1 appears to mediate Cl /Cl exchange during the transport cycle, but SGLT1 does not. These experimental observations provide a novel insight into the mechanistic similarity of these transport molecules despite their phylogenetic distance.

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Role of Cl\textsuperscript{−} in Electrogenic Na\textsuperscript{+}-coupled Cotransporters GAT1 and SGLT1
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