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Conformational basis for the Li^+-induced leak current in the rat γ-aminobutyric acid (GABA) transporter-1

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The rat γ-aminobutyric acid transporter-1 (GAT-1) was expressed in *Xenopus laevis* oocytes and the substrate-independent Li^+-induced leak current was examined using two-electrode voltage clamp. The leak current was not affected by the addition of GABA and was not due to H^+ permeation. The Li^+-bound conformation of the protein displayed a lower passive water permeability than that of the Na^+- and choline (Ch^+)-bound conformations and the leak current did not saturate with increasing amounts of Li^+ in the test solution. The mechanism that gives rise to the leak current did not support active water transport in contrast to the mechanism responsible for GABA translocation (~330 water molecules per charge). Altogether, these data support the distinct nature of the leak conductance in relation to the substrate translocation process. It was observed that the leak current was inhibited by low millimolar concentrations of Na^+ (the apparent affinity constant, $K'_{0.5}$ = 3 mM). In addition, it was found that the GABA transport current was sustained at correspondingly low Na^+ concentrations if Li^+ was present instead of choline. This is consistent with a model in which Li^+ can bind and substitute for Na^+ at the putative 'first' apparently low-affinity Na^+ binding site. In the absence of Na^+, this allows a Li^+-permeable channel to open at hyperpolarized potentials. Occupancy of the 'second' apparently high-affinity Na^+ binding site by addition of low millimolar concentrations of Na^+ restrains the transporter from moving into a leak conductance mode as well as allowing maintenance of GABA-elicited transport-associated current.

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The major role of the γ-aminobutyric acid (GABA) transporters is termination of the synaptic response by re-uptake of GABA released into the synaptic cleft during neuronal activity. Four different GABA transporter subtypes have been described (GAT-1, GAT-2, GAT-3 and the betaine–GABA transporter-1 (BGT-1)), which are characterized by distinct localization patterns in the mammalian body and central nervous system (for review see Borden, 1996). GAT-1 was the first cloned member of the family of Na^+–Cl^- neurotransmitter transporters (Guastella *et al.* 1990). The uptake process is driven by the transmembrane Na^+ gradient with the co-transport of two Na^+ and one Cl^- ions, thereby rendering the translocation electrogenic (Radian & Kanner, 1983; Keynan & Kanner, 1988). Many electrophysiological studies of heterologously expressed GAT-1, both in mammalian cell lines and in *Xenopus laevis* oocytes, have been carried out and four current-generating modes of the transporter have been described: the Na^+-coupled GABA transport, the leak current, the capacitive Na^+-dependent transient currents, and a not fully documented uncoupled substrate-induced channel activity (Kavanaugh *et al.* 1992; Mager *et al.* 1993, 1996; Cammack *et al.* 1994; Cammack & Schwartz, 1996; Risso *et al.* 1996; Bismuth *et al.* 1997; Lu & Hilgemann, 1999; Forlani *et al.* 2001; MacAulay *et al.* 2001*a*).

The GABA transporter and several related transporters sustain an inward uncoupled leak current in the absence of their substrates. The cationic permeability differs for the different family members, with Li^+, and to a smaller extent Cs^+, being the only ions found to permeate through GAT-1 (Mager *et al.* 1996; Bismuth *et al.* 1997; MacAulay *et al.* 2001*a*). The dopamine and serotonin transporters (DAT and SERT) are less restrictive, allowing permeation of Na^+, K^+, Li^+ and possibly H^+ (Mager *et al.* 1994; Cao *et al.* 1997; Sonders *et al.* 1997). The molecular mechanism underlying the leak currents remains poorly understood. It has been suggested that the leak current in the neurotransmitter transporters is a channel-mode conductance (Cammack & Schwartz, 1996; Lin *et al.* 1996) and that it might (Sonders & Amara, 1996; Petersen & DeFelice, 1999;) or might not (Mager *et al.* 1994) share a common permation pathway

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with the substrate. In contrast, the Na^+ leak current in the functionally related Na^+-coupled glucose transporter (SGLT) was suggested to involve the same pathway as and a similar mechanism to the Na^+-coupled glucose transporter (Loo *et al.* 1999).

Recently, we have structurally and functionally probed the GAT-1 by introducing engineered Zn^{2+} binding sites in the transporter molecule. Intriguingly, we observed that although Zn^{2+} binding at one site resulted in strong inhibition of both GABA translocation and the Li^+-induced leak conductance, Zn^{2+} binding to a closely related site only blocked translocation without any effect on the leak current (MacAulay *et al.* 2001*a*). It was therefore suggested that the leak current represents a unique operational mode of the transporter involving conformational changes and/or states different from those of the substrate translocation process. In the present paper, we have obtained additional new insight into the molecular basis of the leak current of the GABA transporter. We have used the *Xenopus laevis* expression system and two-electrode voltage clamp to assess the transporter-mediated currents and volume measurements to monitor the water transport properties of the GAT-1. Most significantly, we observe that the mechanism underlying the leak current is distinct from that underlying the GABA-induced current and that the leak current is inhibited by low millimolar concentrations of Na^+ ($K'_{0.5}$ = 3 mM). In addition, we find that transport is sustained at correspondingly low Na^+ concentrations if Li^+ is present instead of choline. The data suggest that Li^+ can replace Na^+ at the putative 'first' apparently low-affinity Na^+ binding site while Na^+ occupancy of the putative 'second' apparently high-affinity Na^+ binding site is sufficient to restrain the transporter from moving into a leak conductance mode.

METHODS

Molecular biology and oocytes

The rGAT-1 construct was cloned into a vector optimized for oocyte expression (pNB1) as earlier described (MacAulay *et al.* 2001*a*). The cDNA was linearized downstream of the poly-A segment and *in vitro* transcribed with the T7 RNA polymerase using the mCAP mRNA capping kit (Stratagene, La Jolla, CA, USA) and 50 ng cRNA was injected into defolliculated *Xenopus laevis* oocytes (MacAulay *et al.* 2001*a*). *Xenopus* oocytes were collected under anaesthesia (Tricain, 2 g l^{-1}) and the frogs were observed for a period of 3 h after the operation. After the final collection the frogs were humanely killed by decapitation. The surgical procedures complied with Danish legislation and were approved by the controlling body under the Ministry of Justice. The oocytes were incubated in Kulori medium (90 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.4) at 19 °C for 3–7 days before experiments were performed.

[^{3}H]GABA uptake experiments in oocytes

The uptake experiments were performed in 24-well plates with 100 μM GABA and 50 nM [^{3}H]GABA (4-amino-*n*-[2, 3-^{3}H]butyric acid, 81 Ci $mmol^{-1}$, Amersham, Little Chalfont, UK) added to a total of 400 μl test solution (0–100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.4, NaCl substituted with equimolar LiCl or ChCl). Oocytes were incubated for 30 min at room temperature, washed 3 times in 1 ml test solution with 100 mM ChCl (100 mM ChCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.4), and dissolved in 200 μl 10 % SDS. Before counting, 2.0 ml scintillation fluid were added to the samples.

Electrophysiology

The oocytes were impaled by two microelectrodes in recording solution containing 100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM Hepes (pH 7.4). In substitution experiments, sodium ions were replaced by equimolar lithium or choline ions. The data presented are subtractive currents, i.e. ($I_{Na+GABA} - I_{Na}$) or ($I_{Li} - I_{Ch}$). Two-electrode voltage clamp recordings were performed at room temperature with a Dagan clampator interfaced to an IBM-compatible PC using a DigiData 1200 A/D converter and pCLAMP 6.0 (Axon Instruments). Electrodes were pulled from borosilicate glass capillaries to a resistance of 0.5–2 MΩ and were filled with 1 M KCl.

Volume measurements

The volume measurements have previously been described in detail (Zeuthen *et al.* 1997; Meinild *et al.* 1998). The impaled oocyte was observed from below via a low magnification objective and a charge-coupled device camera. To achieve a high stability of the oocyte image, the upper surface of the bathing solution was determined by the flat end of a perspex rod, which also provided an illuminated background. Images were captured directly from the camera to the random access memory of a computer. The oocyte was focused at the circumference and assumed to be spherical. The volume was recorded and calculated on-line at a rate of one point per second with an accuracy of 3 in 10 000. The osmotic water permeability, L_p, was calculated per true membrane surface area (Loo *et al.* 1996), which is about 9 times the apparent area due to membrane foldings (Zampighi *et al.* 1995). The data were corrected for the batch-specific L_p of the native oocytes. L_p values are given in units of cm s^{-1} (osmol l^{-1})$^{-1}$ and were equal to $J_{H_2O}/A\,\Delta\pi$, where J_{H_2O} is the water flux, A is the surface area of the oocyte, and $\Delta\pi$ is the osmotic difference. The coupling ratio of the GAT-1 is taken as the number of water molecules cotransported per unit charge by the protein during GABA transport. Accordingly, the coupling ratio equals $F\,J_{H_2O}\,(V_w\,I_s)^{-1}$, where J_{H_2O} is the water flux, V_w is the partial molal volume of water (18 cm^3 mol^{-1}), I_s is the clamp current induced by application of GABA, and F is Faraday's constant. The coupling ratio was calculated by linear regression of the data from each oocyte and the average of these numbers is stated in the text.

Calculations

The data were analysed by linear and non-linear regression analysis using Prism 3.0 from GraphPad Software (San Diego, CA, USA). All numbers are given as means ± S.E.M. with n equal to the number of oocytes tested unless otherwise stated.

RESULTS

Current–voltage relationship

Addition of 100 μM GABA to GAT-1-expressing *Xenopus laevis* oocytes under voltage clamp (−50 mV) yielded currents in the range 100–350 nA. The GABA transport is strictly dependent on Na^+ as the cotransported cation

(Radian & Kanner, 1983; Keynan & Kanner, 1988) but in the absence of GABA and Na^+, addition of Li^+ generates a large inward current (Fig. 1 and Mager *et al.* 1996; Bismuth *et al.* 1997). As seen in Fig. 1, the *I–V* relationship of the GABA-induced current was distinct from that of the Li^+-induced leak current, which showed stronger inward rectification and occurred only at membrane potentials more negative than ~ −75 mV. At membrane potentials more negative than ~ −135 mV the leak current was larger than the GABA-induced current. The addition of GABA had no effect on the Li^+ current (Fig. 1). No Na^+ leak current was apparent in the GAT-1-expressing oocytes, as reported earlier (Mager *et al.* 1993; Loo *et al.* 1999; MacAulay *et al.* 2001*a*) and non-injected oocytes supported no Na^+/Li^+ leak current (data not shown and Fig. 1). The specific inhibitor of GAT-1, SKF89976A (50 μM), partly inhibited the Li^+-induced leak current of GAT-1 (about 50 %) and 100 μM Zn^{2+} inhibited the leak current of a Zn^{2+}-sensitive mutant of GAT-1 to the same extent (MacAulay *et al.* 2001*a*).

pH dependence

The Li^+-bound conformation of GAT-1 may support Li^+ flux and/or it may allow permeation of other ions, such as H^+. The *I–V* relationship of the leak current was not affected by changes in the pH of the LiCl solution (data not shown). At a clamp potential of –160 mV the leak current obtained at pH 6.5 was 103 ± 6 % of the current obtained in control solution at pH 7.5. At pH 8.5 the leak current was 116 ± 12 % of control (n = 6). These data suggest that H^+ is not the major permeant ion in a Li^+ test solution. It has not been possible to determine if Li^+ carries all the current, as the leak current does not reverse at the tested clamp potentials.

Activation energy and saturation profile

The leak currents of the neurotransmitter transporters have been suggested to be a channel mode of conductance (Cammack & Schwartz, 1996; Lin *et al.* 1996) as opposed to that of the SGLT in which a transporter mode has been proposed (Loo *et al.* 1999). In an attempt to obtain more information about the mechanism by which Li^+ permeates through the transporter, we measured the Arrhenius activation energy (E_a) of the transport processes. The E_a values were determined from the slope of the Arrhenius plot (Fig. 2). The E_a value of the leak current (obtained in the range 15–27 °C) was 26 ± 1 kcal mol^{-1} at –80 mV (109 ± 4 kJ mol^{-1}; n = 4), which is not statistically different from that of the GABA-induced current, 23 ± 2 kcal mol^{-1} at –50 mV (96 ± 8 kJ mol^{-1}; n = 5).

We tested for saturation of the leak current at increasing concentrations of Li^+ at different clamp potentials (data not shown). At the most negative clamp potential (−160 mV) there was a barely detectable saturation of the current, whereas the current was a linear function of the Li^+ concentration at less negative potentials.

Water permeability measurements

The existence of a passive water permeability (L_p) through the transporter has been demonstrated previously for the

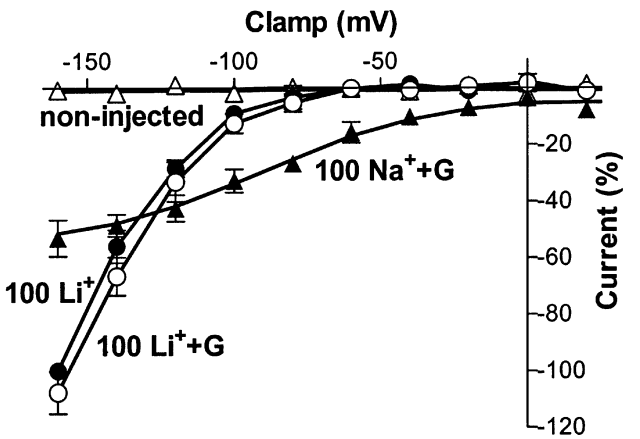

Figure 1. Li^+-induced leak current *versus* GABA-induced current

Non-injected and GAT-1-expressing oocytes were clamped to a holding potential of –50 mV before the membrane potential was jumped to the test potential for 300 ms (from +40 to –160 mV with intervals of 20 mV). Data are presented as a percentage of the Li^+-induced leak current obtained in the GAT-1-expressing oocytes with 100 mM LiCl at –160 mV and have been averaged for 5 oocytes. ▲, GABA-induced current ($I_{Na+GABA} - I_{Na}$); ●, the leak current ($I_{Li} - I_{Ch}$); ○, the leak current in the presence of 100 μM GABA ($I_{Li+GABA} - I_{Ch}$); and △, the Li^+-induced leak current in a non-injected oocyte.

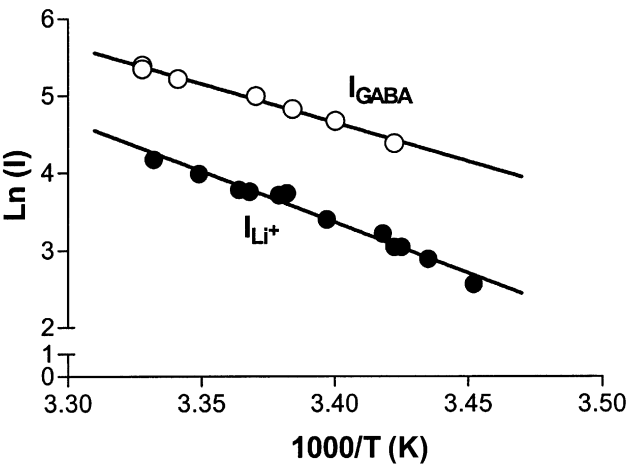

Figure 2. Arrhenius activation energy of the leak current and the GABA-induced current

For the GABA-induced current, GAT-1-expressing oocytes were clamped to a holding potential of –50 mV in Na^+ solution and 100 μM GABA was added to the test solution at different bath temperatures (I_{GABA}). For the leak current, the oocytes were clamped to –80 mV in Ch^+ solution which was replaced with the Li^+ solution to obtain the leak current (I_{Li}) at the different bath temperatures. Data are presented as Ln of the current obtained by either GABA or Li^+ as a function of temperature (K). The present experiment is a representative example of 4.

GAT-1 as well as for several other Na^+-coupled transporters (Zeuthen, 1991; Zeuthen *et al.* 1996; Loike *et al.* 1996; Loo *et al.* 1996, 1999; Meinild *et al.* 2000; MacAulay *et al.* 2001*b*). Since an alteration of this permeability reflects a change in transporter conformation, we compared the L_p value in the presence of Na^+, Li^+ or Ch^+. The water permeability measurements were performed in the two-electrode voltage clamp set-up with simultaneous monitoring of the oocyte from beneath with a sensitive camera, which gives an accurate read-out of the volume of the oocyte (Zeuthen *et al.* 1997; Meinild *et al.* 1998). Application of a hyperosmotic gradient in the surrounding test solution (which contained Na^+, Li^+ or Ch^+, but no GABA) caused the oocyte to shrink as water osmotically escaped the cytoplasm of the oocyte (Fig. 3*A*; see Methods for the calculation of the L_p). In agreement with earlier studies (Loo *et al.* 1999), we observed in GAT-1 an inherent passive water permeability (L_p) as reflected by the ability of the GAT-1-expressing oocyte to shrink at a higher rate than the non-injected oocyte. Subtraction of the contribution from the non-injected oocyte membrane ($2.19 \pm 0.23 \times 10^{-6}$ cm s^{-1} (osmol l^{-1})$^{-1}$, $n = 4$) allowed for

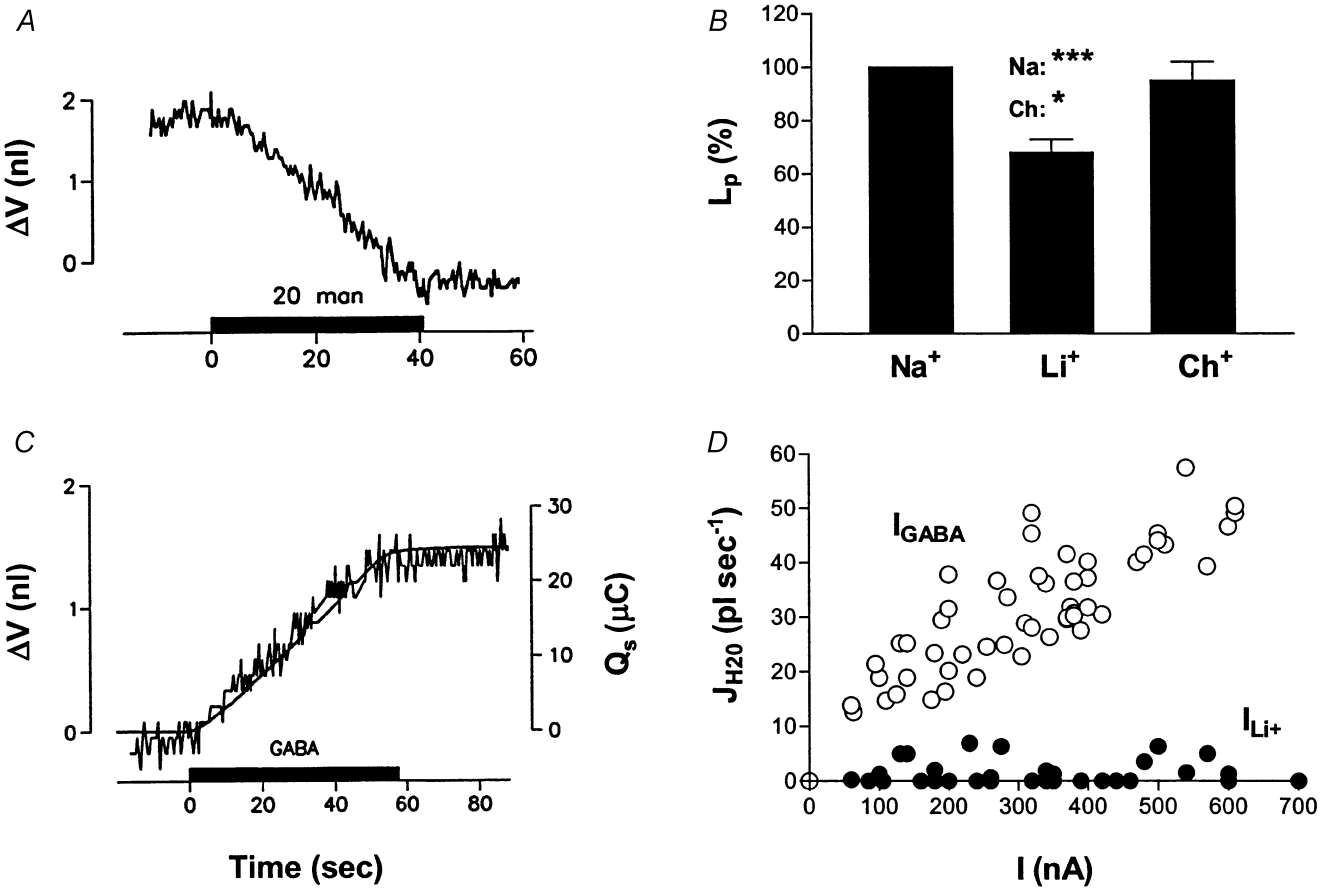

Figure 3. Water transport properties of GAT-1

A, a GAT-1-expressing oocyte was clamped to –30 mV and was superfused for 40 s with a test solution of the same ionic composition as the control solution (no GABA) but with the addition of 20 mosmol l^{-1} mannitol (man) to obtain a hyperosmolar solution (filled bar). ΔV is the change in volume of the oocyte. The L_p was calculated from the rate of shrinkage of the oocyte volume (see Methods). *B*, the oocytes were bathed in a control solution containing either 100 mM Na^+, Li^+ or Ch^+ as indicated and were superfused with the hyperosmolar test solution for 40 s. The L_p was calculated for each oocyte as a percentage of the L_p obtained in 100 mM Na^+. The data are presented as an average of these percentages ($n = 5$). * $0.01 < P < 0.05$; *** $P < 0.001$. The contribution from the native oocyte membrane has been subtracted. *C*, a GAT-1-expressing oocyte was clamped to –50 mV and 100 μM GABA was isotonically added to the test solution (filled bar). Accordingly, there was no osmotic driving force across the membrane. The jagged line in the figure represents the volume of the oocyte and the straight line represents the total amount of charges translocated by the GABA transport (Q_s). *D*, GAT-1-expressing oocytes were clamped to varying potentials (from –30 to –140 mV). The leak current (I_{Li}) obtained with 100 mM Li^+ ($I_{Li} - I_{Ch}$) or the GABA current I_{GABA} obtained with 100 μM GABA ($I_{Na+GABA} - I_{Na}$) gave currents in the range 50–700 nA ($n = 6$–7). The accompanying water flux (J_{H_2O}) is plotted *versus* this current for the leak current (●) and the GABA-induced current (○). See Methods for calculation of the coupling ratio.

a determination of the L_p of the expressed GABA transporters (in the present study $2.92 \pm 0.57 \times 10^{-6}$ cm s^{-1} (osmol $l^{-1})^{-1}$ in Na^+ ($n = 5$)). The L_p of the non-injected oocyte was not affected by the choice of cation in the solution (data not shown). The passive water permeability of GAT-1 is completely abolished in the presence of the inhibitor SKF89976A (Loo *et al.* 1999). Interestingly, GAT-1 displayed a significantly smaller L_p when Li^+ was present in the solution (68 ± 5 % of the L_p obtained in the Na^+ solution, $n = 5$) as compared with when Na^+ or Ch^+ was the main cation present (Fig. 3*B*). The water permeability observed in the presence of Ch^+ was not significantly different from the L_p in the presence of Na^+ (95 ± 7 %, $n = 5$), although it was significantly different from the L_p obtained in Li^+. The L_p was determined for each oocyte with all three cations, which made the oocyte its own control; therefore differences in the size of the oocytes can be neglected. The difference among the data was significant even when the contribution from the non-injected oocyte was not subtracted from the L_p obtained with the GAT-1-expressing oocytes, which indicates that variability between batches does not affect the confidence of the calculated results. These data support the notion that the Li^+-bound state of GAT-1 is structurally distinct.

Active water transport

Several cotransporters have been shown not only to possess a passive water permeability but also to transport water along with their substrate in a secondarily active mode (with coupling ratios of 50–500 water molecules per charge; for review see Zeuthen, 2000; Zeuthen & MacAulay, 2002). Active water transport has been shown previously in GAT-1 (Loo *et al.* 1996), although the exact coupling ratio was not determined. As seen in Fig. 3*C* (jagged line), the volume of the clamped oocyte increased linearly with time in the presence of GABA. It should be noted that there is no osmotic driving force across the membrane under these experimental conditions. The straight line is the integrated GABA-induced current, which reflects the total amount of charges entering the cell. Comparison of these two traces indicates a fixed amount of water molecules entering the cell per translocated charge. The coupling ratio was calculated from the slope of the volume trace (the water flux; see Methods), and was a linear function of the GABA-induced current (Fig. 3*D*). The calculated coupling ratio was 330 ± 49 water molecules per charge ($n = 7$). The increase in current (along the abscissa in the Fig. 3*D*) was obtained by varying the clamp potential from –30 to –140 mV. Li^+-induced leak currents of the same amplitude did not give rise to a similar water transport (the same oocytes were used to obtain both the GABA-induced current and the Li^+-induced leak current). In fact, little water followed the current, 33 ± 19 water molecules per charge, which was not significantly different from zero ($n = 6$), and there was no increase in water flux with increasing current. These data underline the distinctive nature of the leak conductance in comparison to the substrate-transporting mode.

Effect of Na^+ on the leak current

We wished to explore the effect of Na^+ on the Li^+-induced leak current of GAT-1 by generating *I–V* curves with

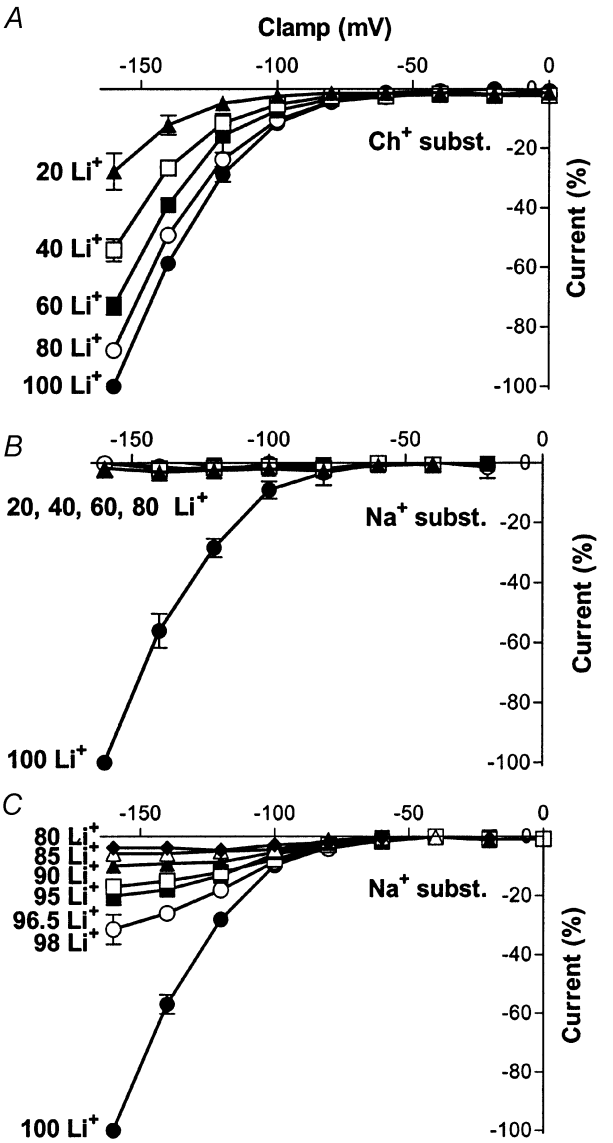

Figure 4. The effect of Na^+ on the Li^+-induced leak current

GAT-1-expressing oocytes were clamped to a holding potential of – 50 mV before the membrane potential was jumped to the test potential for 300 ms (0 to –160 mV with intervals of 20 mV) at different Li^+ concentrations. Data are presented as a percentage of the leak current obtained with 100 mM LiCl at –160 mV and have been averaged for 5 oocytes. *A*, Li^+ was substituted with equimolar Ch^+ and the leak current ($I_{Li} - I_{Ch}$) at different Li^+ concentrations are plotted. ▲, 20 mM Li^+; □, 40 mM Li^+; ■, 60 mM Li^+; ○, 80 mM Li^+; ●, 100 mM Li^+. *B*, Li^+ was substituted with equimolar Na^+, otherwise as above. *C*, Li^+ was substituted with equimolar Na^+, as in *B*, but the Li^+ concentrations were as follows: ◆, 80 mM Li^+; △, 85 mM Li^+; ▲, 90 mM Li^+, □, 95 mM Li^+; ■, 96.5 mM Li^+; ○, 98 mM Li^+; ●, 100 mM Li^+.

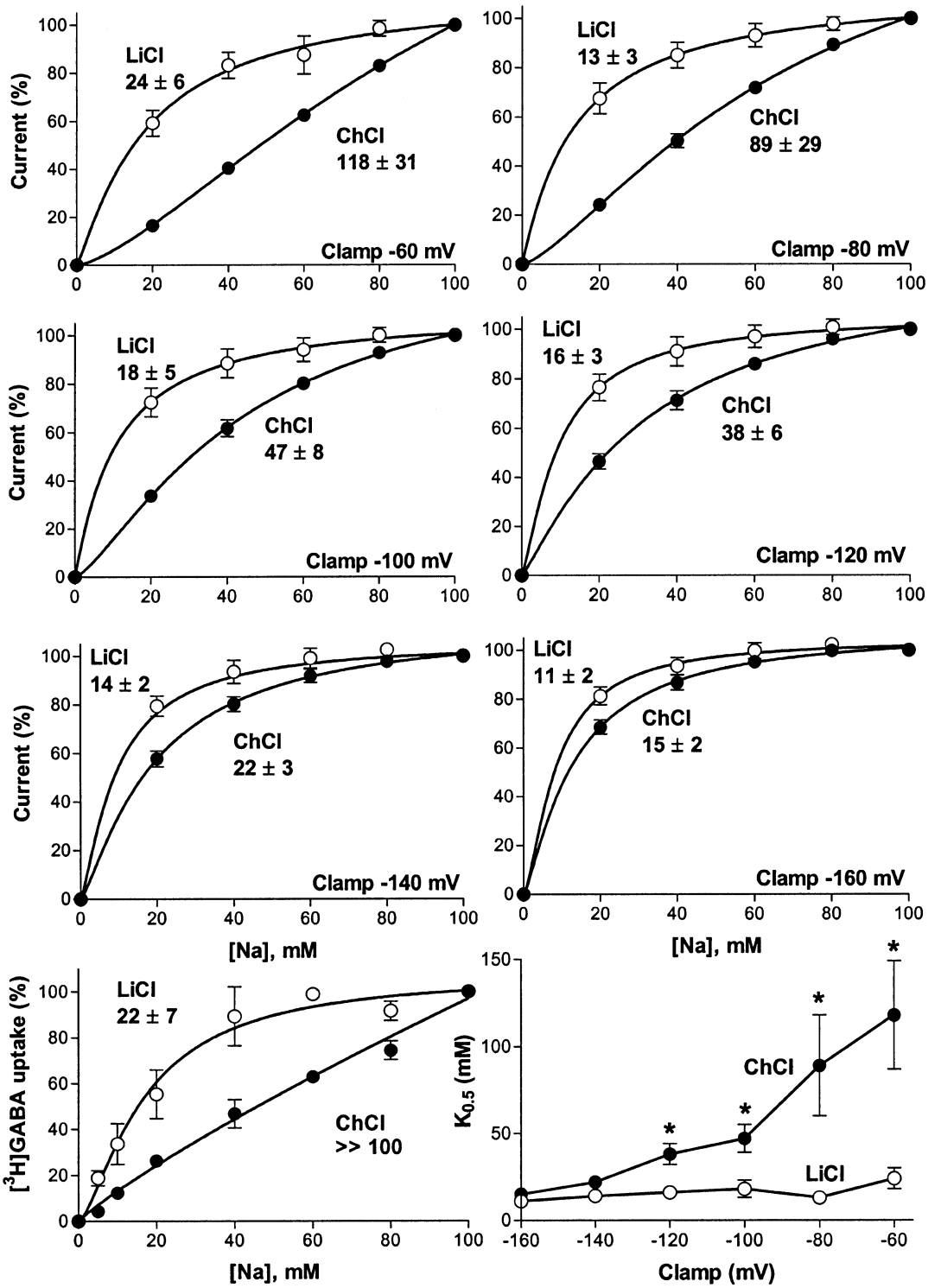

Figure 5. Apparent Na^+ affinity with different substituting cations

GAT-1-expressing oocytes were clamped to a holding potential of –50 mV before the membrane potential was jumped to the test potential for 300 ms (0 to –160 mV with intervals of 20 mV) at different Na^+ concentrations +/- 100 μM GABA (Ch^+ or Li^+ substitution). Data are presented as the GABA-induced current (% of the current obtained at 100 mM Na^+) with Li^+ substitution (○) or with Ch^+ substitution (●) at different clamp potentials as stated. Data are average of 4 oocytes. The data were fitted to the Hill equation and the resulting $K'_{0.5}$ values (mM) are stated in the figure and plotted in the lower right panel. * $0.01 < P < 0.05$. The identical experimental conditions were repeated with [^{3}H]GABA uptake in unclamped oocytes where the data are presented as the percentage of the uptake at 100 mM Na^+. The average of 3 experiments (performed in quadruplicate) is shown in the lower left panel with the $K'_{0.5}$ values of the experiment. The Ch^+-substituted Na^+ curve did not reach saturation at the Na^+ concentrations used, so a reliable $K'_{0.5}$ could not be calculated for this curve.

increasing amounts of Li^+ (0–100 mM), substituted with equimolar Ch^+ (Fig. 4*A*) or Na^+ (Fig. 4*B* and *C*). With Ch^+ as the substituting cation, the leak current increased essentially linearly with increasing Li^+ concentration at all potentials tested (Fig. 4*A*). With Na^+ as the substituting ion, however, this was not the case. The presence of even small concentrations of Na^+ reduced the leak current substantially (compare Fig. 4*A* and *B*). In Fig. 4*C*, even smaller concentrations of Na^+ were used to determine the concentration of Na^+ that led to 50 % inhibition of the leak current: 2.7 ± 0.1 mM at –120 mV ($n = 5$). These data show that Na^+ has an inhibitory effect on the Li^+ -induced leak current.

GABA transport is dependent on the binding of two Na^+ ions prior to GABA translocation (Radian & Kanner, 1983; Keynan & Kanner, 1988). Modelling of the GABA transporter led Hilgeman & Lu (1999) to propose two different affinities by which these two Na^+ ions bind to the transporter. According to this model, the transporter releases its substrate to the cytoplasm of the cell, after which an apparently low-affinity Na^+ binding site (K_d of 920 mM) opens up facing the outside of the membrane. Na^+ binding to this apparently low-affinity 'first' Na^+ binding site leads to the formation of the outward-facing conformation by a voltage-dependent step and during this process a 'second' apparently high-affinity binding site (K_d of 10 mM) becomes accessible from the extracellular side, leading to binding of the second Na^+ ion and subsequently to GABA binding and translocation (Hilgemann & Lu, 1999). The sequential and co-operative binding of the two sodium ions was reflected in a characteristic sigmoidal Na^+ dependence curve of the GABA-induced current with a Hill coefficient of 1.4 ± 0.1 at –120 mV ($n = 5$, data not shown and Martin & Smith, 1972; Keynan *et al.* 1992; Mager *et al.* 1993).

A conceivable explanation for the above data would be that Li^+ interacts with the first, apparently low-affinity cation binding site in the absence of Na^+, allowing not only the transporter to go into a leak-conducting mode but also leading to exposure of the second apparently high-affinity Na^+ binding site. Binding of Na^+ to this site could then lead to a conformational change causing inhibition of the leak current with a half-maximal effect at 2.7 mM. Thus, Na^+ may bind to the second site with the same apparently high affinity whether it is to 'pull' the transporter out of its leak-conducting mode or whether it is to support GABA-induced current. This leads to the question: if Li^+ can substitute for the first Na^+ ion, and still allow for the second Na^+ to bind, can the Li^+–Na^+ transporter complex support GABA binding and translocation? We determined the Na^+ dependence of the GABA-induced current with the substituting ion being either Ch^+ or Li^+. As seen from the upper six panels in Fig. 5, the GABA-induced current reached saturation at lower Na^+ concentrations when Li^+ was the substituting ion than when Ch^+ replaced the Na^+, that is, lower concentrations of Na^+ were required to obtain half-maximal GABA currents when Li^+ was present in the bath (for $K'_{0.5}$ values, see Fig. 5). We verified that this current was indeed due to GABA transport by performing the identical experiment with [^{3}H]GABA uptake into unclamped oocytes (Fig. 5, lower left panel). This finding indicates that Li^+ inclusion in the buffer markedly reduces the voltage dependence of the apparent Na^+ affinity and thereby suggests a contribution of Li^+ to the Na^+ activation of the GABA-induced current. The Li^+-induced leak current does not contribute to the generated current under these experimental conditions since no Li^+-induced leak current is observed in the presence of 20 mM Na^+ (see Fig. 4). The lowest Na^+ concentration used therefore was 20 mM (lower concentrations were used in the uptake experiment, where the leak current is not an issue). As GABA translocation is strictly dependent on the presence of Na^+ (data not shown and Radian & Kanner, 1983; Keynan & Kanner, 1988), the GABA-induced current is set to zero in the absence of Na^+. The difference between the $K'_{0.5}$ values obtained with Ch^+ and Li^+ decreased as the membrane potential became more hyperpolarized (lower right panel in Fig. 5). While the $K'_{0.5}$ values for Na^+ with Li^+ substitution did not change significantly with the membrane potential ($P > 0.05$, two-tailed t test, $n = 4$), the voltage-dependent apparent Na^+ affinity obtained with Ch^+ substitution markedly increased at more negative membrane potentials (Fig. 5 and Mager *et al.* 1993). Altogether, with Li^+ as the substituting ion, the transporter appears to sense a high cation concentration, even at low Na^+ concentrations, consistent with the notion that Li^+ may substitute for the first apparently low-affinity Na^+ binding in the GABA translocation cycle.

DISCUSSION

Originally a Na^+-coupled cotransporter was thought of as a protein whose sole role was the translocation of its substrate, often against large electrochemical gradients. However, increasing evidence suggests that transport proteins show resemblance to ion channels by also carrying currents unrelated to translocation of their substrate. The glutamate transporters have for example been shown to carry a large glutamate-gated Cl^- conductance and can thus also be considered substrate-gated anion channels (Fairman *et al.* 1995; Wadiche *et al.* 1995; Eliasof & Jahr, 1996). In addition, several cotransporters, among them the GABA transporters as well as the monoaminergic transporters, were shown to support uncoupled leak currents (Umbach *et al.* 1990; Mager *et al.* 1994, 1996; Galli *et al.* 1995; Vandenberg *et al.* 1995; Sonders *et al.* 1997). The leak permeability differs between the transporters with GAT-1 being permeable to Li^+ and to a lesser extent Cs^+, but not to Na^+ (Mager *et al.* 1996; Bismuth *et al.* 1997; Loo *et al.* 1999;

MacAulay *et al.* 2001*a*), whereas other functionally related transporters, such as SERT, DAT, the noradrenaline transporter (NET), the glutamate transporter-1 or the excitatory amino acid transporter-1 (EAAT1) and SGLT, also sustain Na^+ leak currents (Umbach *et al.* 1990; Mager *et al.* 1994; Vandenberg *et al.* 1995; Galli *et al.* 1995; Sonders *et al.* 1997). This Na^+ leak current is often smaller than the Li^+-induced leak current (Mager *et al.* 1994; Sonders *et al.* 1997; Panayotova-Heiermann *et al.* 1998; Petersen & DeFelice, 1999). In this study, we ruled out the possibility of Li^+ serving a permissive role for subsequent H^+ permeation in GAT-1, although H^+ has been shown to permeate GAT-1 and SERT in a NMDG test solution (Cao *et al.* 1997). Under their experimental conditions, with both Na^+ and Li^+ absent, protons may replace the role of Li^+ (although at less negative potentials, −40 mV) or they may permeate via a proton wire through a water-filled pore (Cao *et al.* 1997). Cl^- is not carried through the leak current pathway in SERT and DAT (Lin *et al.* 1996; Sonders *et al.* 1997), although its presence in the test solution is necessary to obtain maximal leak currents in SERT, DAT and GAT-1 (Lin *et al.* 1996; Mager *et al.* 1996; Sonders *et al.* 1997).

The substrate translocation in the monoaminergic transporters and GAT-1 is strictly dependent on the presence of Na^+ and cannot transport their substrates with Li^+ as the cationic ligand (Radian & Kanner, 1983; Keynan & Kanner, 1988; Gu *et al.* 1994; Galli *et al.* 1995, 1997; Lin *et al.* 1996; Sonders *et al.* 1997; Petersen & DeFelice, 1999), although it appears as if substrates can interact with the Li^+-bound state in SERT and DAT (Mager *et al.* 1994; Sonders *et al.* 1997; Petersen & DeFelice, 1999). This transporter–substrate interaction inhibits the Li^+ leak current in these two transporters, whereas the leak current of GAT-1 is completely unaffected by the presence of substrate. The Li^+-bound conformation may therefore not support GABA binding.

Mechanism of the Li^+-induced leak current

Based on a high Arrhenius activation energy (19 kcal mol^{-1} (79 kJ mol^{-1})), a Hill coefficient of 2 and the same apparent Na^+ affinity (2.5 mM) of the leak current and the glucose transport (Loo *et al.* 1999), it was suggested for the SGLT that the leak current is carried through this transporter in the 'transporter mode', i.e. the leak is a consequence of the transporter moving through its transport cycle even in the absence of substrate. Another possibility is that the current arises as a channel mode of conductance as was suggested for SERT and GAT-1 (Cammack & Schwartz, 1996; Lin *et al.* 1996). Previously, we have observed distinct Zn^{2+} sensitivities of the GABA-induced current and the Li^+-induced leak current in a mutant GAT-1 containing a bidentate Zn^{2+} binding site between transmembrane segments 7 and 8 (T349H/Q374C; MacAulay *et al.* 2001*a*). Based on these findings we were able to conclude that either the conformational changes responsible for the Li^+ conductance are different from those involved in GABA translocation and/or the conformational states adopted by the Li^+-bound transporter are distinct from those adopted in the presence of Na^+–GABA. The current data provide additional support for an altered conformational state of the Li^+-bound transporter, as reflected in the reduced passive water permeability of the transporter in the presence of Li^+ as compared with that in the presence of Na^+ or Ch^+. Most probably this lower water permeability is a result of a smaller aqueous pore in the Li^+-bound conformation. Of notable interest, the SGLT did not show this Li^+-induced reduction in the passive water permeability (Loo *et al.* 1999). However, covalent modification with sulfhydryl-reactive methanethiosulphonate (MTS) reagents of the closely related SERT and the glycine transporter has shown a distinct Li^+-bound conformation, similar to the findings in GAT-1, suggesting that it is not the lack of Na^+ binding that renders the conformational occupancy distinct but it is the Li^+ binding *per se* (Chen *et al.* 1997; Lopez-Corcuera *et al.* 2001; Ni *et al.* 2001). The distinct nature of the leak current and the substrate translocation process is also supported by the number of mutated or modified transporters in which the leak current is intact but the transport current is abolished (Mager *et al.* 1996; Bismuth *et al.* 1997; Yu *et al.* 1998; MacAulay *et al.* 2001*a*).

Several Na^+- and H^+-coupled cotransporters have been shown to translocate water across the membrane together with their substrates. This has been found for the K^+–Cl^- cotransporter (Zeuthen, 1994), the lactate transporter MCT-1 (Zeuthen *et al.* 1996), SGLT (Loo *et al.* 1996; Meinild *et al.* 1998), the dicarboxylate transporter NaDC-1 (Meinild *et al.* 2000), the glutamate transporter EAAT1 (MacAulay *et al.* 2001*b*), GAT-1 (Loo *et al.* 1996), and the plant H^+-amino acid transporter APP5 (Loo *et al.* 1996). In each of these cotransporters, water is transported with a fixed coupling ratio with a value in the range of 50–500 water molecules per charge. The water transport is independent of external parameters, such as ligand concentrations, osmolarity and temperature, and even takes place uphill, against an imposed water–chemical gradient favouring water transport the opposite way (Zeuthen, 1994; Meinild *et al.* 1998, 2000; MacAulay *et al.* 2001*b*). These studies suggest that the active and passive water transport are two independent modes of transport that proceed in parallel. The active water transport is stoichiometrically coupled to the substrate translocation and is *not* due to a build-up of an osmotic gradient as Na^+ and other ligands are transported into the cell (Zeuthen *et al.* 2002; for review see Zeuthen, 2000; Zeuthen & MacAulay, 2002). The GABA transport led to the translocation of 330 ± 49 water molecules per translocated charge ($n = 7$). If GABA transport leads to translocation of only one charge

(Kavanaugh *et al.* 1992), the coupling ratio reflects the number of water molecules being transported per turnover. If two charges are being translocated per GABA molecule, as was recently suggested (Loo *et al.* 2000), it follows that 660 water molecules are transported per turnover. It is noted that the water flux as a function of current does not appear to be a straight line through 0.0. A previous study on EAAT1 showed a similar pattern in the *presence* of the permeable anion, whereas the water flux was a linear function of the glutamate transport (through 0.0) in the *absence* of the permeable anion (MacAulay *et al.* 2001*b*). In analogy to this, the GABA-induced current may be made up of two components – the transport-associated current and an uncoupled current. The existence of a substrate-induced uncoupled current component has been proposed for GAT-1 (Cammack *et al.* 1994; Risso *et al.* 1996) and for the monoaminergic transporters (Mager *et al.* 1994; Galli *et al.* 1995, 1997; Sonders *et al.* 1997; Petersen & DeFelice, 1999).

Currents of the same amplitude as the GABA-induced current could be obtained with the leak current, yet no significant water flux was observed ($n = 6$). This clearly distinguishes the mechanism of Li^+ permeation from that of the GABA translocation. As mentioned above, it has been shown that the water transport is *not* driven by the osmotic build-up of ions and substrate (Meinild *et al.* 1998; MacAulay *et al.* 2001*b*; Zeuthen *et al.* 2001, 2002). Even so, it could be argued that four molecules are transported into the cytoplasm per charge translocated by the GABA transport while only one Li^+ enters the cytoplasm per charge during the leak-current process, and that this might cause the increased water flux with the GABA transport. One should then multiply the number of water molecules transported with the leak current by four ($33 \times 4 = 132$) in order to compare with the number of water molecules translocated with GABA into the cell (330). Thus, osmotic build-up would still not explain the difference in the water transport properties of these two current modes.

The E_a of the leak current in GAT-1 was not significantly different from that of the GABA-induced current. At first this would indicate the involvement of large conformational changes in the mechanism with which the leak current takes place. However, as the leak current barely showed any saturation with increased Li^+ concentration and did not carry any water, we propose that the permeation of Li^+ takes place in a channel mode of conductance but that the actual opening of the pore requires conformational changes. In support of this, the voltage dependence of the leak current is quite steep and the permeation does not take place until the membrane potential is more hyperpolarized than −75 mV. The driving force for Li^+ would in itself allow Li^+ to permeate at much more depolarized potentials, which suggests that at hyperpolarized potentials, Li^+ leads to an increase in the single-channel open probability, as was proposed for SERT (Lin *et al.* 1996). It follows that in GAT-1, the conformational change leading to channel opening does not take place until the membrane potential is hyperpolarized below −75 mV. The high E_a may then reflect upon the opening of the channel and not on the permeation through the pore (Hille, 2001). In analogy with this, the *Shaker* K^+ channel has low activation energies for the conducting current and high activation energies for the opening and closing of the channel (Nobile *et al.* 1997).

Conformational basis of the leak current

The GABA transporter translocates two Na^+ ions per GABA ion (Radian & Kanner, 1983; Keynan & Kanner, 1988) and by a model proposed by Hilgeman & Lu (1999), these two Na^+ ions bind to the transporter with distinct affinities in a co-operative manner. According to this model, the apparent affinity of the first Na^+ binding site is around 900 mM and that of the second Na^+ binding site around 10 mM. The Na^+ activation curve showed a strong voltage dependence of the apparent Na^+ affinity from around 15 mM at −160 mV, 40 mM at −120 mV to > 100 mM at −60 mV (Fig. 5 and Mager *et al.* 1993). This voltage dependence may well reflect on the binding of the first Na^+ as this binding step has been associated with the voltage-dependent return step of the empty transporter from inward-facing to outward-facing (Parent *et al.* 1992; Hilgemann & Lu, 1999). Interestingly, Na^+ inhibited the Li^+-induced leak current with a half-maximal effect at 2.7 mM Na^+, suggesting that the binding of Na^+ (with an apparent affinity constant of around 2.7 mM) constrains GAT-1 in a conformation that does not support a leak current. An intriguing explanation is that Li^+ is able to substitute for the first Na^+ ion and thereby allow Na^+ to bind with the apparently high affinity that is characteristic of the second Na^+ binding site. As Li^+ replaced Na^+ in the first binding site, GABA transport took place with a significantly higher apparent Na^+ affinity than when Na^+ was substituted with Ch^+ (Fig. 5), as has also been found in the glutamate transporter, GLT-1 (Grunewald & Kanner, 1995). At potentials from −60 to −120 mV, the apparent affinity for Na^+ was significantly different with the two different cation substitutes (Ch^+ or Li^+). The apparent Na^+ affinities obtained with the two different substituting cations approached each other at the more hyperpolarized test potentials (−140 and −160 mV), most probably because the apparent Na^+ affinity of the voltage-dependent binding of the first Na^+ is so high at this potential that the two Na^+ binding sites most likely approach the same apparent Na^+ affinity, and the Li^+ substitution is no longer stimulatory.

Altogether, we propose that Li^+ can bind to the first cation binding site of the transporter (C_1Li) as depicted in the simplified model in Fig. 6. At hyperpolarized potentials, a

Li^+-permeable channel opens (C_1LiO) and gives rise to the leak current. Na^+ may bind to the Li^+-bound state (C_2LiNa) in a similar manner as it would bind to the 'normal' Na^+-bound state ($C_1Na_1 \rightarrow C_2Na_2$) before GABA (S) binds to either of those two states (C_3LiNaS or C_3Na_2S) and the complex gets translocated (C_4LiNaS or C_4Na_2S). In theory, the pore may also be permeable to Na^+, as is the case for DAT, SERT, NET, SGLT and EAAT1 (Umbach *et al.* 1990; Mager *et al.* 1994; Galli *et al.* 1995; Vandenberg *et al.* 1995; Sonders *et al.* 1997), but since low concentrations of Na^+ transfer the protein into the C_2Na_2 conformation which is not permeable, no Na^+ permeation would be detected. In support of this, covalent modification with sulfhydryl-reactive methanethiosulphonate (MTS) reagents of the first external loop in GAT-1 renders the transporter permeable to Na^+ as well as Li^+ (Yu *et al.* 1998), which could be interpreted as the transporter getting 'stuck' in the C_1 conformation and thereby allowing Na^+ to permeate. Non-additive Na^+- and Li^+-induced leak currents have also been observed in SERT (Petersen & DeFelice, 1999; Ni *et al.* 2001) and an idea similar to the one presented in this paper was introduced (Ni *et al.* 2001). The authors suggested that Na^+ stabilized a conformation of the protein that was different from that of the Li^+-bound conformation. Another possibility is that Na^+ and Li^+ may interact in a common pore with anomalous mole fractions, as was suggested for the *Drosophila* SERT, with 6 mM Na^+ inhibiting the Li^+ current down to 50 % (Petersen & DeFelice, 1999), instead of the 3 mM found in the present study with GAT-1.

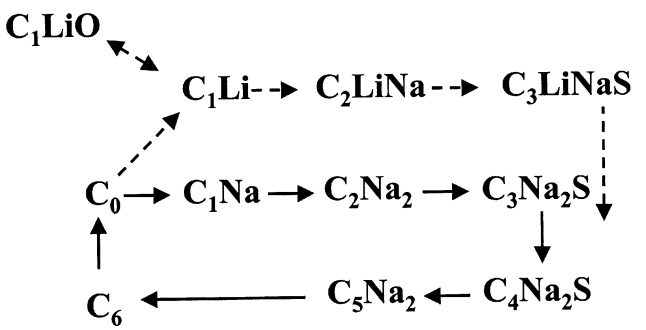

Figure 6. The GAT-1 reaction scheme

The simplified GAT-1 reaction scheme shows the empty inward-facing transporter (C6) returning to the empty outward-facing state (C_0) where 2 Na^+ are bound to the transporter sequentially ($C_0 \rightarrow C_1Na \rightarrow C_2Na_2$) before substrate (S) is bound ($C_2Na_2 \rightarrow C_3Na_2S$) and the complex is translocated (C_4Na_2S). An alternative pathway is shown with dotted arrows where Li^+ can replace the first Na^+ and enter into a conformationally distinct state (C_1Li) from which the Li^+ leak channel may open (C_1LiO). Na^+ can bind to the second apparently high-affinity Na^+ binding site (C_2LiNa) and the transporter can no longer sustain the Li^+-induced leak current. According to our model, the Li^+–Na^+- bound complex binds substrate (C_3LiNaS) and the translocation takes place.

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