Residues in the Extracellular Loop 4 Are Critical for Maintaining the Conformational Equilibrium of the \( \gamma \)-Aminobutyric Acid Transporter-1\( ^* \)

Received for publication, December 20, 2002, and in revised form, April 22, 2003
Published, JBC Papers in Press, May 22, 2003, DOI 10.1074/jbc.M213023200

Nanna MacAulay†¶§§, Anne-Kristine Meinild§§, Thomas Zeuthen¶, and Ulrik Gether¶

From the ³Department of Medical Physiology and the ³Department of Pharmacology, The Panum Institute, University of Copenhagen, DK-2200 Copenhagen N, Denmark

We mutated residues Met\(^{345} \) and Thr\(^{349} \) in the rat \( \gamma \)-aminobutyric acid transporter-1 (GAT-1) to histidines (M345H and T349H). These two residues are located four amino acids apart at the extracellular end of transmembrane segment 7 in a region of GAT-1 that we have previously suggested undergoes conformational changes critical for the transport process. The two single mutants and the double mutant (M345H/T349H) were expressed in Xenopus laevis oocytes, and their steady-state and presteady-state kinetics were examined and compared with wild type GAT-1 by using the two-electrode voltage clamp method. Oocytes expressing M345H showed a decrease in apparent \( \gamma \)-aminobutyric acid affinity, an increase in apparent affinity for \( Na^+ \), a shift in the charge/voltage (\( Q/V_m \)) relationship to more positive membrane potentials, and an increased \( Li^+ \)-induced leak current. Oocytes expressing T349H showed an increase in apparent GABA affinity, a decrease in apparent \( Na^+ \) affinity, a profound shift in the \( Q/V_m \) relationship to more negative potentials, and a decreased \( Li^+ \)-induced leak current. The data are consistent with a shift in the conformational equilibrium of the mutant transporters, with M345H stabilized in an outward-facing conformation and T349H in an inward-facing conformation. These data suggest that the extracellular end of transmembrane domain 7 not only undergoes conformational changes critical for the translocation process but also plays a role in regulating the conformational equilibrium between inward- and outward-facing conformations.

The \( \gamma \)-aminobutyric acid (GABA) transporters belong to a large family of \( Na^+ /Cl^- \) coupled neurotransmitter transporters that includes the transporters for several other neurotransmitters such as serotonin, dopamine, noradrenaline, and glycine. These transporters are involved in re-uptake of the neurotransmitter at the synaptic terminals and thereby contribute to termination of the neuronal response. The transport proteins are predicted to contain 12 transmembrane domains (TMs) (1) with cytosolic amino and carboxyl termini (2). The GABA re-uptake is coupled to the cotransport of \( 2 Na^+ \) and \( 1 Cl^- \), which renders the transport process electrogenic (3, 4). Several electrophysiological studies of GAT-1 heterologously expressed in either mammalian cell lines or in Xenopus laevis oocytes have been carried out, and four current-generating modes of the transporter have been described; they are the \( Na^+ \)-coupled substrate-induced current, capacitive \( Na^+ \)-dependent presteady-state currents, a \( Li^+ \)-induced leak conductance, and a not fully documented un-coupled substrate-induced channel activity (5–18).

It is a general assumption that the \( Na^+/Cl^- \)-dependent transporters operate by an alternating access mechanism, where the transporter interchanges between a series of “outward-facing” conformations, in which the substrate binding sites are accessible to the extracellular medium, and a series of “inward-facing” conformations, in which the binding sites are accessible to the intracellular environment. However, relatively little is known about the conformational changes associated with the binding and translocation of GABA, \( Na^+ \), and \( Cl^- \)(19, 20). Several residues in the transmembrane domains (6, 20–25) and in the extracellular loops (26–28) have been proposed to be involved in substrate binding and/or translocation in GAT and the monoamnergic transporters. Previously, we have obtained evidence in both GAT-1 (14) and the homologous dopamine transporter, DAT (29–31), that the external ends of TM 7 and 8 undergo conformational changes that are critical for the translocation process. This conclusion was based on the observation that binding of \( Zn^{2+} \) to bidentate \( Zn^{2+} \) binding sites engineered between TM 7 and 8 in both GAT-1 and DAT results in potent non-competitive inhibition of substrate transport. This is consistent with the ability of \( Zn^{2+} \) to restrain movements between the two helices and/or of the connecting fourth extracellular loop.

In this study we have investigated two mutants in the GAT-1 situated only four residues apart at the external end of TM 7. The residues were initially mutated during our attempt to transfer engineered \( Zn^{2+} \) binding sites from the dopamine transporter to GAT-1. Contrary to DAT (31), the combined mutation of the two residues Met\(^{345} \) and Thr\(^{349} \) to histidines did not result in \( Zn^{2+} \) sensitivity of GAT-1 (data not shown). Instead, we show here that the individual mutation of these two residues results in characteristic and distinct phenotypes that are consistent with oppositely directed shifts in the conformational equilibrium. It is therefore proposed that the TM 7/8 microdomain in this class of transporters not only may undergo conformational changes during the transport process but also may play a role in maintaining an appropriate confor-
mational equilibrium of the transporter, possibly through intramolecular interactions.

EXPERIMENTAL PROCEDURES

Molecular Biology and Oocytes—The rat GAT-1 cDNA was mutated by PCR-derived site-directed mutagenesis and cloned into a vector optimized for oocyte expression (pNB1) as earlier described (14). 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), and 50 ng cRNA was injected into defolliculated X. laevis oocytes (14). The oocytes were incubated in K+ medium (90 mM NaCl, 1 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH 7.4) at 19°C for 3–7 days before experiments were performed.

Electrophysiology—The two-electrode voltage clamp method was used to control the membrane potential and monitor the current in oocytes expressing WT and mutant transporters, as earlier described (14). Generally, the membrane potential (V_m) of the oocyte was held at −50 mV, and the experimental chamber was continuously perfused by a NaCl solution containing 100 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, pH 7.4. In Na⁺ substitution experiments, Na⁺ was replaced by equimolar choline ions, and in the leak current experiments equimolar Li⁺ replaced Na⁺. 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 6.0/8.0 (Axon Instruments). Currents were low-pass-filtered at 500 Hz and sampled at 2 kHz. Electrodes were pulled from borosilicate glass capillaries to a resistance of 0.5–2 mΩ and filled with 1 M KCl.

The transporter-specific GABA-induced current (I_{GABA}) was obtained by subtracting the current in NaCl solution from the current in NaCl solution + GABA (I_{Na/GABA} − I_{Na}), whereas the leak current was obtained by subtracting the current in ChCl solution (100 mM ChCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, pH 7.4) from the current in LiCl solution (I_{Li/GABA} − I_{Li}). For steady-state kinetic analysis, the I_{max} was measured at various membrane potentials and external substrate concentrations (SI), and at each voltage, the I_{GABA} versus the [S] relations were fitted to the Michaelis-Menten equation, I = \frac{I_{max} \times [S]}{[S] + K_{S}}, where [S] is the substrate concentration, I_{max} is the maximal current for saturating [S], and K_S is the apparent affinity constant. The apparent GABA affinity was determined at 100 mM external Na⁺ by varying the GABA concentration, and Na⁺ activation was determined at a fixed (saturating) GABA concentration by varying the external Na⁺ concentration. For determination of the presteady-state transient currents, a pulse protocol was used where V_m initially was held at −40 mV and then jumped to a series of test potentials (from +40 to +160 mV with 20-mV increments) for 500 ms before returning to V_m. At each test potential, the transporter-specific presteady-state transient currents (I_{Na/GABA} − I_{Na}) were obtained by subtraction of the current traces in ChCl solution from those obtained in NaCl solution. The charge movement, Q, was obtained by integrating the ON transient currents at different potentials with respect to time. The charge-voltage (Q/V_m) relations were fitted to a Boltzmann function, Q = Q_{50 delays} \times \exp\left(\frac{V_m - V_{50 delays}}{\kappa}\right), where Q_{50 delays} = Q_{50 delays} and \kappa are the Q at depolarizing and hyperpolarizing limits. V_{50 delays} is the Faraday constant, R is the gas constant, T is the absolute temperature, V_m is the membrane potential where there is 50% charge transfer, and \kappa is the apparent valence of the movable charge (32). The time constants, \tau, were obtained by fitting the current traces to the equation I_{transient}(t) = I_{max} \times \exp\left(-\frac{t}{\tau}\right) + I_s, where I_{transient} is the total current, I_{max} is the transporter-specific current, I_s is the capacitative current, and \tau is the steady-state current, and t is the time.

3H[GABA Uptake Experiments in Oocytes—The uptake experiments were performed in 24-well plates with 100 μM 3H[GABA (RBI, Natick, MA) and 50 mM 4-aminoo-n-2,3,4-[3H]butyric acid (3H[GABA), 88 Ci/mmol (Amersham Biosciences) added to a total of 400 μl of uptake buffer (100 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, pH 7.4). Oocytes were incubated for 30 min at room temperature, washed 3 times with 1 ml of wash buffer with choline chloride (100 mM ChCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, pH 7.4), and dissolved in 200 μl of 10% SDS. Before counting, 2 ml of scintillation fluid (Aquasol-M, LKB Wallac) was added to the sample, and radioactivity was measured using a liquid scintillation counter (SL3800, LKB Wallac).

Calculations—The data were analyzed by nonlinear regression analysis using Prism 3.0 (GraphPad Software, San Diego, CA), Clampfit (Axon Instruments Inc., Union City, CA), and SigmaPlot (Version 6.0, SPSS Inc., Chicago, IL). All numbers are given as means ± S.E., with n equal to the number of oocytes tested. The current range of the oocytes expressing the various constructs was taken as a measure of the range in expression level and is stated in the figure legends and in the table.

RESULTS

GABA Affinity and pH Dependence—Met345H and Thr349H, located at the extracellular end of TM 7 in GAT-1, were individually and together mutated to histidines, resulting in the mutants M345H, T349H, and M345HT349H. All three mutants were functional as assessed upon expression in X. laevis oocytes and subsequent uptake experiments and two-electrode voltage clamp analysis. The apparent GABA affinity (K_{0.5}) was significantly altered by the histidine mutations (Fig. 1, filled symbols). In the T349H mutation, the K_{0.5} decreased from 22 ± 4 μM in the WT (n = 7) to 4 ± 1 μM (n = 5) at a membrane potential of −60 mV, whereas M345H displayed a reduced apparent affinity as reflected in a K_{0.5} value of 220 ± 16 μM (n = 5). The double mutant T349H/M345H showed an apparent affinity intermediate of the two single mutations (K_{0.5} = 45 ± 3 μM, n = 5). Contrary to M345H and T349H/M345H, the K_{0.5} values of the WT and T349H were voltage-dependent, i.e. the apparent GABA affinity decreased upon hyperpolarization.

The effect of histidine substitutions were compared with substitutions with an alanine and a cysteine in position 345 and a cysteine in position 349. The effects of these substitutions were less pronounced and are, therefore, not dealt with in detail; M345C showed slightly reduced apparent GABA affinity at V_m = −60 mV (−2-fold change as opposed to the −10-fold in M345H, data not shown), whereas the apparent affinity of M345A was in between that of the WT and the M345H (−6-fold change, data not shown). The apparent GABA affinity of the T349C mutant transporter was slightly decreased compared with that of the WT (−2-fold change), which was exactly the opposite effect of the T349H (data not shown).

From the IV’ plots in Fig. 2 (filled symbols), it is apparent that the voltage dependence of the GABA-induced current was changed in the mutant transporters; T349H showed increased voltage dependence compared with the WT, as seen from the steep slope in the IV’ relation. The current does not saturate at the clamp potentials employed in these experiments, and very little current is seen at potentials more depolarized than −100 mV. In contrast, M345H and M345HT349H displayed less voltage dependence than the WT. This is indicated by the early saturation of the IV’ relation as the membrane potential of the oocytes is increasingly hyperpolarized and by the presence of a substrate-induced current even at 0 mV, especially for M345H.

We analyzed the effect of lowering the pH from 7.4 to 5.5 (open symbols in Figs. 1 and 2). Such a pH change would be expected to cause protonation of the inserted histidines and thereby introduce a positive charge. In the WT we observed no change in the voltage-dependent apparent GABA affinity upon lowering the pH (Fig. 1), and the magnitude of the current and the shape of the IV’ relation in the presence of saturating GABA concentration was not affected at the potentials tested. For example, at a membrane potential of −150 mV the current at pH 5.5 was 109 ± 3% compared with that seen in the control solution of pH 7.4, n = 5 (Fig. 2). This is in agreement with previous studies (10). In the mutants, however, we found a different pattern. For M345H, the reduced pH caused an increase in the apparent affinity for GABA (Fig. 1), which was completely voltage-insensitive at pH 5.5 as well as at pH 7.4. The K_{0.5} for GABA was pH-dependent in both T349H and M345HT349H. In T349H at pH 5.5, the K_{0.5} was increased at hyperpolarized potentials compared with pH 7.4. Oppositely, the K_{0.5}GABA at pH 5.5 in M345H/T349H decreased at depolarized potentials and also became voltage-dependent. For all three mutant transporters the magnitude of the GABA-induced currents was markedly increased at pH 5.5 (open symbols in
At a membrane potential of 

\[ \text{pH} 11002 \]

150 mV the current for 

\[ M345H \]

at pH 5.5 was 148 ± 6% (\[ n \] 5) of that found at pH 7.4 and 157 ± 18% (\[ n \] 4) for T349H. An even larger effect was observed in the double mutant (258 ± 20% of the current obtained at pH 7.4, \[ n \] 5), suggesting an additive effect on the transport-associated current upon protonation of both M345H and T349H. This increase in GABA-induced current at lower pH did not reflect an increase in an uncoupled transport-associated current as shown by uptake experiments with

\[ [3H]GABA \]. The \[ [3H]GABA \] uptake of WT was reduced to 90 ± 3% at pH 5.5 compared with that at pH 7.4 (\[ n \] 4), whereas the \[ [3H]GABA \] uptake of M345H/T349H was 128 ± 5 (\[ n \] 5), \( p < 0.001 \), Student’s t test. The uptake experiments were performed under unclamped conditions, in which the membrane potential approaches the reversal potential of the transporter; therefore, the numbers are not directly comparable with those

\[ GABA \] and the mutant transporters.

The GABA-induced current (\( I_{\text{Na, GABA}} - I_{\text{Na}} \)) was measured at membrane potentials from –40 mV to –160 mV with 20-mV increments with varying concentrations of GABA (from 0 to 5 mM) and a Na\(^+\) concentration of 100 mM. The GABA-induced current plotted as a function of the GABA concentration was fitted to the Michaelis-Menten equation to derive values for \( K_{0.5} \). The \( K_{0.5,\text{GABA}} \) is plotted as a function of the membrane potential in an average of 5–7 oocytes expressing each construct. The range of the GABA-induced current (saturating GABA concentration at –60 mV) was 110–380 nA for the WT, 225–690 nA for M345H, 35–145 nA for T349H, and 65–210 nA for M345H/T349H.

Filled symbols represent data obtained at pH 7.4, and open symbols represent data obtained at pH 5.5. When not visible, the error bars are within the symbol. Student’s t test; *\( p < 0.05 \).

\[ [3H]GABA \] and the mutant transporters.

The GABA-induced current (\( I_{\text{Na, GABA}} - I_{\text{Na}} \)) was measured as described in Fig. 1 (3 mM GABA) at pH 7.4 (black symbols) or pH 5.5 (white symbols). The data are presented as a representative example of five experiments. The data are summarized in the last panel as the GABA-induced current at pH 5.5 compared with the current obtained at pH 7.4 at a membrane potential of –150 mV, Student’s t test; *\( p < 0.05 \); ***, \( p < 0.001 \).
Conformational Equilibrium of GAT-1

Na⁺ was replaced with equimolar Ch⁺, and the GABA-induced currents were obtained with 3 mM GABA at varying clamp potentials. The number of experiments is stated in parentheses. NA, non-applicable, signifies a $K_{\text{Na}}$ too large to be determined with the Michaelis-Menten equation.

| $I_{\text{GABA}}$ (−50 mV) | $K_{\text{Na}}$ (nA) at different clamp potentials | Na⁺ (mM) ± S.E. |
|--------------------------|-----------------------------------|------------------|
| WT | 180−300 | 220 ± 145 (5) | 87 ± 17 (5) |
| M345H | 190−600 | 21 ± 6 (4) | 8 ± 2 (4) |
| T349H | 50−85 | NA (4) | NA (4) |
| M345H + T349H | 110−150 | 34 ± 9 (4) | 9 ± 2 (4) |

**Fig. 3.** Apparent Na⁺ affinity of GAT-1 and the mutant transporters. The Na⁺ activation of GABA-induced currents ($I_{\text{GABA}} - I_{\text{GABA}}$) was measured at a membrane potential of −120 mV with varying concentrations of Na⁺ (0−100 mM with CHCl substitution), whereas the GABA concentration was kept constant and saturating at 3 mM. The GABA-induced current plotted as a function of the Na⁺ concentration was fitted to the Michaelis-Menten equation to derive the $K_{\text{Na}}$. The data are presented as the percentage of the current obtained at 100 mM Na⁺, with an average of 4−5 oocytes expressing each construct with a GABA-induced current (saturating GABA concentrations at −50 mM) of 180−300 nA for the WT, 190−600 nA for M345H, 50−85 nA for T349H, and 110−150 nA for M345H/T349H. When not visible, the error bars are within the symbol. The $K_{\text{Na}}$ values are shown in Table I.

above, in which the GABA-induced current was measured under voltage clamp (−150 mV). As seen From Fig. 2, the percentage increase in GABA-induced current at low pH increases with more negative clamp potentials. At a membrane potential of −20 mV (which is a feasible membrane potential during an uptake experiment), the pH-induced increase in current is comparable with that obtained in the uptake experiments.

**Na⁺ Affinity**—The Na⁺ activation curves performed at −120 mV for the WT, M345H, T349H, and M345H/T349H are shown in Fig. 3, and the $K_{\text{Na}}$ values obtained at different membrane potentials ($V_{\text{m}}$) are summarized in Table I. The apparent Na⁺ affinity for the WT was 44 ± 6 mM (n = 5) and was highly voltage-dependent, in agreement with previous studies (Table I and Refs. 15 and 16). Interestingly, the apparent Na⁺ affinity of the two individual mutants (M345H and T349H) were affected in opposite directions. In T349H the apparent affinity was reduced compared with the WT ($K_{\text{Na}}$ = 200 ± 64 mM at −120 mV, n = 4) and showed an even more pronounced voltage dependence. In contrast, M345H displayed increased apparent Na⁺ affinity ($K_{\text{Na}}$ = 6 ± 1 mM, n = 4). Similarly, the double mutant M345H/T349H displayed increased apparent affinity for Na⁺ ($K_{\text{Na}}$ = 6 ± 1 mM, n = 4). Note that the Na⁺ activation curve for the GABA-induced current in both the WT and the T349H mutant did not show saturation at potentials more depolarized than −80 and −120 mV, respectively, and could therefore not be fitted by the Michaelis-Menten equation. This indicates a strong voltage dependence of the Na⁺ binding and/or the associated conformational changes. On the contrary, the apparent Na⁺ affinity of the M345H and T349H/M345H transporters barely showed voltage dependence within our range of measurements (Table I).

**Transient Currents**—The GAT-1 and related transporters give rise to substrate-independent capacitive transient currents upon shifts in the membrane potential (10, 14, 16, 33, 34). These currents are thought to arise from conformational changes associated with Na⁺ binding and dissociation and may, therefore, be used as a functional read-out of the partial reactions of the transport cycle involving these steps. To perform these experiments, the membrane potential was held at −40 mV and jumped to the test potentials (from 40 mV to −160 mV with intervals of 20 mV) for 500 ms and returned to the holding potential for another 500 ms before the next test potential was applied (Fig. 4, upper panels). Because these currents are generally entirely capacitive (16, 33, 34), the time integral of the transient OFF current is identical to that of the ON current (data not shown). The $Q/V_{\text{m}}$ relationships shown in the lower panel of Fig. 4 were obtained from the ON currents for WT, M345H, T349H, and M345H/T349H. The $Q/V_{\text{m}}$ for the WT transporter was −36 ± 2 mV (n = 5), which is in agreement with previously published data (10, 14, 16). The $Q/V_{\text{m}}$ relationship was shifted markedly toward more negative membrane potentials in T349H, as reflected by a $V_{\text{m}}$ of −85 ± 7 mV, n = 5, whereas it was shifted toward more positive potentials in M345H ($V_{\text{m}}$ = 17 ± 3 mV, n = 6). The $V_{\text{m}}$ of the double mutant (M345H/T349H) was in between that of the two single mutants ($V_{\text{m}}$ = −10 ± 6 mV, n = 5), whereas it was shifted toward more positive potentials in M345H ($V_{\text{m}}$ = 17 ± 3 mV, n = 6). The $V_{\text{m}}$ constant (r) for the relaxation of the ON currents were also examined. For the WT transporter, the $r_{\text{max}}$ was 98 ± 5 ms (n = 4), which is in accordance with previously reported values (7, 10). This time constant was increased significantly in T349H (230 ± 17 ms, n = 5, p < 0.001, Student’s t test). We also observed an apparent, although not significant, increase in $r_{\text{max}}$ for M345H and M345H/T349H (158 ± 23 ms (n = 6) and 132 ± 15 ms (n = 5), respectively).

**Leak Current**—The GAT-1 sustains substrate-independent leak currents at hyperpolarized potentials in the presence of Li⁺ (6, 14, 15, 17). As seen from Fig. 5, upper panel, the clamp potential at which the leak current commences differed for the WT and the mutants. The T349H mutant required increased hyperpolarization (compared with that of the WT) in order for the leak conductance to initiate, whereas the M345H mutant supported leak current at a more depolarized potential than the WT. The double mutant (M345H/T349H) behaved more or less like the WT with the requirement of an intermediate hyperpolarization. A remarkable feature of the M345H/T349H mutant was the significantly increased ratio between the magnitude of the leak current and the GABA-induced current: 6.7 ± 0.6 for M345H/T349H compared with 2.3 ± 0.1 for the WT (at $V_{\text{m}}$ = −150 mV), n = 5. The leak $I_{\text{GABA}}/I_{\text{leak}}$ ratio of the single mutants approached that of the WT, although the ratio for M345H was significantly higher (3.0 ± 0.2 for M345H and...
GABA by stepping the membrane potential from a function of time. These currents were obtained in the absence of H11006.0.5 V the Boltzmann equation to derive the value for Qmax of the transporter, after normalization to a Qmax of 1. The Q/Vm curves were fitted to the Boltzmann equation to derive the value for V1/2. Data are shown as the average of 4–6 oocytes expressing each of the constructs with a GABA-induced current of (saturating GABA concentrations at –150 mV; Li+–induced leak current are in brackets): 390–580 (885–1365) nA for the WT, 395–550 nA for M345H, 45–145 nA for T349H, and 100–210 nA for M345H/T349H.

1.9 ± 0.2 for T349H, n = 5); see Fig. 5, lower panel. At a clamp potential of –90 mV, the GABA-induced current exceeded that of the Li+–induced leak current in the WT (leak:GABA ratio = 0.5 ± 0.1) and in the T349H (0.3 ± 0.1). The leak current of M345H and M345H/T349H was still larger than the GABA-induced current, with ratios of 1.6 ± 0.1 and 1.8 ± 0.2, respectively.

FIG. 4. Charge/voltage relations of GAT-1 and the mutant transporters. Upper panels, transporter-specific transient currents as a function of time. These currents were obtained in the absence of GABA by stepping the membrane potential from +40 mV to –160 mV for 500 ms from a holding potential of –40 mV. The charge movement (Q) was obtained by integration of these transporter-specific transient currents with time at each membrane potential tested (see “Experimental Procedures”) and was plotted as a function of the membrane potential, after normalization to a Qmax of 1. The Q/Vm curves were fitted to the Boltzmann equation to derive the value for V1/2. Data are shown as the average of 4–6 oocytes expressing each of the constructs with a GABA-induced current of (saturating GABA concentrations at –60 mV) 235–540 nA for the WT, 395–550 nA for M345H, 45–145 nA for T349H, and 100–210 nA for M345H/T349H.

DISCUSSION

In the present paper we have identified two residues that may play an important role in the maintenance of the proper conformational equilibrium of the GABA transporter. Mutation of two residues (M345H or T349H) at the top of TM7 generated transporters with oppositely directed pre-steady-state and steady-state kinetics, which in the double mutant (M345H/T349H) were sometimes additive and sometimes approached the kinetics of the WT transporter. In previous studies of the GAT-1 and the homologous DAT we have obtained evidence that this region of the transporter may undergo conformational changes during the substrate translocation process (14, 29–31). As discussed below, the present data support that this part of the transporter, in addition to undergoing conformational changes during transport, also play a critical role in regulating the distribution between distinct structural states in the reaction cycle.

Introduction of histidines at residues Met345 and Thr349 caused changes in the apparent ligand affinity constants. M345H caused a reduction in apparent GABA affinity and an increase in apparent Na+ affinity and T349H caused an increase in apparent GABA affinity and a reduction in apparent Na+ affinity. It is important to note that the shift in voltage dependence of the mutant transporters may contribute to the change in apparent affinities, since the GABA-induced currents do not always fully saturate at the most negative clamp potentials applied in this study. Ideally, the K0.5 for Na+ should be obtained at completely saturating voltages and the K0.5 for GABA should be obtained at completely saturating voltages and Na+ concentrations. We have applied the highest concentrations of Na+ (100 mM) and the most hyperpolarized potentials (–160 mV) at which the oocytes, at least in our hands, can survive repetitive recordings. These conditions (–160 mV/100 mM Na+) are saturating for M345H and M345H/T349H; the apparent Na+ affinities are virtually identical (4 versus 3 mM, Table I), the IV curves approach saturation in both constructs (Fig. 2), but the K0.5 for GABA is 320 versus 50 μM (Fig. 1).

The apparent GABA affinity was not as drastically changed in constructs with more conservative amino acid substitutions, such as M345A, M345C, and T349C (data not shown), suggest-
Conformational Equilibrium of GAT-1

Fig. 6. Schematic representation of the transport cycle of GAT-1. The upper part of the figure represents the outward-facing conformations, and the lower part represents the inward-facing conformations. Arrows indicate the favored conformations for M345H and T349H.

M345H

\[ C_1 \rightarrow C_2Na_2 \rightarrow C_3Na_2GABA \]

\[ C_6 \leftarrow C_5Na_2 \rightarrow C_4Na_2GABA \]

T349H

The increase in GABA transport at low pH caused by the single mutations was additive in the double mutant. One explanation could be that protonation of the histidines would increase the rate of the forward conformational change from the outward-facing conformations to the inward-facing conformations for M345H, and for T349H, from the inward-facing conformations to the outward-facing conformations. Thereby, the rate of both steps could be increased in the double mutant, causing M345H/T349H to considerably increase its turnover at low pH. Several studies point to this region as being involved in conformational changes associated with substrate translocation (10, 14, 19, 26, 27). Amino acid residues may interact with and/or repulse each other depending on the charge of the residues.
idues, and these interactions may affect the interhelical movements underlying the conformational changes taking place in the reaction cycle. The external loops in the C-terminal part of the transporters have been proposed earlier to be pH-sensing; acidic pH increased serotonin-induced current in rat serotonin transporter, whereas human serotonin transporter was unaffected. Residues 490 and 493 in the external loop 5 in rat serotonin transporter were responsible for this effect of pH (35). Mutating K448E in the homologous loop in rat GAT-1 conferred pH sensitivity to the otherwise insensitive GABA-induced current (10). Altogether these findings suggest that conformational changes underlying substrate translocation in transport proteins are sensitive to the charge of the residues residing in the loop regions of the transporters.

The mutant transporters and the WT supported Li\(^{+}\)-induced, substrate-independent leak currents, although the ratio between the Li\(^{+}\) leak current and the GABA-induced current was altered in the mutants. The leak current mode of the double mutant was increased severalfold over that of the GABA-induced current, which is probably partly because of its low GABA translocation turnover rate and partly because of its increased leak current. M345H also had an increased ratio compared with the WT, although not as pronounced as M345H/T349H. According to our recent study, the leak current takes place after Li\(^{+}\) binding to the transporter at the first cation binding site in the transporter cycle (15). As the C\(_6\) \(\rightarrow\) C\(_1\) \(\rightarrow\) C\(_{\text{Na}}\) steps are voltage-dependent in M345H and M345H/T349H and the M345H mutation causes a poise of the transporters toward the outward-facing conformations, these two transporters would more readily enter into the cation-bound leak current conformation. Therefore these transporters (compared with the WT) sustain an increased leak current, which initiates at more depolarized potentials (Fig. 5). T349H, on the other hand, favors the inward-facing conformation, and it takes increased hyperpolarization for the transporter to enter into the cation-bound state. Therefore, the leak current represents a smaller component than the GABA-induced current in this mutant transporter.

In conclusion, our study provides further support that the TM 7 region plays a critical role for the function of Na\(^{+}\)/Cl\(^{−}\)coupled neurotransmitter transporters. In this study, we identify two positions in this part of the transporter that upon mutation cause opposite shifts in the steady-state conformational equilibrium. This occurs most likely via disruption of particular amino acid interactions in this micro-domain after introduction of bulky histidine side chains. Obviously, we cannot in the absence of high resolution structural information deduce the specific structural correlates reflecting the observed phenotypes. However, the distinct effects on the steady-state equilibrium by mutation of two residues situated four amino acids apart in a conformationally sensitive region of the transporter support a hypothesis where a domain around the external end of TM 7 serves a critical role in controlling conformational changes and the conformational equilibrium of the transporter. It is obvious, however, that other domains within the transporter molecule also may contribute to the control of these processes. In the homologous dopamine transporter, we have recently shown evidence that a tyrosine (Tyr\(^{355}\)) situated in the intracellular loop connecting TM 6 and 7 might be indispensable for maintaining the transporter in a conformation in which extracellular substrates can bind and initiate transport (36).

Acknowledgments—The GAT-1 clone was a kind gift from Baruch Kanner. We are grateful for the technical assistance of B. Lynderup and T. Soland.

REFERENCES
1. Guastella, J., Nelson, N., Nelson, H., Czyzyk, L., Keynan, S., Miedel, M. C., Davidson, N., Lester, H. A., and Kanner, B. I. (1990) Science 240, 1303–1306
2. Mahbush, N. J., and Kanner, B. I. (1992) J. Biol. Chem. 267, 2563–2568
3. Keynan, S., and Kanner, B. I. (1988) Biochemistry 27, 12–17
4. Radian, R., and Kanner, B. I. (1983) Biochemistry 22, 1236–1241
5. Binda, F., Bossi, E., Giovannardi, S., Forlini, G., and Peres, A. (2002) FEBS Lett. 512, 303–307
6. Bisti, K. M., Rudnick, G., and Stephan, M. M. (1998) J. Biol. Chem. 273, 16096–16102
7. Bossi, E., Giovannardi, S., Binda, F., Forlini, G., and Peres, A. (2002) J. Biol. Chem. 277, 14047–14052
8. Cammock, J. N., Rakhlin, S. V., and Schwartz, E. A. (1994) Neuron 13, 949–960
9. Cammock, J. N., and Schwartz, E. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 723–727
10. Forlini, G., Bossi, E., Ghirardelli, R., Giovannardi, S., Binda, F., Bonadiman, L., Ielmini, L., and Peres, A. (2001) J. Physiol. (Lond.) 536, 479–494
11. Hirayama, B. A., Diez-Sampedro, A., and Wright, E. M. (2003) Br. J. Pharmacol. 134, 484–495
12. Kanner, B. I., Bendahan, A., Pantanowitz, S., and Su, H. (1994) J. Biol. Chem. 269, 22007–22013
13. Lu, C. C., and Hilgemann, D. W. (1999) Gen. Physiol. 114, 429–444
14. MacAulay, N., Bendahan, A., Loland, C. J., Kanner, B. I., Zeuthen, T., and Gether, U. (2001) J. Biol. Chem. 276, 40476–40485
15. MacAulay, N., Zeuthen, T., and Gether, U. (2002) J. Physiol. (Lond.) 544, 447–458
16. Mager, S., Naeve, J., Quick, M., Labraca, C., Davidson, N., and Lester, H. A. (1993) Neuron 10, 177–188
17. Mager, S., Kleinberger-Doron, N., Keshet, G. I., Davidson, N., Kanner, B. I., and Lester, H. A. (1996) J. Neurosci. 16, 4584–4598
18. Pantanowitz, S., Bendahan, A., and Kanner, B. I. (1993) J. Biol. Chem. 268, 8540–8546
19. Pantanowitz, S., Bendahan, A., and Kanner, B. I. (1993) J. Biol. Chem. 268, 3222–3225
20. Penado, K. M., Rudnick, G., and Stephan, M. M. (1998) J. Biol. Chem. 273, 28098–28106
21. Kanner, B. I., Bendahan, A., Pantanowitz, S., and Su, H. (1994) FEBS Lett. 369, 191–194
22. Tamura, S., Nelson, H., Tamura, A., and Nelson, N. (1995) J. Biol. Chem. 270, 28712–28718
23. Yu, N., Cao, Y., Mager, S., and Lester, H. A. (1998) FEBS Lett. 428, 174–178
24. Loland, C. J., Norregaard, L., and Gether, U. (1999) J. Biol. Chem. 274, 36928–36934
25. Norregaard, L., Frederiksen, D., Nielsen, E. O., and Gether, U. (1998) EMBO J. 17, 4266–4273
26. Norregaard, L., Vissers, I., Loland, C. J., Ballesteros, J., Weinstein, H., and Gether, U. (2000) Biochemistry 39, 15836–15846
27. Lee, D. D. P., Hazama, A., Supelison, S., Turk, E., and Wright, E. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7792–7795
28. Parent, L., Supplisson, S., Luo, D. D. F., and Wright, E. M. (1992) J. Membr. Biol. 125, 63–79
29. Wadiche, J. I., Arizza, J. L., Amara, S. G., and Kanner, B. I. (1995) Neuron 14, 1019–1027
30. Cao, Y., Li, M., Mager, S., and Lester, H. A. (1998) J. Neurosci. 18, 7739–7749
31. Loland, C. J., Norregaard, L., Litman, T., and Gether, U. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 1683–1688
Residues in the Extracellular Loop 4 Are Critical for Maintaining the Conformational Equilibrium of the \( \gamma \)-Aminobutyric Acid Transporter-1
Nanna MacAulay, Anne-Kristine Meinild, Thomas Zeuthen and Ulrik Gether

J. Biol. Chem. 2003, 278:28771-28777.
doi: 10.1074/jbc.M213023200 originally published online May 22, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M213023200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 36 references, 20 of which can be accessed free at http://www.jbc.org/content/278/31/28771.full.html#ref-list-1