The Interaction of the γ-Aminobutyric Acid Transporter GAT-1 with the Neurotransmitter Is Selectively Impaired by Sulphydryl Modification of a Conformationally Sensitive Cysteine Residue Engineered into Extracellular Loop IV

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The (Na⁺ + Cl⁻)-coupled γ-aminobutyric acid (GABA) transporter GAT-1 keeps synaptic levels of this neurotransmitter low and thereby enables efficient GABAergic transmission. Extracellular loops (II, IV, and V) have been shown to contain determinants for GABA selectivity and affinity. Here we analyze the role of extracellular loop IV in transport by cysteine scanning mutagenesis. Fourteen residues of this loop have been replaced by cysteine. GABA transport by eight of the fourteen mutants is markedly more sensitive to inhibition by membrane-impermeant methane thiosulfate reagents than wild-type. Mutant A364C has high activity and is potently inhibited by the sulphydryl reagent. GABA transport by the A364C/C74A double mutant, where the only externally accessible cysteine residue of the wild-type has been replaced by alanine, is also highly sensitive to the sulphydryl reagents. Maximal sensitivity is observed in the presence of the cosubstrates sodium and chloride. A marked protection is afforded by GABA, provided sodium is present. This protection is also observed at 4 °C. The non-transportable analogue SKF100330A also protects the double mutant against sulphydryl modification in the presence of sodium but has the opposite effect in its absence. Electrophysiological analysis shows that upon sulphydryl modification of this mutant, GABA can no longer induce transport currents. The voltage dependence of the transient currents indicates an increased apparent affinity for sodium. Moreover, GABA is unable to suppress the transient currents. Our results indicate that part of extracellular loop IV is conformationally sensitive, and its modification selectively abolishes the interaction of the transporter with GABA.

Most neurotransmitters are removed from the synaptic cleft by sodium- and chloride-dependent neurotransmitter transporters, and this process is essential to maintain efficient synaptic transmission (for a review see Refs. 1 and 2). These transporters form a large family, including transporters for biogenic amines and amino acids and four different GABA transporters. One of the best examples of the importance of these neurotransmitter transporters comes from studies of dopamine transporter knock-out mice; the decay of extracellular dopamine in brain slices of such mice is about 100 times longer than normal (3).

GAT-1, the first member of the family to be identified, was purified to homogeneity in a form active upon reconstitution (4) and subsequently cloned (5). The transporter catalyzes electrogenic sodium:chloride:GABA cotransport with a stoichiometry of 2:1:1 (6–9). It should be noted, however, that there is still some dispute on this issue. In a recent report (10), it has been proposed that during sodium-coupled GABA transport obligatory chloride/sodium exchange takes place. GAT-1, as well as other members of the family, is predicted to have twelve transmembrane domains linked by hydrophilic loops with the amino and carboxyl termini residing inside the cell (5). Studies on the serotonin transporter SERT indicate that the theoretical topological model is correct (11). Mutagenesis studies, in particular on GAT-1 and SERT, but also on other members of the family, suggest the importance of transmembrane domains I and III in the interaction of the transporters with the neurotransmitters, as well as with sodium and chloride (12–16).

GAT-1 has 15 endogenous cysteine residues of which only three are located on extracellular loops. Studies on the related dopamine and serotonin transporters indicate that the equivalent residues of cysteine 164 and 173 located in the second extracellular loop form a disulfide bond (17, 18) and would not react with sulphydryl reagents. Thus cysteine 74, located in the first extracellular loop, is the only cysteine residue that reacts with impermeant MTS reagents. It appears that modification of this residue causes only modest inhibition of GABA transport in GAT-1 even when relatively high concentrations of MTSET are used (19). The reagent (2-aminoethyl)methane thiosulfonate (MTSEA) has a definite membrane permeability and can react with cysteine 399, which is located on the intracellular loop connecting transmembrane domains VIII and IX (20). The accessibility of cysteine 399 is dependent on the conformation of the transporter (20).

Replacement of residues in extracellular loop IV has been shown to influence the apparent affinity of GAT-1 for GABA (21). Moreover, extracellular loop IV does not tolerate deletions of single amino acid residues (22). In this report we have addressed the question of whether this loop may undergo conformational changes during the transport cycle. Cysteine residues have been engineered at various positions of this loop, and...
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EXPERIMENTAL PROCEDURES

Generation and Subcloning of Mutants—Mutations were made by site-directed mutagenesis of the wild-type GAT-1 in the vector pBlue-Script SK(−) (Stratagene) according to the Kunkel method as described (23, 24). Briefly the parent DNA was used to transform Escherichia coli CJ236 (dut−, ung−). From one of the transformants, single-stranded unincorporating DNA was isolated upon growth in uridine-containing medium according to the standard protocol from Stratagene using helper phage R408. This yields the sense strand, and consequently mutagenic primers were designed to be antisense. Mutants A364C/S and L366C/S were subcloned into a construct containing C7A-GAT-1 in the pOG1 vector using the unique restriction enzymes NheI and PinI. The pOG1 vector is an oocyte expression vector that contains a 5′-untranslated Xenopus β-globin sequence, the T7 RNA promoter, and a 3′-poly(A) signal. The above mutants and the other mutants described in this study were also subcloned into a construct containing wild-type GAT-1 in pBlueScript SK(−) using the above two restriction sites. The coding and non-coding strands were sequenced between the above two restriction sites.

Cell Growth and Expression—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 200 units/ml penicillin, 200 μg/ml streptomycin, and 2 mM glutamine. Infection with recombinant vaccinia/T7 virus vTF7-3 (25) and subsequent transfection with plasmid DNA, as well as GABA transport, was done as published previously (26). In the experiments described in Figs. 1–3 and 7, the expression vector was pBlueScript SK(−), and in the other figures the mutations subcloned in the pOG1 vector were used.

Cell Surface Biotinylation—Labeling of wild-type and mutant transporters at the cell surface of the HeLa cells, using sulfo-SulfoNHS-SS-biotin (Pierce), was done exactly as described (27).

Inhibition Studies with Sulphydryl Reagents—Before the transport measurements, the cells adhering to 24-well plates were washed with the transport medium containing 150 mM choline chloride instead of NaCl. Each well was then incubated at room temperature with 200 μl of the preincubation medium (the different compositions are indicated in the figure legends). After 5 min, the medium was aspirated, and the cells were washed twice with 1 ml of the transport solution. Subsequently they were assayed for [3H]GABA transport at 37 °C, unless indicated otherwise. The hydrophilic methanethiosulfonate reagents used during the preincubation were purchased from Toronto Research Chemicals, Inc. or from Anatrace, Inc. The concentration of these reagents given in the different experiments was stated optimized according to the experimental conditions or the mutants used. For instance, in Fig. 7, different concentrations were used for the different mutants, because some are more sensitive than others.

cRNA Transcription, Injection, and Oocyte Preparation—Capped run-off cRNA transcripts were made from transporter constructs in pOG1, linearized with SacII, using mMessage mMACHINE (Ambion). Oocytes were removed from anesthetized Xenopus laevis frogs and treated with collagenase (type 1A; Sigma number C-9891) until capillaries were absent and injected with 50 nl of undiluted cRNA the same day. Oocytes were washed twice with 1 ml of the transport solution. Subsequently they were assayed for [3H]GABA transport at 37 °C, unless indicated otherwise. The hydrophilic methanethiosulfonate reagents used during the preincubation were purchased from Toronto Research Chemicals, Inc. or from Anatrace, Inc. The concentration of these reagents given in the different experiments was stated optimized according to the experimental conditions or the mutants used. For instance, in Fig. 7, different concentrations were used for the different mutants, because some are more sensitive than others.

Oocyte Electrophysiology—Oocytes were placed in the recording chamber, penetrated with two micropipettes (backfilled with 2 M KCl, resistance varied between 0.5 and 2 megohms), and voltage clamped using a GeneClamp 500 amplifier (Axon Instruments) and digitized using Digidata 1200A (Axon Instruments), both controlled with the pClamp6 suite (Axon Instruments). Currents were acquired with clampex03.03 at 10 kHz every 0.5 ms and low pass-filtered online. Oocytes were stepped from −135 mV to +40 mV in 25-mV increments, usually without holding potential, unless stated otherwise in the figure legends. Each potential was held clamped for 500 ms. The membrane potential was measured relative to an extracellular Ag/AgCl electrode in the recording chamber. Recording solution contained 96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, and 5 mM Hepes, pH 7.4.

RESULTS

Cysteine Scanning Mutagenesis of Extracellular Loop IV—Extracellular loop IV, which connects transmembrane domains VII and VIII, encompasses residues 341–374 (Fig. 1A). To screen for those positions where introduction of a cysteine residue is tolerated in terms of activity, yet renders the transporter sensitive to sulfhydryl reagents, we have initially mutated amino acids throughout the loop with intervals ranging from two to five residues. Cysteine replacement at positions 364 and 366 results in high levels of sodium-dependent [3H]GABA uptake.
HeLa cells were washed and incubated in NaCl with or without 1 mM MTSET for 5 min as described under “Experimental Procedures” prior to ([3H]GABA uptake. Data are given as percentages of inhibition of values obtained in the absence of MTSET and are averages (± S.E.) of three experiments each performed in triplicate.

The impact of cysteine replacement is mostly because of the previous group but significantly more sensitive to MTSET (Fig. 2). A346C, T349C, A354C, A364C, and E370C, the reduced transporters retaining significant activity, which is sensitive to sulfhydryl modification, the additional mutations at positions 362, 363, and 365 were made in a second round of mutagenesis. Mutants G362C and F365C have low activity (Fig. 1B), which, however, is highly sensitive to MTSET (Fig. 2). A346C, T349C, and E370C, which have high transport activity, are less sensitive than the previous group but significantly more sensitive to MTSET than the wild-type (one-way ANOVA, p < 0.05). Increased sensitivity to MTSET is also observed with S359C (Fig. 2), which has lower activity than the wild-type (Fig. 1B). Interestingly, mutant A358C is stimulated, rather than inhibited, by treatment with MTSET (Fig. 2). Thus, of the twelve mutants that show measurable activity, nine are functionally impacted by the sulfhydryl reagent.

The total sample of cells transfected with the vector alone are nonspecific bands, documented previously (27). The ratio of the intensities of the specific bands from biotinylated and total samples (after subtraction of the corresponding region from the lanes containing the samples of cells expressing the vector alone) of the mutants G343C, G362C, L366C, and F365C is similar to that of the wild-type and A346C. This latter mutant has almost the same activity as the wild-type (Fig. 1B). The values are 1.17 ± 0.41, 1.18 ± 0.14, 1.25 ± 0.2, and 1.13 ± 0.19 for the mutants, respectively, and 1.42 ± 0.17 and 1.35 ± 0.17 for wild-type and A346C (n = 4; one-way ANOVA, p < 0.05). The ratios are larger than one, because only a small fraction of the total samples was run on the gel (see legend to Fig. 3). The average surface expression of the mutants as percent of that of the wild-type are 101.0 ± 42.8, 47.8 ± 30.3, 82.6 ± 31.6, 82.6 ±
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31.5, and 73.9 ± 37.3 for A346C, G343C, G362C, L363C, and F365C, respectively (n = 4). According to statistical analysis using a one-way ANOVA with a post hoc Dunnett multiple comparison test of significance, the surface expression of the mutants is not significantly different from that of the wild-type (p < 0.05). Thus, even though there is a large variation experiment to experiment in surface changes, our overall impression is that not only with the inactive mutants G343C and L363C, but also with mutants G362C and F365C, which have less than 10% of the transport activity of the wild-type (Fig. 1B), these differences in transport cannot be attributed to surface changes.

Because A364C has the highest transport activity (Fig. 1B) of the mutants potently inhibited by MTSET (Fig. 2), we have focused on the characterization of this inhibition in the presence and absence of the substrates of the transporter. Cysteine 74 has been found to be the major determinant for MTSET sensitivity in GAT-1 (19). To rule out the possibility that the introduction of a cysteine in position 364 causes an increased accessibility of the endogenous cysteine at position 74, mutation A364C has been subcloned into the background where this cysteine is replaced by an alanine, and we have done the same with L366C. The sensitivity of the double mutants A364C/C74A (Fig. 4A) and of L366C/C74A (see Fig. 7) to MTSET indicates that it is probably the introduced cysteine that is the target for modification by the sulfhydryl reagent. Moreover, mutant A364S/C74A is not inhibited by MTSET (Fig. 4A) rendering unlikely the possibility that the introduction of a cysteine residue at position 364 exposes a previously inaccessible endogenous cysteine residue.

The modification of A364C/C74A by MTSET appears to cause a reduction of V_{max} rather than a decrease in the apparent substrate affinity of the transporters. Cells expressing the double mutant have been treated by 0.15 mM MTSET, so that an inhibition of around 85% is achieved. Analysis of the initial rate of transport as a function of the GABA concentration shows that the K_{m} for GABA is very little changed by the treatment: 1.7 ± 1.0 µM (n = 3) without and 1.6 ± 0.2 µM (n = 3) after treatment with the sulfhydryl reagent. On the other hand the value of V_{max} is reduced from 8.5 ± 0.8 to 2.5 ± 0.9 pmol/min/well (n = 3). In the batch of HeLa cells used in these experiments the V_{max} of the untreated A364C/C74A was 45.2 ± 13.7% that of the wild-type.

Effect of Substrates on the Accessibility of the Cysteine Introduced at Position 364—The inhibition of the transport activity of A364C/C74A is dependent on the composition of the external medium. Inhibition by 0.4 mM MTSET is significantly larger (p < 0.05, one-way ANOVA) in the presence of the cosubstrate sodium than in its absence (choline or lithium replacement) (Fig. 4A). The effect of the other cosubstrate, chloride (glucuronate replacement), is less pronounced than that of sodium. In the experiments depicted in Fig. 4A, we show the effects of the composition of the external medium at a single concentration of MTSET. However, the effects are observed regardless of the concentration used (Fig. 4B). The substrate itself, GABA, affords a marked protection against the inhibition of 3H[GABA transport of A364C/C74A by MTSET (Fig. 5A). Such a protection is not observed by l-aspartate, which is not a substrate (data not shown). The protection by GABA is only observed in the presence of sodium (Fig. 5A). In the presence of lithium or choline the protection by GABA does not take place (Fig. 5, A and B). Protection by GABA is maximal when chloride is present, as well (Fig. 5A), resulting sometimes in higher levels of transport than those observed after MTSET treatment in choline chloride or lithium chloride (Fig. 5B). The protection by GABA is also observed at 4 °C (Fig. 6). At this temperature the conformational changes of the transporter are slowed down as evidenced by the fact that the transport activity at 2–4 °C is only 5 ± 1.5% (n = 4) that at 37 °C.

To determine whether the inhibition of A364C/C74A is because of the generation of a positively charged side group at position 364, we have studied the effect of MTSES. This reagent has almost the same dimensions as MTSET but bears a negative charge. The A364C/C74A mutant is also inhibited by MTSES, and the characteristics of this inhibition (Fig. 5C) are similar to that by MTSET (Fig. 5A).

Several other cysteine mutants in extracellular loop IV have similar characteristics of inhibition by MTSET as A364C/C74A. Also in mutant L366C/C74A the inhibition in sodium medium is more than that in choline, and GABA affords significant protection in the presence of sodium (Fig. 7). The same trend, but less pronounced, is observed with mutants S359C and A346C. On the other hand there is very little effect of the medium composition on the inhibition of E370C by MTSET (Fig. 7).

To address the question of whether the protection by GABA against inhibition by MTSET reagents is because of a physical blockade by the substrate to the access to the cysteine at position 364, we have examined the effect of SKF100330A on this inhibition (Fig. 8). SKF100330A is a non-transportable hydrophobic GABA analogue that acts as a high affinity competitive inhibitor of transport (28). Because of its hydrophobic-
ity it is relatively difficult to wash out the SKF100330A from cells expressing the wild-type (20), and it is even more difficult to do so in the A364C/C74A mutant. Nevertheless in many experiments a recovery of more than 40% of the transport activity has been observed upon washing of the cells expressing the double mutant. This is based on the comparison between transport values obtained after incubation of the cells with and without SKF100330A followed by washing in sodium or choline medium. In these experiments the effect of SKF100330A on inhibition of A364C/C74A by MTSET is protective in the presence of sodium chloride (Fig. 8). Strikingly, in the absence of sodium (choline substitution), SKF100330A markedly potentiates the inhibition of the double mutant by MTSET (Fig. 8), and the same is observed in the presence of lithium (data not shown).

Electrophysiological Analysis of the Impact of Sulfhydryl Modification of A346/C74A—The inactivation of [3H]GABA

Fig. 6. Concentration dependence of the inhibition of A364/C74A by MTSET at 4 °C. HeLa cells expressing A364C/C74A were washed with NaCl solution at 2–4 °C and incubated at this temperature for 5 min in the same solution at 2–4 °C with (filled circles) or without (open circles) 1 mM GABA containing the indicated concentrations of MTSET. After one wash with NaCl solution at 4 °C and another at 37 °C, transport was measured at the latter temperature.

transport of A364C/C74A expressed in HeLa cells by MTSET and MTSES is also observed on the sodium- and chloride-dep

Fig. 7. Dependence of inhibition by MTSET on sodium, chloride, and GABA in A346C, S359C, L366C/C74A, and E370C. Conditions are the same as described in the legend for Fig. 5. Graph shows percentage of [3H]GABA uptake activity remaining after treatment with MTSET relative to the untreated samples in choline chloride (empty bars), sodium chloride (filled bars), or sodium chloride with 1 mM GABA (striped bars). MTSET concentrations used were 1 mM with A346C and E370C and 0.05 mM with S359C and L366C/C74A. Data shown are averages of three experiments done in triplicate (± S.E.).
sodium. Upon jumping back to the holding potential, transients of opposite direction and magnitude are observed. Modification by MTSES results in transients that are now predominantly outward, as if at −25 mV all the transporters have already bound sodium (Fig. 10B). Consistent with the increased affinity for sodium is the fact that progressive reduction of the external sodium concentration causes the transients of the MTSES-modified A364C/C74A transporters to become symmetric and even inward at 6 mM sodium (Fig. 11). At 2 mM external sodium the currents seemingly disappear; apparently at this concentration very little sodium binds even at the most negative potential (−135 mV) in the voltage-jump protocol (Fig. 11).

Similar transient currents are obtained when in sodium medium the currents in the presence of 30 μM of the blocker SKF100330A are subtracted from those in its absence (Fig. 10C). On the other hand GABA cannot interact with the modified transporter, and unlike the non-transportable GABA analogue SKF100330A it cannot isolate the sodium-dependent transient currents (Fig. 10D). Moreover, 1 mM GABA cannot prevent the ability of SKF100330A, at concentrations as low as 5 μM, to suppress the sodium-dependent transient currents (n = 4, data not shown). In the control experiments it can be seen that no effects by MTSES are observed in the A364C/C74A mutant (Fig. 10, E–H). It should be noted that the currents shown in Fig. 10, D and H represent the currents in sodium and GABA subtracted from those in the presence of sodium alone and have the opposite sign of those shown in Fig. 9, B and D, where the currents in sodium are subtracted from those in sodium and GABA. Similar effects in the A364C/C74A mutants are observed by MTSET, except that the increase in sodium affinity by the reagent is less dramatic; the transient currents become symmetrical after treatment with MTSET, and again MTSET has no effect on the A364S/C74A mutant (data not shown).

**DISCUSSION**

The data presented indicate that the accessibility of residue 364 from EL-4 changes during the transport cycle of GAT-1, and similar changes are observed with residues 346, 359, and 366. Thus it appears that at least part of the extracellular loop is conformationally sensitive. The scheme presented in Fig. 12 is helpful to explain the observations made in this study. For reasons of simplicity the role of chloride is not indicated. In the absence of sodium (upper left corner), membrane-impermeant MTS reagents added from the extracellular medium have a limited accessibility to the cysteine residue introduced at position 364 (marked as −SH) (see Figs. 2, 4, and 5). Upon binding of sodium a conformational change occurs (step 1) so that the affinity for GABA is increased (13), and cysteine 364 becomes more accessible (see Figs. 2, 4, and 5). In the presence of sodium, GABA can bind (step 2), bringing the transporter into a state where the access to position 364 is again more restricted, presumably because of a physical blockade by GABA. This explains the protection by GABA (Fig. 5) and the non-transportable competitive blocker SKF100330A (Fig. 8), which is only observed in the presence of sodium. In the presence of sodium and SKF100330A the transporter is stabilized in this outward-facing state (upper right corner), where we depict the sodium site as occluded, as it has been shown that this blocker promotes sodium occlusion (13). In the presence of sodium and GABA, the transporter does not stop at the sodium-occluded state, but the transport step occurs (step 3). Release of sodium and GABA to the inside (step 4) results in the unloaded transporter with its binding sites facing inward (lower left corner). Upon return of the unloaded transporter to the outside, a new transport cycle can start (Fig. 12). To explain the opposite effect on accessibility of A364C/C74A to MTSET by SKF100330A in the absence of sodium (Fig. 8), we propose that the empty transporter can bind SKF100330A also in the absence of sodium. One possibility is that the transporter binds the blocker in a different way in the absence of sodium, perhaps involving a hydrophobic inhibitor site (29). This could result in a different conformational change, increasing the accessibility of the engineered cysteine. GABA itself does not bind in the absence of sodium, and therefore it cannot confer protection under these conditions (Fig. 5).

Position 364 does not appear to be near the sodium binding sites, because sodium potentiates the inhibition by MTS reagents rather than protects against them (see Figs. 2, 4, and 5). Moreover sodium can still bind to the mutant transporter after the modification (see Figs. 10 and 11). The increased apparent affinity of A364C/C74A for sodium upon modification by the MTS reagents (see Figs. 10 and 11) can be explained as follows. In the absence of GABA the transporters are shuttling back and forth between the empty inward-facing form (lower left corner of Fig. 12) and the outward-facing sodium-bound form of the transporter via steps 5 and 1 (Fig. 12). Modification of cysteine 364 either by positively charged MTSET or negatively charged MTSES may stabilize the sodium-bound form, resulting in an increased apparent sodium affinity. The scheme presented here is simplified in the sense that the role of chloride is not indicated, and it also does not address the question of whether the two sodium binding sites are equivalent. Thus we cannot rule out more complex scenarios such as one that the increased sodium affinity after treatment of A364C/C74A is a result of the retention of one binding site of high affinity and the obliteration of the site of lower affinity.

In the scheme depicted in Fig. 12, we have placed the GABA binding site not far away from the cysteine introduced at position 364 for the following reasons. Significant protection by GABA is observed in nominally chloride-free medium, even though GABA transport is almost fully inhibited under these conditions (26). Moreover, protection by GABA is still observed when inactivation by MTSET is measured at 2–4 °C (Fig. 6). At this temperature transport is almost totally inhibited, presumably reflecting the temperature dependence of the translocation step (step 3). The fact that GABA nevertheless protects at this temperature supports the idea that this protection occurs at a step closely linked to GABA binding. It is of interest to note that the extent of inhibition of transport of A364C/C74A by 0.4
mM MTSET at 4 °C is reduced (see Figs. 5 and 6). A possible explanation for this could be that at 4 °C step 5 may be slowed very much, and those transporters facing inward almost cannot react with the sulfhydryl reagent. The better protection by GABA at 2–4 °C (see Figs. 5 and 6) may indicate that the temperature dependence of the reaction rate of MTSET with the transporter is steeper than that of the rate constant of GABA binding. The protection against sulfhydryl reagents by the substrate at 4 °C has also been observed with residue A395C of the glutamate transporter EAAT-1 (30). On the other hand a long range conformational change has been inferred to underline the temperature-sensitive protection of the accessibility of cysteine 357 of the serotonin transporter SERT by serotonin and cocaine (31). Consistent with the idea that position 364 is close to the GABA binding site is the fact that upon sulphydryl modification, not only GABA uptake is blocked (see Figs. 2 and 4–8), but GABA neither gives rise to steady state currents (Fig. 9) nor suppresses the sodium-dependent transient currents (Fig. 10). On the other hand, SKF100330A can still bind after sulphydryl modification (Fig. 10). This may be because of the hydrophobic groups of the blocker that could enable it to bind to the transporter, despite the modification of

**Fig. 9. Effect of MTSES on GABA-induced currents by oocytes expressing A364C/C74A.** Currents recorded in the absence of GABA during 500-ms voltage pulses from -135 to +40 mV were subtracted from currents recorded during superfusion of 1 mM GABA in the sodium-containing recording solution. The prepulse potential was -25 mV. The stippled line indicates 0 current. Representative oocytes expressing A364C/C74A (A and B) and A364S/C74A (C and D) are shown, before (A and C) and after (B and D) a 2-min incubation with 0.2 mM MTSES. Data shown are from representative oocytes (n = 7) from four batches of oocytes for A364C/C74A and of n = 4 from three batches of oocytes for A364S/C74A.

**Fig. 10. Effect of MTSES treatment on sodium-dependent transient currents in A364CC74A.** Currents of typical oocytes expressing A364C/C74A (A–D) or A364S/C74A (E–H) were recorded using the same voltage jump protocol as for Fig. 9. Sodium-dependent currents (obtained by subtraction of currents obtained in the presence of choline from those in the presence of sodium) were recorded before (A and E) and after treatment with MTSES (0.2 mM, 2 min) (B and F). Sodium-dependent currents were also recorded in the presence of SKF100330A (30 μM) after treatment with MTSES (0.2 mM, 2 min) (C and G). The currents shown in these panels are obtained by subtraction of currents obtained in the presence of SKF100330A and sodium from those in the presence of sodium without the blocker. Currents in sodium and GABA subtracted from those in sodium alone are shown for the MTSES-treated A364C/C74A (D) and A364S/C74A (H)-expressing oocytes.
cysteine 364. In fact, the binding of the blocker to the mutant appears to be even stronger than to the wild-type, as seen by difficulties to remove it in washout experiments. Thus, the effect of mutations in extracellular loop IV on the apparent GABA affinity (21) is likely because of a direct effect on the GABA binding pocket.

Recent experiments indicate that residues 347 and 374 of GAT-1, located at the extracellular ends of transmembrane domains VII and VIII, respectively, are close in space (33). Creation of zinc or cadmium binding sites at these positions renders GABA transport sensitive to these divalent cations. Another way to prevent the relative movement of these positions to each other, namely the formation of a disulfide bond between them, also results in inhibition of transport (33). Similar observations have been made previously on the dopamine transporter DAT (34, 35). Therefore, it is possible that the region encompassing transmembrane domains VII and VIII fulfills an important role in transport by all members of the family of sodium- and chloride-dependent neurotransmitter transporters. The findings presented here indicate that this may be because of conformational changes in at least part of extracellular loop IV, connecting these transmembrane domains. In addition, it is possible that movement of these transmembrane domains takes place, as well, during transport. In fact, it has been proposed that in the related serotonin transporter SERT, transmembrane domain VII is involved in propagating the conformational changes caused by ion binding (36). During the revision of this paper additional evidence for the importance of extracellular loop IV emerged; introduction of a histidine at positions 345 and 349 of GAT-1 shifted the sodium dependence in opposite directions (37).

Overall, our results support the idea that sodium binding to GAT-1 causes a change in the accessibility of extracellular loop IV, exposing positions 364 and several other residues. Subsequent GABA binding causes the residues at these positions again to become less exposed, possibly by direct occlusion. We speculate that when the cysteine introduced at position 364 is modified by sulfhydryl reagents, GABA can no longer bind and that this is the main reason for the block of transport.

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REFERENCES
1. Kanner, B. I. (1984) J. Exp. Biol. 169, 227–249
2. Nelson, N. (1998) J. Neurochem. 71, 1785–1803
3. Giros, B., Jaber, M., Jones, S. R., Wightman, R. M., and Caron, M. G. (1996) Nature 379, 606–612
4. Radin, R., Bendahan, A., and Kanner, B. I. (1986) J. Biol. Chem. 261, 15437–15441
5. Guastella, J., Nelson, N., Nelson, H., Czyzyk, L., Keynan, S., Miedel, M. C., Davidson, N., Lester, H. A., and Kanner, B. I. (1990) Science 249, 1303–1306
6. Keynan, S., and Kanner, B. I. (1988) Biochemistry 27, 12–17
7. Kavanaugh, M. P., Arriza, J. L., North, R. A., and Amara, S. G. (1992) J. Biol. Chem. 267, 22007–22009
8. Mager, S., Naeve, J., Quirk, M., Labarca, C., Davidson, N., and Lester, H. A. (1992) Neuron 10, 177–188
9. Lu, C. C., and Hilgemann, D. W. (1999) J. Gen. Physiol. 114, 429–444
10. Loo, D. D. P., Eskandari, S., Boorer, K. J., Sarkar, H. K., and Wright, E. M. (2000) J. Biol. Chem. 275, 37414–37422
11. Chen, J. G., Liu-Chen, S., and Rudnick, G. (1998) J. Biol. Chem. 273, 12675–12681
12. Pantanowitz, S., Bendahan, A., and Kanner, B. I. (1993) J. Biol. Chem. 268,
13. Mager, S., Kleinberger-Doron, N., Keshet, G. I., Davidson, N., Kanner, B. I., and Lester, H. A. (1996) *J. Neurosci.* **16**, 5404–5414
14. Bismuth, Y., Kavanaugh, M. P., and Kanner, B. I. (1997) *J. Biol. Chem.* **272**, 16096–16102
15. Chin, J. G., Sachpatzidis, A., and Rudnick, G. (1997) *J. Biol. Chem.* **272**, 28321–28327
16. Barker, E. L., Moore, K. R., Rakshan, F., and Blakely, R. D. (1999) *J. Neurosci.* **19**, 4705–4717
17. Wang, J. B., Moriaki, A., and Uhl, G. R. (1995) *J. Neurochem.* **64**, 1416–1419
18. Chen, J. G., Liu-Chen, S., and Rudnick, G. (1997) *Biochemistry* **36**, 1479–1486
19. Bennett, E. R., and Kanner, B. I. (1997) *J. Biol. Chem.* **272**, 1203–1210
20. Golovanovskyy, V., and Kanner, B. I. (1999) *J. Biol. Chem.* **274**, 23020–23026
21. Tamura, S., Nelson, H., Tamura, A., and Nelson, N. (1995) *J. Biol. Chem.* **270**, 28712–28715
22. Kanner, B. I., Bendahan, A., Pantanowitz, S., and Su, H. (1994) *FEBS Lett.* **356**, 191–194
23. Kunkel, T. A., Roberts, J. D., and Zarkour, R. A. (1987) *Methods Enzymol.* **154**, 367–383
24. Kleinberger-Doron, N., and Kanner, B. I. (1994) *J. Biol. Chem.* **269**, 3063–3067
25. Fuerst, T. R., Niles, E. G., Studier, F. W., and Moss, B. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 8122–8126
26. Keynan, S., Suh, H.-J., Kanner, B. I., and Rudnick, G. (1992) *Biochemistry* **31**, 1974–1979
27. Bennett, E. R., Su, H., and Kanner, B. I. (2000) *J. Biol. Chem.* **275**, 34106–34113
28. Ali, F. E., Blendirell, W. E., Dandridge, P. A., Frayer, J. S., Garvey, E., Girard, G. R., Kaiser, C., Ku, T. W., Lafferty, J. J., Monsammy, G. I., Oh, H. J., Rush, J. A., Settler, P. E., Stringer, D. D., Vinalovsky, J. W., Volpe, B. W., Yungier, L. M., and Zishie, C. L. (1985) *J. Med Chem.* **28**, 653–660
29. Hirayama, B. A., Diez-Sampedro, A., and Wright, E. M. (2001) *Br. J. Pharmacol.* **134**, 484–495
30. Seal, R. P., and Amara, S. G. (1998) *Neuron* **21**, 1487–1498
31. Androutsellis-Theotokis, A., Ghassemi, F., and Rudnick, G. (2001) *J. Biol. Chem.* **276**, 45933–45938
32. Deleted in proof
33. MacAulay, N., Bendahan, A., Loland, C. J., Zeuthen, T., Kanner, B. I., and Gether, U. (2001) *J. Biol. Chem.* **276**, 40476–40485
34. Norregaard, L., Fredriksen, D., Nielsen, E. O., and Gether, U. (1998) *EMBO J.* **17**, 4266–4273
35. Loland, C. J., Norregaard, L., and Gether, U. (1999) *J. Biol. Chem.* **274**, 36928–36934
36. Kamdar, G., Penade, K. M. Y., Rudnick, G., and Stephan, M. M. (2001) *J. Biol. Chem.* **276**, 40438–40445
37. MacAulay, N., Meinild, A.-K., Zeuthen, T., and Gether, U. (2003) *J. Biol. Chem.* **278**, 28771–28777