To determine whether glycine residues play a role in the conformational changes during neurotransmitter transport, we have analyzed site-directed mutants of the γ-aminobutyric acid (GABA) transporter GAT-1 in a domain containing three consecutive glycines conserved throughout the sodium- and chloride-dependent neurotransmitter transporter family. Only cysteine replacement of glycine 80 resulted in the complete loss of [3H]GABA uptake, but oocytes expressing this mutant exhibited the sodium-dependent transient currents thought to reflect a charge-moving conformational change. When sodium was removed and subsequently added back, the transients by G80C did not recover, as opposed to wild type, where recovery was almost complete. Remarkably, the transients by G80C could be restored after exposure of the oocytes to either GABA or a depolarizing pre-pulse. These treatments also resulted in a full recovery of the transients by the wild type. Whereas in wild type lithium leak currents are observed after prior sodium depletion, this was not the case for the glycine 80 mutants unless GABA was added or the oocytes were subjected to a depolarizing pre-pulse. Thus, glycine 80 appears essential for conformational transitions in GAT-1. When this residue is mutated, removal of sodium results in “freezing” the transporter in one conformation from which it can only exit by compensatory changes induced by GABA or depolarization. Our results can be explained by a model invoking two outward-facing states of the empty transporter and a defective transition between these states in the glycine 80 mutants.
coding and non-coding strands were sequenced between the above two restriction sites.

\(^{\text{[3H]GABA}}\) Transport in HeLa Cells—HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 200 units/ml penicillin, 200 \(\mu\)g/ml streptomycin, and 2 mM glutamine. HeLa cells plated on 24-well plates were infected with recombinant vaccinia/T7 virus vTF7-3 (23) and transfected with cDNA (pBluescript SK+ with wild type or mutant transporter inserted downstream to the T7 promoter) using the transfection reagent DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N,N-trimethylammonium methyl sulfate) as described (24). Uptake of \(^{\text{[3H]GABA}}\) into the cells was assayed 18–24 h posttransfection. The wells were washed twice with a solution containing 150 mM choline chloride, 5 mM KP, pH 7.4, 0.5 mM MgSO\(_4\), and 0.3 mM CaCl\(_2\). Each well was then incubated with 0.4 \(\mu\)Ci of \(^{\text{[3H]GABA}}\) in a NaCl transport solution (150 mM NaCl, with KP, MgSO\(_4\), and CaCl\(_2\) as above). Transport reactions were carried out for 10 min at room temperature, and the assay was terminated by washing the cells twice with ice-cold NaCl transport solution. Cells were lysed with 1% SDS, and radioactivity was measured by liquid scintillation counting.

Inhibition Studies with Sulfhydryl Reagents—Before the transport measurements, the cells adhering to 24-well plates were washed twice with 1 ml of the transport medium containing 150 mM choline chloride instead of NaCl. Each well was then incubated at room temperature with 200 \(\mu\)l of the preincubation medium containing the sulfhydryl reagent (the different compositions are indicated in the legend to Fig. 1) and 150 mM NaCl was added to the medium. After 5 min in the medium was aspirated, and the cells were washed twice with 1 ml of the transport solution. Subsequently they were assayed for \(^{\text{[3H]GABA}}\) transport at room temperature (22–26 °C) unless indicated otherwise. The hydrophilic methanethiolosulfonate reagents used during the preincubation were purchased from Toronto Research Chemicals, Inc.

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 Inc.). Oocytes were removed from anesthetized Xenopus laevis frogs and treated with collagenase (type 1A, Sigma C-9891) until most of the oocytes were removed from anesthetized pOG1 linearized with SacII using mMessage mMachine (Ambion Inc.). Transport reactions were carried out with KPi, MgSO\(_4\), and CaCl\(_2\) as above). Transport reactions were carried out with 1 ml of the transport medium containing 150 mM choline chloride, 5 mM KP, MgSO\(_4\), and CaCl\(_2\) as above). Transport reactions were carried out with 1 ml of the transport medium containing 150 mM choline chloride, 5 mM KP, MgSO\(_4\), and CaCl\(_2\) as above). Transport reactions were carried out with 1 ml of the transport medium containing 150 mM choline chloride, 5 mM KP, MgSO\(_4\), and CaCl\(_2\) as above).

Oocyte Electrophysiology—This was done as described (16). Oocytes were placed in the recording chamber, penetrated with two agarase (1%)-cushioned micropipettes (back-filled with 2 M KCl, resistance varied between 0.5 and 2 megohms), and voltage-clamped using GeneClamp 500 (Axon Instruments, Inc.) and digitized using Digidata 1200A (Axon Instruments, Inc., Union City, CA), both controlled with the pClamp6 suite (Axon Instruments, Inc.). Currents were acquired with Clampex 6.03 and low-pass filtered at 10 kHz every 0.5 ms. Oocytes were stepped from –140 mV to +60 mV in 25-mV increments using –25 mV as the holding potential. Each potential was held clamped for 500 ms. In the experiments depicted in Fig. 5, the voltage was stepped in 9-mV increments from –160 mV to +11 mV in the case of C74A and from –100 mV to +71 mV for G80C/C74A. In all experiments, after each voltage jump the oocytes were held at –25 mV for 500 ms before jumping to the next voltage. The membrane potential was measured relative to an extracellular Ag+/AgCl electrode in the recording chamber. The recording solution (ND) contained 96 mM NaCl, 2 mM KC1, 1 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), and 5 mM HEPES, pH 7.4. In substitution experiments sodium ions were replaced with equimolar choline or lithium. All electrophysiological experiments shown in the figures are in the absence of MTSET.

RESULTS

Cysteine Scanning Mutagenesis—The amino acid residues of the consensus sequence NGGGAF (residues 77–82 in GAT-1), located in the first extracellular loop and conserved throughout the sodium- and chloride-dependent neurotransmitter transporter family, were replaced one at a time by cysteine. This was done in the background of the C74A mutant, because cysteine 74 is the only cysteine in wild type GAT-1 sensitive to modification by impermeant methanethiolosulfonate reagents. Using

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5-min preincubation with a 0.2 mM concentration of impermeant positively charged thiol reagent MTSET; in the case of G78C/C74A, G79C/C74A, and A81C/C74A, \(^{3}H\)GABA uptake was inhibited, whereas that by N77C/C74A was stimulated by the reagent (Fig. 1B). Similar results with the four mutants were obtained upon preincubation with a 1 mM concentration of the negatively charged (2-sulfonatoethyl)methanesulfonate (data not shown). Even though the mutants are in the background of C74A, one possibility that explains the effects by MTSET is that one or more of the mutants might expose a previously inaccessible endogenous cysteine residue. However, the fact that introduction of an alanine or serine residue at these positions did not render the transporters sensitive to MTSET (Fig. 1B) strongly suggests that the introduced cysteines themselves are modified by the sulfhydryl reagent.

To assess if the reactivity of N77C, G78C, G79C, and A81C is influenced by the conformation of the transporter, the effect of the three substrates on the sensitivity of the mutants to MTSET was determined. In the case of N77C/C74A the stimulation by MTSET in sodium-containing medium was less than that in its absence. In the presence of sodium, GABA did not have much of an effect, but removal of chloride resulted in a diminished stimulation (Fig. 1C). In G78C/C74A there was not much of an effect of the composition of the external medium on sensitivity to the sulfhydryl reagent, but both in G79C/C74A and A81C/C74A there was a significant protection by sodium, and this protection was diminished either in the presence of GABA or when chloride was removed (Fig. 1C).

Effect of GABA on Sodium Transients—Because the lack of \(^{3}H\)GABA transport of G80C/C74A and F82C/C74A is not due to defective targeting to the plasma membrane, it is possible that one or both of the mutants have a defect in one of the steps of the transport cycle but still may be able to execute some of the other steps. This possibility was addressed by analyzing the ability of the mutant transporters upon expression in *X. laevis* oocytes to carry out reactions linked to the transport cycle. One of those is the capacitative sodium-dependent transient current that is thought to represent a charge-moving conformational change subsequent to sodium binding to GAT-1 (Refs. 13, 16, 17, and 19 and Fig. 2). When the membrane potential of oocytes expressing wild type GAT-1 was jumped to a range of voltages from −140 to +60 mV (for 500 ms at each voltage) from a holding potential of −25 mV, transient currents were observed in sodium-containing medium (Fig. 2A) but not in sodium-free medium (Fig. 2B). These sodium-dependent transients were inward when the jumps were to potentials more negative than the holding potential and outward when the jumps were to potentials more positive than −25 mV. When the voltage was returned to the holding potential, transients of identical magnitude but opposite sign were observed. These transients are many orders of magnitude slower than expected for the rate of diffusion-limited sodium binding. The transients are rather a readout of sodium binding to the transporters and are thought to represent sodium-dependent charge movements by the transporter through the membrane electric field during the “on” steps and back during the “off” steps (13, 16, 17, 19). It was also shown that when external sodium concentrations were progressively lowered, the contribution of the inward charge movements during jumps from the same holding potential became more and more pronounced, as if at the holding potential less and less sodium was bound to the transporter. Thus at lower extracellular sodium, more negative membrane potentials are required for the transporters to bind sodium. As expected, when after prior sodium removal the oocytes expressing wild type GAT-1 were perfused again with this cation, the transient currents returned (Fig. 2C). The addition of nontransportable solutes, such as L-aspartate, did not have an effect on the transients (Fig. 2D). As observed previously (13, 16, 17, 19), the addition of GABA resulted in an almost complete disappearance of the transient current, which now became a maintained steady-state current (Fig. 2E). This is because, rather than sodium-dependent charge movements into and out of the membrane electric field, now sodium and GABA are transported across the membrane in an electrogenic manner. After GABA was removed, the transients were observed again (Fig. 2F).

Although no transient currents were observed with the F82C/C74A mutant, the G80C/C74A transporters did exhibit sodium-dependent transients, but in contrast to the wild type, in 96 mM external sodium they were only observed when the voltage was jumped to positive potentials from a holding potential of −25 mV (Figs. 2A and 3A) as if at the holding potential all the mutant transporters were in the sodium-bound state. Indeed lowering the sodium concentration resulted in the transients becoming more and more symmetrical and eventually inward (data not shown, but see Fig. 4). These transients reflect capacitative charge movements because upon return to −25 mV, transients of similar size but opposite sign were observed (Fig. 3A). In the absence of sodium (choline replacement) no transients by G80C were observed (Fig. 3B). Remarkably, when after extensive perfusion with the sodium-free medium, the G80C/C74A oocytes were perfused again with sodium Ringer for as long as 10 min, almost no transients were detected (Fig. 3C), and this remained the same even after the perfusion with sodium was continued for an hour (data not shown). Similar results were obtained when sodium was re-

**Fig. 2. Sodium-dependent transient currents mediated by G74A.** Currents in sodium-containing medium (ND) supplemented with 30 μM SKF100330A (last measurement) were subtracted from those obtained in the absence of the blocker during 500-ms voltage jumps from −140 to +60 mV (steps of +25 mV). The perfusion medium contained sodium (ND) before (A) and after (C) perfusion with choline-containing medium (20–30 ml, B). Subsequently the ND medium was supplemented with 1 mM L-aspartate (D) or 1 mM GABA (E) and finally again with ND (F). Traces from an oocyte typical of nine are shown.
moved by perfusion with N-methyl-D-glucamine (data not shown). Interestingly, exposure to 1 mM GABA caused a marked and immediate recovery of the transients (Fig. 3E).

Half-maximal recovery of the transients was obtained at around 100 μM GABA. This effect of GABA was specific because 1 mM l-aspartate was not able to bring about any recovery of the transients (Fig. 3D). Subsequent removal of GABA caused a further increase of the transients (Fig. 3F). The recovery of the transients upon exposure to GABA was completely dependent on the simultaneous presence of sodium; after exposure to GABA in lithium- or choline-containing media followed by washout of the GABA, the sodium-dependent transients did not recover (data not shown). On the other hand the recovery of the transients by GABA was also observed in the absence of chloride (gluconate substitution, data not shown). Observations similar to those on G80C/C74A were also made when glycine 80 was replaced by alanine and, albeit to a much lesser extent, when the replacement was by serine or threonine. When glycine 80 was replaced by proline or aspartate, no sodium-dependent transients were observed regardless of the presence of GABA (data not shown). This was not due to a targeting defect, at least not in the G80P/C74A mutant, as evidenced by surface-biotinylation experiments (data not shown).

The phenomenon of poor recovery of the transients after removal of sodium was neither observed with G78C/C74A nor with G79C/C74A (data not shown).

It is important to see the full extent of the transients to quantify the phenomenon. However, even at +60 mV the full extent of the transients was not yet observed since their size was still increasing as a function of the voltage (Fig. 3). It is difficult to determine the full extent of the transients of G80C/C74A at 96 mM sodium because at the holding potential all the transporters are in the sodium-bound form, and jumping to potentials more positive than +100 mV is required to obtain the maximal charge movement (data not shown). Such very positive potentials were not well tolerated by most batches of oocytes. At reduced sodium concentrations, less positive potentials should be required to cause dissociation of sodium from the transporters. Therefore, the effect of GABA was determined in a solution containing only 10 mM sodium. Before perfusion with the 10 mM sodium-containing solution, the oocytes were perfused first with a sodium-free solution. The results are given as non-subtracted records to show more clearly the opposite effect of GABA on the transient currents by C74A (Fig. 4, A and B) and the mutants (Fig. 4, C–F). In 10 mM sodium, transients in the G80C/C74A and G80A/C74A mutants were almost undetectable (Fig. 4, C and E), but exposure to 1 mM GABA resulted in the induction of the transients (Fig. 4, D and F). As predicted, the transients by G80C/C74A at 10 mM sodium were symmetrical, in contrast to the outward transients measured at 96 mM sodium (Fig. 2). The size of the transients saturated in the voltage range used (Fig. 4, D and F). Again, the addition of 1 mM l-aspartate did not cause the induction of any transients (data not shown). In the absence of GABA reintroduction of sodium at 10 mM to oocytes that expressed the parent construct C74A resulted in the appearance of transients (Fig. 4A). Because of the reduced sodium concentration, most of the charge movement was observed when the voltage was jumped to negative potentials (compare with Fig. 2 where 96 mM sodium is used). The addition of GABA (Fig. 4B, see also Fig. 2E) resulted in an almost total disappearance of...
apparent when comparing the steady-state currents at negative potentials in Fig. 4B with those in Fig. 4A. For instance, in this experiment the GABA-induced current at $-140$ mV was $-279.5$ nA. In the case of other mutants at position 80, also in the presence of 10 mM sodium small transients could be induced in the serine and threonine replacement mutants by GABA but not in those where glycine 80 was replaced with proline or aspartate (data not shown). The induction of the transient currents in G80A/C74A and G80C/C74A was dependent on the GABA concentration. In the case of G80A/C74A, the charge movements were maximal at 1 mM. At 10 mM GABA a small but significant decrease of the $Q_{\max}$ was observed, $87 \pm 3\%$ that at 1 mM ($n = 6$), apparently due to the emergence of GABA-induced steady-state currents (see also Fig. 6). At 0.1 mM GABA, the $Q_{\max}$ was $40 \pm 4\%$ that at 1 mM GABA ($n = 6$). In the case of G80/C74A, the $Q_{\max}$ at 0.1 mM GABA was only $9 \pm 4\%$ that at 1 mM, whereas at 10 mM its size increased to $216 \pm 25\%$ that at 1 mM GABA ($n = 5$). This indicates that the G80C/C74A mutant has an $\sim 10$- fold lower apparent affinity for GABA than G80A/C74A.

Our observations on the dependence of the transient currents on the sodium concentration and voltage indicate that the glycine mutants have an increased apparent sodium affinity. In the voltage range used there was no saturation of the charge movements (representing sodium dissociation) of G80C/C74A in response to depolarization at 96 mM sodium (Fig. 2), and the same was true for sodium binding to C74A in response to hyperpolarization at 10 mM sodium (Fig. 4A). To directly compare the $Q/V$ relationship of G80C/C74A with that of C74A we employed an intermediate sodium concentration (20 mM, Fig. 5). The curve of the mutant was right-shifted by around 80 mV relative to that of the control; $V_{1/2}$ for C74A was $-86.6 \pm 2.3$ mV ($n = 10$), $-8.4 \pm 2.6$ mV for G80C/C74A ($n = 4$), and $-11.7 \pm 1.7$ mV for G80A/C74A ($n = 3$). Importantly, the $Q/V$ relationship of the G80C/C74A mutant after the removal of sodium and its subsequent re-addition was similar to that before sodium removal (Fig. 5A). The same was true after addition and subsequent removal of L-aspartate and GABA (Fig. 5A). Similar observations were made with G80A/C74A (data not shown).

The charge movements in 20 mM sodium were compared under four consecutive conditions, 1) before sodium removal, 2) after sodium removal by perfusion with sodium-free solution, 3) after prior perfusion with 20 mM sodium and 1 mM L-aspartate, and 4) after prior perfusion with 20 mM sodium and 1 mM GABA. For each oocyte the size of the charge movements from each condition was normalized to that of condition 1, and the data are presented in Fig. 5, B and C, represent the average normalized values for each condition of all oocytes tested. The data indicate that GABA primarily influenced the number of the G80C/C74A transporters capable to carry out the charge-moving conformational change (Fig. 5B). Interestingly, at 20 mM sodium, also the recovery of the transients by C74A after sodium removal was not entirely complete, namely $76 \pm 5\%$ of the $Q_{\max}$ before sodium removal ($n = 10$) (Fig. 5C). Remarkably, after exposure to GABA and its subsequent removal, the transients recovered to their original size, $105 \pm 4\%$ ($n = 10$) of the $Q_{\max}$ before sodium removal (Fig. 5C). At 96 mM sodium the phenomenon was less pronounced, and the corresponding values were $91 \pm 2$ and $106 \pm 4\%$, respectively ($n = 7$). The data collected in this figure allow us to estimate the expression levels of the G80C/C74A mutant and the wild type. Before sodium removal the $Q_{\max}$ for the mutant in 20 mM sodium was $5.74 \pm 1.2$ nC ($n = 4$). However, GABA stimulated the charge movements by around 3.5-fold (Fig. 5B) so that under optimal conditions $Q_{\max}$ of the mutant was around 20 nC, which is compared with a $Q_{\max}$ of 24.00 $\pm 0.25$ nC ($n = 10$) for C74A.
Steady-state GABA Currents—In the 96 mM sodium-contami-
ing medium, small steady state currents induced by 1 mM GABA were observed with G80A/C74A (Fig. 6A). At −140 mV these were −80.6 ± 5.7 nA (n = 5) for the mutant and −502 ± 38 nA (n = 5) for C74A. In the case of the G80A/C74A mutant, when GABA-induced steady-state currents were measured after sodium was removed and subsequently was added back, they were found to be 102.3 ± 3.7% (n = 5) at −140 mV of the corresponding currents monitored before sodium removal. Up-
take of [3H]GABA under voltage clamp at −80 mV showed charge/flux ratios of 1.62 ± 0.09 for C74A (n = 5) and 1.56 ± 0.13 for G80A/C74A (n = 4). These values are in between the proposed charge/flux ratios of 1 (11–14) and 2 (15) for GABA transport, but it is obvious that the mutation does not cause a change in the stoichiometry of coupled GABA transport. The apparent affinity for GABA in G80A/C74A was markedly re-
duced relative to C74A (Fig. 6A). In the case of G80C/C74A, inward currents were almost undetectable at 1 mM GABA, but at 10 mM very small inward currents were observed (data not shown). Again, this indicates that the apparent GABA affinity of G80C/C74A is substantially lower than that of G80A/C74A. In the case of G80S/C74A and G80T/C74A no GABA-induced currents could be observed even at 10 mM. The sodium concentra-
tion dependence of the GABA-induced currents by G80A/
C74A was shifted toward much lower concentrations than in
C74A. At 2 mM sodium the currents induced by 10 mM GABA in
G80A/C74A were 50.3 ± 4.7% that of those at 96 mM sodium at
−140 mV, whereas in C74A the corresponding value was 7.2 ± 3.0% (n = 3). The GABA-induced current by C74A increased at
more negative voltages, possibly due in part to an increased
binding of sodium to the transporter. Consistent with this idea
is that this voltage dependence in the mutant, which has a
much higher apparent affinity for sodium, is much more shal-
low (Fig. 6B). The percentage of the size of the GABA-induced
 currents at +60 mV relative to those at −140 mV was 56.1 ± 4.5% for G80A/C74A (n = 5) and 5.4 ± 1.6% for C74A (n = 5),
respectively. The size of the currents at −140 mV was −145 ± 12 nA for G80A/C74A and −490 ± 36 nA for C74A (n = 5).

Lithium Leak Currents—The lithium-leak current is another transportable GABA analogue 100330A also was not able
charge/flux ratios of 1.62 and 63.6 ± 0.8% inhibition by 1 mM sodium for G80A/C74A and G80C/C74A, respectively, at −140 mV (n = 3). Measurements of charge/flux ratios at −80 mV showed that no
[3H]GABA uptake took place in lithium-containing media (data
not shown).

Even though in G80C/C74A sensitivity of [3H]GABA trans-
port to MTSET could not be determined because of the lack of
activity (Fig. 1A), we could assess the impact of the sulphydryl reagent on the GABA-dependent lithium-leak currents and sodium-transient currents. Neither of these currents was sen-
tive to 1 mM MTSET regardless of if the preincubation was
performed in choline or sodium containing media with or with-
out 1 mM GABA (data not shown).

Effect of Depolarization on Transient and Leak Currents—
The data presented thus far indicate that GABA is able to
correct defective conformational changes in the glycine 80
mutants. Because the binding order of sodium and GABA could
be random, it is possible that GABA exerts its effect by bypassing
the affected step in the transport cycle. We considered the
possibility that the membrane potential may perhaps directly
influence this affected step. During our voltage-jump protocols
the oocytes were held at varying membrane potentials for 500
ms. After perfusion with a choline-containing medium, oocytes
expressing G80A/C74A and G80C/C74A were exposed to a
medium containing 10 mM sodium. As documented in Fig. 4,
no transients were observed under these conditions unless GABA
was added. Before monitoring the transient currents at the
holding potential of −25 mV, the oocytes were held clamped for
2 min at various potentials beginning at −85 mV. At positive
potentials, the transients recovered in the absence of GABA
(Fig. 8). The size of the recovery was time-dependent, reaching
the maximum at 2 min, and this time dependence was similar at
+35 and +65 mV (data not shown). The size of the transients
by C74A after 96 mM sodium was added back to the oocytes and
was increased by the depolarization pre-pulse by 35 ± 7% (n = 3); moreover, when the oocytes were held in the lithium-
containing media for 2 min at +65 mV, leak currents could
subsequently be monitored in the absence of GABA: −549 ± 66 nA
for G80A/C74A (n = 3) and −374 ± 76 nA for G80C/C74A (n = 4), both at −140 mV. When, after the depolarizing pre-pulse the
leak currents were monitored at several time points after the
return to the holding potential (−25 mV), a progressive decay
was observed with a half-time of −45 s (data not shown).

The same was true for the transients but only when they were
monitored at low sodium concentrations; at 10 mM sodium the
half-time of decay was around 60 s, but at 96 mM sodium no
measurable decay was observed even after keeping the oocytes
at the holding potential for 4 min (data not shown). As will be
In this study we have shown that mutation of glycine 80 of GAT-1, a residue conserved throughout the SLC6 transporter family, results in a remarkable phenotype. Unlike the wild type, the sodium-dependent transient currents of G80C (Figs. 3 and 4) and G80A (Fig. 4) do not recover when, after sodium removal, this cation is added back. However, the addition of GABA corrects this defect (Figs. 3E and 4, D and F), and the ability to carry out these transients persists after removal of GABA (Fig. 3F). Sodium removal is also a prerequisite for the measurement of the lithium leak current because sodium is a potent inhibitor of this current (16, 20). Indeed, in the G80C and G80A mutants the lithium leak currents are almost undetectable, but also here they emerge in the presence of GABA (Fig. 7). Thus, it appears that after removal of sodium, the mutant transporters are "frozen" in a state from which they cannot exit to undergo the conformational changes involved in the transient and leak currents. This idea is further underscored by the observation that not only GABA but also a prolonged depolarization (Fig. 8) can restore both activities, as if either of these treatments can release the mutant transporters from their frozen state.

The frozen state appears to be outward-facing, since extracellular sodium and GABA can bind to it and cause the mutant transporters to regain the ability to mediate the transients. However, there exists yet another outward-facing state of the mutant transporters that is reached after the depolarizing steps in the voltage jump protocol (Fig. 3A, no prior sodium removal). Jumping to positive potentials causes the dissociation of sodium from the transporter and the outward charge movement. When after 500 ms the potential is stepped back to the holding potential, sodium rebinds, as evidenced by the ensuing opposite charge movement (Fig. 3A). To distinguish between these two outward-facing states, the frozen state and the one able to bind sodium and undergo the charge movement, they will be referred to as T and T*, respectively. The T* state is believed to represent a genuine intermediate of the transport cycle (13, 16, 17, 19). Importantly, T* also represents the leak mode of the transporter, but when sodium binds to it and is occluded, the leak pathway is inhibited (16, 20). Regarding the T* state we know that addition of sodium and GABA to G80A/C74A, after prior sodium removal, results in steady-state transport currents of the same magnitude as those recorded before sodium removal. Thus, there must be a sodium- and GABA-dependent transition between T and the translocation complex even though the transition from T to T* is attenuated (Fig. 3). These features are incorporated into a model of which we present two variants, which both can explain the observations on the glycine 80 mutants (Fig. 9, A and B). These variants have the same basic features, and the only difference between them is that in Fig. 9A, the T state is reached by the transporter when the empty inward-facing transporter reori-

**FIG. 7. Effect of GABA on lithium-dependent currents.** Shown are I-V relationships of lithium currents after subtraction of the currents in ND without the addition (□), with 10 mM L-aspartate (○) or 10 mM GABA (▲). In each of the panels the currents are normalized to those obtained in the presence of 10 mM GABA at −140 mV. The sizes of the lithium currents in C74A were −76 ± 137 nA and −644 ± 28 nA without and with 10 mM GABA, respectively (n = 3). The corresponding values for G80A/C74A were −23 ± 9 and −192 ± 403 nA and for G80C/C74A −20 ± 5 and −417 ± 54 nA, respectively. A, GAT-1-C74A; B, G80A/C74A; C, G80C/C74A.

**FIG. 8. Effect of the pre-pulse potential on the sodium-dependent transients.** Oocytes expressing G80A/C74A and G80C/C74A were re-perfused with the 10 mM sodium containing medium after prior removal of sodium by perfusion with choline chloride medium. After each depolarization pre-pulse (manually adjusted) for 2 min at the indicated potential (starting with the most negative voltage, −85 mV), the membrane potential was readjusted to −25 mV, and sodium-dependent transients were recorded as under “Experimental Procedure.” The data are normalized to the G max (integrated using Clampfit) at +65 mV and are the averages ± S.E. from 3 or 4 oocytes.
depolarization. GABA binding to the mutants enables the transition 
G80A the mutant transporters are stuck in state T and can only slowly 
mutants, the transition from T to T* (dashed arrow) is impaired, and 
outside to yield T or T*. Which also participate in GABA translocation.

For the sake of simplicity we have omitted the second sodium ion and the chloride ion, 
translocation complex T*NaG in the presence of sodium. T* and T*G 
and outward transients can be observed due to the fact that the 
observation on the glycine mutants can be easily explained.

Why do “native” G80A- or G80C-expressing oocytes exhibit the transient currents? These oocytes have been in a sodium-containing medium for 4–5 days since the moment of injection of the cRNA encoding for the mutant transporters. The rate of T to T* transition is slow in the mutants but not zero, and due to the high apparent sodium affinity any mutant transporters that reach the T* state will be immediately trapped in the T*Na state. If the membrane potential is jumped to positive potentials, sodium dissociates from the transporter, again yielding T*. However because the jump is only for 500 ms, this it too 
short for the T* to T transition to occur, and when the potential 
is jumped back to the holding potential all the transporters 
revert from T* to T*Na. However, after a prolonged absence of 
sodium, the transporters do undergo the T* to T transition, and in this case the transients cannot recover without GABA or a long depolarization.

The model predicts that when in the absence of sodium the 
muscle transporters are brought in the T* state by the depolarizing pre-pulse, after return to –25 mV the transporters will 
revert from T* to T in a time-dependent process. This is expected 
to result in a decay of the lithium leak current, and this has indeed been observed. When the depolarizing pre-pulse is 
given in the presence of saturating sodium, the transporters 
will become trapped in the T*Na state and remain there because of their high apparent affinity for sodium. Indeed, the 
transients do not decay when the oocytes are held at –25 mV in the standard sodium-containing medium for up to 4 min. In the presence of low sodium, part of the transporters are in T* and 
part in T*Na, and the decay of the transients is predicted to 
increase as sodium is lowered, exactly as we have observed.

According to the alternative variant (Fig. 9B), reorientation of the inward-facing binding sites of the transporter to the extracellular side yields the T* state. The explanation of all phenomena is exactly the same as above, as long as also here 
the transition from T to T* is impaired in the mutants while the 
reverse transition is not. In this variant entering the T state 
might be considered a side-reaction rather than part of the main translocation path. Nevertheless, sodium and GABA can 
bind to T to yield the translocation complex T*Na,G. Therefore, the 
T* to T transition would rather represent an alternative transport pathway, analogous to a random binding order of substrates in an enzymatic reaction. There is one phenomenon that is more readily explained by the model in Fig. 9B. When 
sodium is removed and subsequently added back, residual 
transients are observed in the mutant (Fig. 3C), and these 
residual transients persist even when sodium removal continues for up to 1 h. According to Fig. 9B the transporters transit 
from T* to T under these conditions, but it is possible that some 
move to the inward-facing conformation instead. When sodium is 
replenished, those transporters emerging from the inward-facing conformation to T* may bind the cation (resulting in 
T*Na), which can explain the residual transients observed in Fig. 3C.

It is important to note that the unusual behavior of the glycine mutant occurs to some extent also in the wild type,
especially when monitored in low sodium (Fig. 5C). This makes sense in terms of the model, regardless of the variant considered. Low sodium promotes the transition from $T^*_{Na}$ to $T^*$, and therefore in low sodium there is an increased probability that the $T$ state is reached. In low sodium the effect of GABA on transients, which are mediated by the wild type, immediately after GABA removal is less than in the mutants since in the wild type the transition from $T$ to $T^*$ is not impaired. The observation that after sodium was added back to oocytes expressing wild type a depolarization pre-pulse resulted in increased transients also suggests that the mutations at glycine 80 enable us to observe more clearly a phenomenon that already exists in the wild type.

It is interesting that proline cannot substitute for glycine 80. Even though proline introduces kinks in $/H9251$-helices, it seems that it is rather the flexibility afforded by glycine that is important. Glycine residues introduced into lactose permease can confer conformational flexibility to this transporter (10), and glycine has been shown to act as a gating-hinge in potassium channels (9). Glycine 80 is the third of three conserved glycines throughout the SLC6 transporter family. Remarkably, only it behaves as if it is required for conformational changes, indicating the uniqueness of this position. It seems likely that also in other transporter families some of the conserved glycine residues may fulfill a role similar to that of glycine 80 in GAT-1.

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