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Transmembrane Domains I and II of the γ-Aminobutyric Acid Transporter GAT-4 Contain Molecular Determinants of Substrate Specificity

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ABSTRACT

The sodium- and chloride-dependent GABA transporters GABA transporter (GAT) 1 to 4 in the central nervous system enable efficient synaptic transmission by removing the neurotransmitter from the cleft. Taurine interacts only weakly with the GABA transporter GAT-4 (IC_{50} ~ 1.6 mM). Glutamate-61 is located in the conserved transmembrane domain I of GAT-4, whereas in the related taurine-transporter taurine transporter (TAUT), glycine occupies the equivalent position. [^{3}H]GABA uptake by the GAT-4 E61G mutant becomes markedly more sensitive to inhibition by taurine (IC_{50} ~ 0.26 mM). Replacement of cysteine-94, located in the conserved transmembrane domain II of GAT-4, to its TAUT counterpart serine, results only in a modest increase in the ability of taurine to inhibit GABA uptake. However, introduction of glycine at this position decreases the IC_{50} for taurine by approximately 8-fold (IC_{50} ~ 0.20 mM). The inhibitory potency of taurine is inversely correlated with the volume of the side chain of the amino acid residue introduced at positions 61 and 94. It is striking that the IC_{50} for taurine of the E61G/C94G double mutant is decreased by approximately 35-fold (IC_{50} ~ 0.05 mM), and this inhibition of GABA transport is competitive. Changes in the inhibitory potency of the mutants described are also observed with β-alanine and GABA, although they are much less pronounced. Our results suggest that determinants on transmembrane domains I and II can influence the specificity of the substrate binding pocket. The size of the side chain at positions 61 and 94 seems to determine the ability of substrate and substrate analogs to interact with the transporter.

Many neurotransmitters are removed from the synaptic cleft by sodium- and chloride-dependent neurotransmitter transporters (for review, see Kanner, 1994; Nelson, 1998). These transporters therefore play a critical role in synaptic transmission. One of the best examples of the importance of these neurotransmitter transporters comes from studies of dopamine transporter knockout mice; the decay of extracellular dopamine in brain slices of such mice is about 100 times longer than normal (Giros et al., 1996). These transporters form a large family and include transporters for biogenic amines and amino acids. Besides GABA transporter (GAT)-1, the first member of the family to be identified (Guastella et al., 1990), it includes three other GABA transporters, namely GAT-2, GAT-3, and GAT-4, according to the nomenclature by the group of Nelson for transporters from mouse brain (Liu et al., 1993). It should be noted that different names have been assigned to basically the same transporters; for instance, GAT-4 from mouse brain (Liu et al., 1993) has also been cloned from rat brain and has been named GAT-B (Clark et al., 1992) and GAT-3 (Borden et al., 1992), respectively.

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The GABA transporters, as well as many other members of this family, are predicted to have 12 transmembrane domains (TMDs) linked by hydrophilic loops with the amino and carboxyl termini residing inside the cell (Guastella et al., 1990). Studies on the serotonin transporter SERT indicate that the theoretical topological model is correct (Chen et al., 1998). Mutagenesis studies, in particular on GAT-1 and SERT but also on other members of the family, suggest the importance of the highly conserved TMD-I and as well as of TMD-III in the interaction of the transporters with their substrates (Pantanowitz et al., 1993; Mager et al., 1996; Bismuth et al., 1997; Chen et al., 1997; Barker et al., 1999; Kanner, 2003). On the other hand, very little is known about the functional role of the highly conserved TMD-II, although it seems to play a role in formation of oligomers during biosynthesis and their subsequent targeting to the plasma membrane (Scholze et al., 2002).

To define the role of TMD-I in GABA transport by the GABA transporters, mutations in several positions of this domain have been analyzed. In this highly conserved domain, position 61 of GAT-4 displays a high degree of variability among the members of this family, which suggested the

ABBREVIATIONS: GAT, GABA transporter; TAUT, taurine transporter; SERT, serotonin transporter; TMD, transmembrane domain; WT, wild-type.

possibility that it is involved in a function specific to each one of the transporters. Moreover, the fact that the β-alanine-sensitive GAT-2 to 4 contain a glutamate residue at this position, whereas the β-alanine-insensitive GAT-1 contains a tyrosine at the equivalent position (Fig. 1, A and B), raised the possibility that this position is involved in determining substrate specificity. Mutation of tyrosine-60 of GAT-1 to glutamate or to threonine results in impaired transport, even though the GAT-1 Y60T mutant exhibits large lithium leak currents (Kanner, 2003). The GAT-1 Y60G mutant, in which tyrosine-60 is replaced by glycine, located in the equivalent position in the taurine transporter (TAUT) (Fig. 1B), is also inactive in transport, but it still exhibits sodium-dependent transient currents. In contrast to WT GAT-1, these transients can be inhibited not only by GABA but also by taurine (N. Melamed and B. I. Kanner, unpublished data). In contrast to GAT-1, mutation of glutamate-61 of GAT-4 to glycine does not lead to inactivation of GABA transport. We found that [^{3}H]GABA uptake by the GAT-4 mutant E61G is markedly more sensitive to inhibition by taurine than is the case in wild-type GAT-4. Moreover, replacement of a cysteine residue from TMD-II by glycine (Fig. 1B) also increases the inhibitory potency of taurine, and in the E61G/C94G double mutant, this increase is even larger. Our data suggest a functional role for TMD-I and TMD-II in shaping the substrate-binding pocket in this family of transporters.

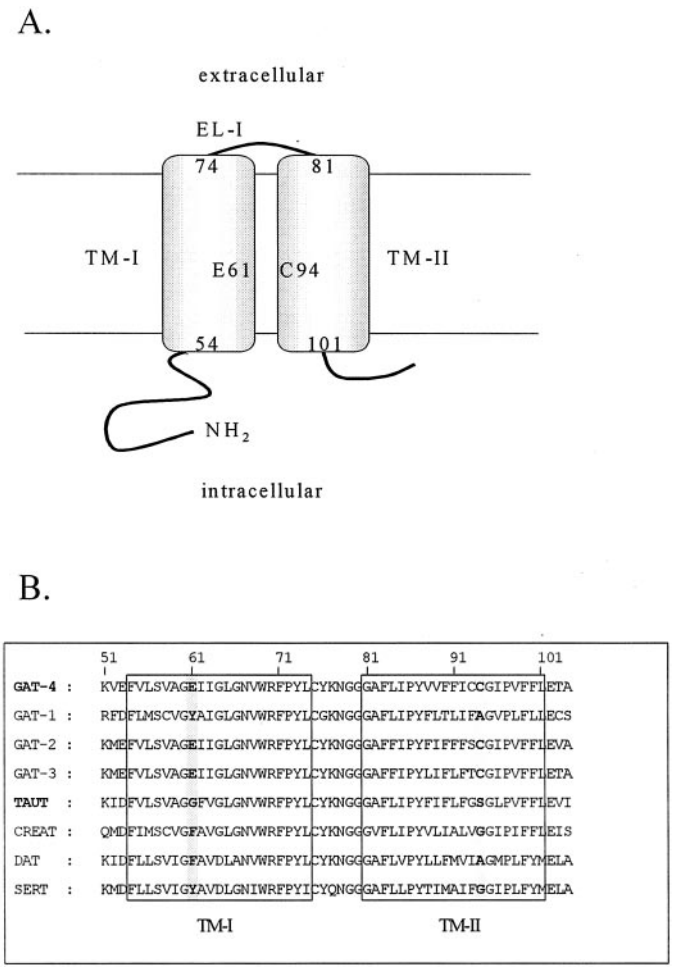

Fig. 1. Schematic diagram and amino acid sequence alignment of transmembrane domains I and II of the sodium- and chloride-dependent transporter family. A, schematic diagram showing transmembrane domains I and II. The positions of glutamate-61 and cysteine-94 of GAT-4 are indicated. The orientation of the domains and distance between them were chosen arbitrarily. B, amino acid sequence alignment for the members of the sodium- and chloride-dependent transporter family. The position numbers indicated are according to GAT-4 sequence. Positions 61 and 94 are highlighted. All sequences are of the cloned transporters from mouse brain. GAT, GABA transporter; TAUT, taurine transporter; CREAT, creatine transporter; DAT, dopamine transporter; SERT, serotonin transporter.

Materials and Methods

Plasmid Constructs. The GAT-4 clone given to us by Dr. Nathan Nelson (Department of Biochemistry, Tel Aviv University, Tel Aviv, Israel) is a shortened version of the originally published cDNA clone (Liu et al., 1993). From the 5′ side, it starts two nucleotides upstream from the start ATG codon. It includes the entire open reading frame and terminates 98 nucleotides after the stop codon at an engineered SpeI site. It was subcloned into the pOG-1 vector between the KpnI and XbaI sites of its multiple cloning site and into the pBluescript SK(−) vector between the KpnI and SpeI sites. pOG-1 is an oocyte expression vector that contains a 5′-untranslated *Xenopus laevis* β-globin sequence, the T7 RNA promoter, and a 3′-poly(A) sequence. Taurine transporter TAUT cDNA (Liu et al., 1992) was also a gift from Dr. Nathan Nelson. It was subcloned into the pOG-1 vector using the EcoRI and XhoI sites and subsequently into the pBluescript SK(−) vector using the ClaI and XbaI sites.

Generation and Subcloning of Mutants. Mutations were made by site-directed mutagenesis of the wild-type GAT-4 and TAUT in the vector pBluescript SK(−) (Stratagene, La Jolla, CA) according to the Kunkel method as described previously (Kunkel et al., 1987; Kleinberger-Doron and Kanner, 1994). In brief, the parent DNA was used to transform *Escherichia coli* CJ236 (*dut*− and *ung*−). From one of the transformants, single-stranded uracil-containing 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; consequently, mutagenic primers were designed to be antisense. All mutants described in this study were subcloned into a construct containing wild-type GAT-4 (using the NcoI and NdeI sites) or the taurine transporter TAUT (using the MscI and BspEI sites) in the pOG-1 vector. The coding and noncoding strands were sequenced between the above two restriction sites.

Uptake Experiments 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 μg/ml streptomycin, and 2 mM glutamine. Infection with recombinant vaccinia/T7 virus vTF7-3 (Fuerst et al., 1986) and subsequent transfection with plasmid DNA, as well as [^{3}H]GABA (100 Ci/mmol; Amersham Biosciences Inc., Piscataway, NJ) or [^{3}H]taurine (23.8 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) uptake, was done as published previously (Keynan et al., 1992). Unless specified otherwise, HeLa cells were incubated for 10 min with the transport solution (20 nM [^{3}H]GABA or 84 nM [^{3}H]taurine in a final volume of 200 μl). In the experiments in which transport kinetics was determined (Figs. 5 and 7), the incubation time was reduced from 10 to 3 min so that true initial rate of transport could be measured.

cRNA Transcription, Injection, and Oocyte Preparation. Capped run-off cRNA transcripts were made from transporter constructs in pOG-1 and linearized with SacII using mMessage mMachine (Ambion, Austin, TX). Oocytes were removed from anesthetized *X. laevis* frogs and treated with collagenase (type 1A; Sigma-Aldrich, St. Louis, MO) until capillaries were absent and injected with 50 nl (50–100 ng) of undiluted cRNA on the same day or the next day. Oocytes were maintained at 18°C in modified Barth's saline: 88 mM NaCl, 1 mM KCl, 1 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 1 mM $CaCl_2$, 0.3 mM $Ca(NO_3)_2$, and 10 mM HEPES, pH 7.5, with freshly added 2 mM sodium pyruvic acid and 0.5 mM theophylline

and supplemented with 10,000 units/l penicillin, 10 mg/l streptomycin, and 50 mg/l gentamicin.

Uptake Experiments in Oocytes. The uptake experiments were performed in 24-well plates with 20 nM [^{3}H]GABA or 84 nM [^{3}H]taurine in 500 μl of oocyte uptake buffer (96 mM NaCl or choline chloride, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.4). Oocytes were incubated with the transport solution for 20 min at room temperature, washed four times in 1 ml of oocyte uptake buffer, and dissolved in 500 μl of 1% SDS. Before counting, 10 ml of scintillation fluid was added to the samples.

Oocyte Electrophysiology. Oocytes were placed in the recording chamber, penetrated with two micropipettes (back-filled with 2 M KCl, resistance varied between 0.5 and 2 MΩ) and voltage clamped using a GeneClamp 500 amplifier (Axon Instruments Inc., Union City, CA) and digitized using Digidata 1200A (Axon Instruments Inc.) 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 holding potential for 500 ms. The membrane potential was measured relative to an extracellular Ag/AgCl electrode in the recording chamber. The recording solution contained 96 mM NaCl, 2 mM KCl, 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. Analysis was performed with Clampfit 6.

Results

Inhibition of [^{3}H]GABA Uptake by Taurine in GAT-4 Mutants. Mutation of glutamate-61 of GAT-4 to glycine, which occupies the equivalent position in the taurine transporter TAUT (Fig. 1B) and to other small amino acid residues, is well tolerated as evidenced by GABA transport activity upon expression in HeLa cells (Table 1). It is remarkable that the data depicted in Fig. 2 illustrate that although in our standard 10-min uptake assay wild-type GAT-4 was only weakly inhibited by taurine (Fig. 2, IC_{50} = 1.63 ± 0.06 mM, n = 8), uptake by the E61G was much more sensitive (Fig. 2, IC_{50} = 0.26 ± 0.02 mM, n = 7). This increased inhibitory potency of taurine seemed to be dependent on the volume of the side chain of the substituent at position 61. The data depicted in Fig. 2 show that E61A also was more sensitive than the wild type, albeit less than E61G (Fig. 2, IC_{50} = 0.69 ± 0.03 mM, n = 4). On the other hand, there was almost no difference in taurine sensitivity among E61C (IC_{50} = 1.36 ± 0.05 mM, n = 4), E61S (IC_{50} = 1.37 ± 0.04 mM, n = 4), and the wild type (Fig. 2). TAUT is also divergent from the GABA transporters at position 62 (GAT-4 numbering, Fig. 1B), which is occupied by phenylalanine in contrast to GAT 2 to 4 (isoleucine) and GAT-1 (alanine). However, the double mutant E61G/I62F was inactive (data not shown); therefore, we could not test the idea that introduction of a phenylalanine at position 62 further enhances the sensitivity of GABA transport by GAT-4-E61G to taurine.

Based on the topology model of this family of transporters, position 61 of TMD-I and positions 93 and 94 of TMD-II may be close in space (Fig. 1A). In addition, except for isoleucine-96, residues 93 and 94 (according to GAT-4 positions) are the only ones in TMD-II that are unique for TAUT and not shared by any of the GABA transporters. In the case of isoleucine-96, it is conservatively replaced in TAUT by leucine and in the other GABA transporters by either isoleucine or valine. Therefore, we explored the possibility that the residues present at position 93 and 94 are also determinants of the sensitivity of [^{3}H]GABA uptake to inhibition by taurine. We mutated cysteines-93 and -94 of GAT-4 to glycine and serine, respectively (C93G/C94S), and because at position 61, small residues are the most effective in increasing the inhibitory potency of taurine (Fig. 2), we also mutated both cysteines to glycine (C93G/C94G). Both double mutants retained significant [^{3}H]GABA uptake activity (Table 1). The sensitivity of [^{3}H]GABA uptake of C93G/C94S to inhibition by taurine was however rather similar to that of the wild type

TABLE 1

[^{3}H]GABA uptake by the WT and mutants in HeLa cells

Uptake of [^{3}H]GABA in HeLa cells expressing wild-type GAT-4 and the indicated mutants was done in NaCl-containing medium as described under *Materials and Methods* at an [^{3}H]GABA concentration of 20 nM. The 100% uptake value for *GAT-4* was 88,236 ± 4103 cpm/10 min/well (~0.18 ± 0.01 pmol/min/well). The background levels of [^{3}H]GABA transport, determined as the uptake by HeLa cells transfected with an empty SK(−) vector, was in the range of 800 to 3200 cpm/10 min/well (~1%–4% of WT uptake). All values are presented as mean (±S.E.) of at least three experiments.

	Uptake
	% of GAT-4
GAT-4	100 ± 0
E61G	77 ± 3
E61A	103 ± 2
E61C	105 ± 4
E61S	81 ± 6
C94G	66 ± 2
C94A	95 ± 4
C94T	45 ± 2
C94M	47 ± 1
C94S	84 ± 7
C93G	95 ± 4
C93G/C94G	66 ± 3
C93G/C94S	88 ± 3
E61G/C94G	41 ± 3
E61G/C94A	79 ± 5
E61G/C94T	57 ± 4
E61G/C94M	45 ± 1
E61G/C94S	68 ± 4
E61G/C93G	65 ± 6
E61G/C93G/C94G	24 ± 2
E61G/C93G/C94S	48 ± 2

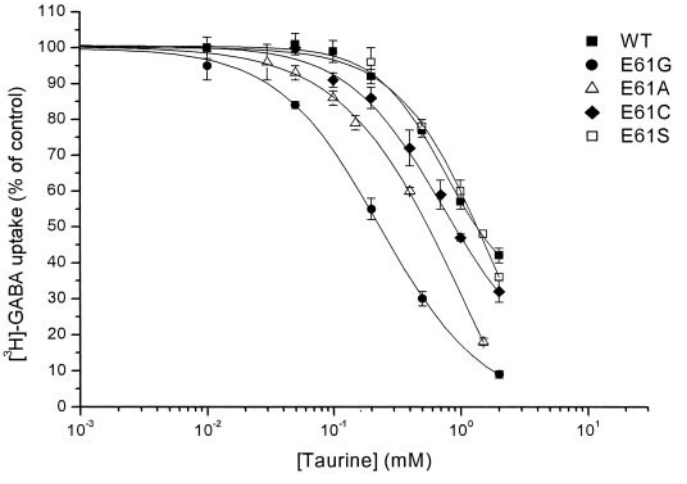

Fig. 2. Effect of substitution mutations in position 61 in TMD-I of GAT-4 on the sensitivity of [^{3}H]GABA uptake to inhibition by taurine. Shown is the inhibition of [^{3}H]GABA uptake by unlabeled taurine in HeLa cells transfected with the WT GAT-4 (■), E61G (●), E61A (△), E61C (◆), and E61S (□). Uptake of [^{3}H]GABA was done in NaCl-containing medium as described under *Materials and Methods* in the presence of the indicated unlabeled taurine concentrations. Values are percentage of activity of each mutant relative to that of the same mutant in the absence of unlabeled taurine and are presented as mean ± S.E. of at least three experiments done in quadruplicate. The respective $IC_{50(taurine)}$ values are 1.63 ± 0.06, 0.26 ± 0.02, 0.69 ± 0.02, 1.36 ± 0.05, and 1.37 ± 0.04.

(Fig. 3). The C93G/C94G mutant showed a remarkable 8-fold increase in the sensitivity to taurine (Fig. 3, IC_{50} = 0.21 ± 0.02 mM, n = 4). This increase is caused by the introduction of a glycine at position 94 rather than 93 (Fig. 3). Moreover, the increase in the inhibitory potency of taurine was further enhanced when the mutations from TMD-I and TMD-II were introduced simultaneously (Fig. 4). The IC_{50} values for taurine of E61G/C93G/C94G and E61G/C94G were 0.04 ± 0.01 mM (n = 4) and 0.05 ± 0.01 mM (n = 7), respectively, which is approximately 35-fold lower than that of wild type GAT-4 (Fig. 4).

To determine whether the inhibition of [^{3}H]GABA uptake by taurine is competitive, transport kinetics of the WT, E61G, and E61G/C93G/C94G were determined in the presence and absence of the taurine concentrations indicated in Fig. 5. Taurine acted as a competitive inhibitor of [^{3}H]GABA uptake in wild type and the mutants (Fig. 5). In the case of WT, the K_i for taurine was 1.3 mM (Fig. 5A), and the corresponding values for E61G and E61G/C93G/C94G were 0.20 and 0.039 mM, respectively (Fig. 5, B and C). This decrease of approximately 6- and 33-fold in the K_i for taurine in these two mutants parallels the decrease in their IC_{50} values (Figs. 2 and 4). Thus, even though these latter experiments do not report true initial rates of transport (see *Materials and Methods*), the parallel decrease of IC_{50} and K_i values indicates that rank ordering based on IC_{50} values is a reasonable indicator of inhibitory potency.

Effect of Size of the Side Chain Present at Position 94. The inhibitory potency of taurine was found to be the highest when the size of the side chain of the substituted amino acid residue at position 61 was the smallest (Fig. 2). The same was found to be true also for position 94. Thus, potentiation of inhibition of [^{3}H]GABA uptake by taurine was observed when cysteine (which occupies this position in the wild-type GAT-4) was substituted by glycine (Figs. 3 and 6A, IC_{50} = 0.20 ± 0.02 mM, n = 4). Introduction of alanine, whose side chain is larger than that of glycine but smaller than that of cysteine, resulted in a modest increase in the sensitivity to taurine (Fig. 6A, IC_{50} = 1.10 ± 0.06 mM, n = 5). Introduction of the larger threonine further decreased the inhibitory potency of taurine (Fig. 6A, IC_{50} = 5.45 ± 0.53 mM, n = 4), and when the methionine, even larger, was introduced at position 94, the inhibitory potency of taurine was further diminished (Fig. 6A, IC_{50} = 17.75 ± 1.05 mM, n = 4). The same effect of the size of the substituent introduced at position 94 on the inhibitory potency of taurine was also observed in the background of the E61G mutation (Fig. 6B).

To determine whether this position plays a similar role in the taurine transporter TAUT, we have studied mutations at the equivalent position in this transporter. As in GAT-4, replacement of serine-90 by the smaller glycine indeed decreased the K_m for taurine transport, whereas the introduction of the bulkier threonine increased it (Fig. 7). However, these effects were less pronounced than those observed in GAT-4 (Fig. 6A).

Taurine Transport by GAT-4. Even though taurine inhibits [^{3}H]GABA uptake by the high-affinity GAT-4 mutants competitively, taurine could be either transported or act as a blocker, which is only capable of binding to the outwardly facing form of the transporter. In the HeLa cell expression system, there is a relatively high level of endogenous [^{3}H]taurine transport or binding (5000–6000 cpm) under the conditions described under *Materials and Methods*. None of the mutants or the wild-type GAT-4 exhibited significant uptake above this level (data not shown). As a positive control, we used the taurine transporter TAUT, which exhibited [^{3}H]tau-

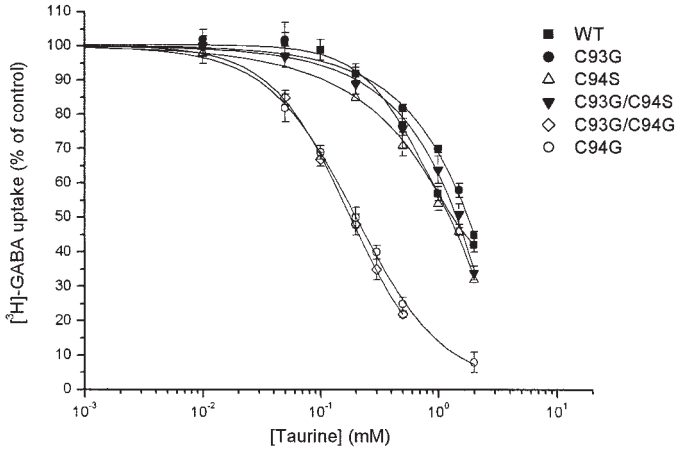

Fig. 3. Effect of substitution mutations in positions 93 and 94 in TMD-II of GAT-4 on the sensitivity of [^{3}H]GABA uptake to inhibition by taurine. Shown is the inhibition of [^{3}H]GABA uptake by unlabeled taurine in HeLa cells transfected with the WT GAT-4 (■), C93G (●), C94S (△), C93G/C94S (▼), C93G/C94G (◇), and C94G (○). Uptake of [^{3}H]GABA was done in NaCl-containing medium as described under *Materials and Methods* in the presence of the indicated unlabeled taurine concentrations. Values are the percentage of activity of each mutant relative to that of the same mutant in the absence of unlabeled taurine and are presented as mean ± S.E. of at least three experiments done in quadruplicate. The respective $IC_{50(taurine)}$ values are 1.63 ± 0.06, 1.82 ± 0.06, 1.25 ± 0.11, 1.43 ± 0.13, 0.21 ± 0.02, and 0.20 ± 0.02.

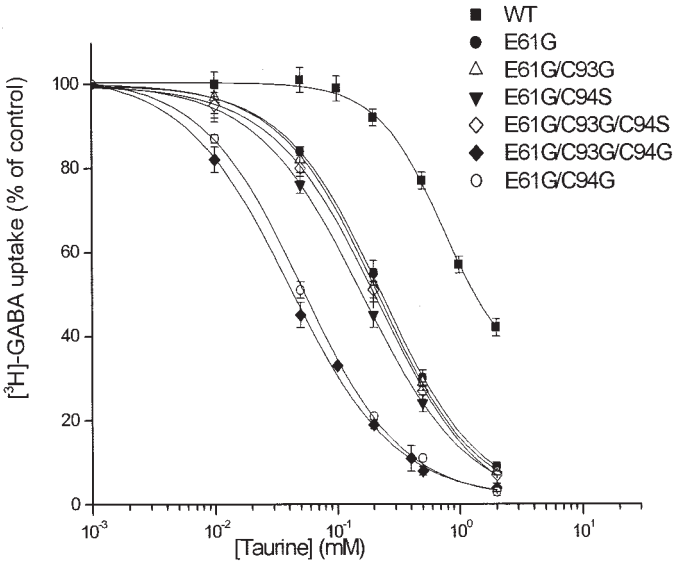

Fig. 4. Effect of double substitution mutations in position 61 in TMD-I and positions 93 and 94 in TMD-II of GAT-4 on the sensitivity of [^{3}H]GABA uptake to inhibition by taurine. Shown is the inhibition of [^{3}H]GABA uptake by unlabeled taurine in HeLa cells transfected with the WT GAT-4 (■), E61G (●), E61G/C93G (△), E61G/C94S (▼), E61G/C93G/C94S (◇), E61G/C93G/C94G (◆), and E61G/C94G (○). Uptake of [^{3}H]GABA was done in NaCl-containing medium as described under *Materials and Methods* in the presence of the indicated unlabeled taurine concentrations. Values are the percentage of activity of each mutant relative to that of the same mutant in the absence of unlabeled taurine and are presented as mean ± S.E. of at least three experiments done in quadruplicate. The respective $IC_{50(taurine)}$ values are 1.63 ± 0.06, 0.26 ± 0.02, 0.23 ± 0.03, 0.17 ± 0.01, 0.22 ± 0.02, 0.04 ± 0.01, and 0.05 ± 0.01.

rine uptake that was approximately 8-fold the background levels (data not shown). Because of the relatively high background levels of [^{3}H]taurine uptake in HeLa cells, we also investigated this process using *X. laevis* oocytes as an expression system, which had a very low background (50–70 cpm). The experiment depicted in Fig. 8A shows that in the presence of sodium, some batches of oocytes expressing wild-type GAT-4 exhibited low (see figure legend), yet significant, [^{3}H]taurine uptake compared with uninjected oocytes. This uptake was sodium-dependent and was not observed when

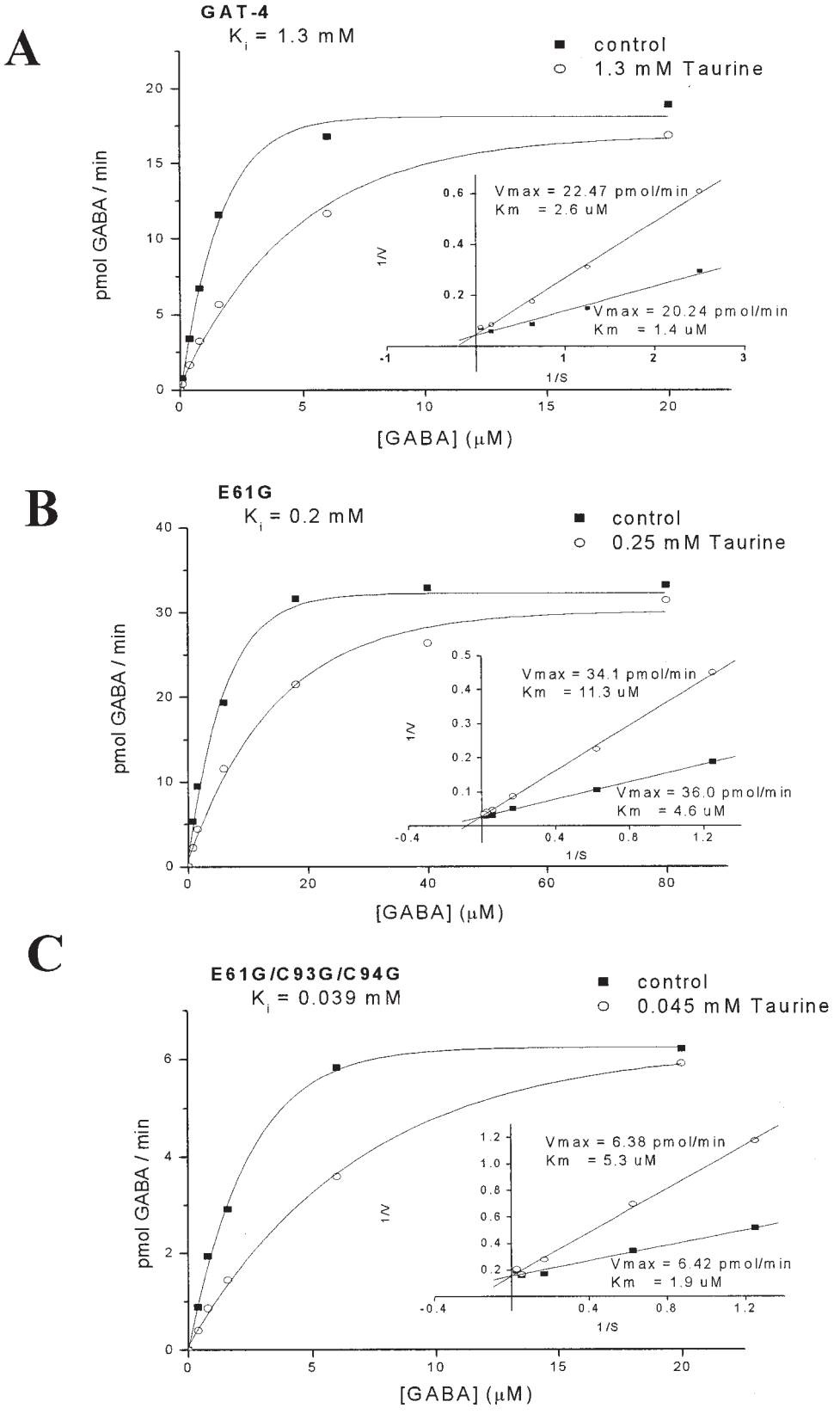

Fig. 5. Effect of taurine on the kinetics of [^{3}H]GABA uptake in HeLa cells. The kinetics of [^{3}H]GABA uptake in HeLa cells assayed for 3 min for GAT-4 (A), E61G (B), and E61G/C93G/C94G (C) were determined as described under *Materials and Methods* in the absence (■) and presence (○) of the indicated taurine concentration. K_m and V_{max} values were determined using linear regression analysis of the Lineweaver-Burk plot (insets). The V_{max} values indicated in the figure are per well. As can be seen, taurine increases the observed K_m values but has almost no effect on V_{max}, which supports taurine being competitive inhibitor. The data represents a typical experiment of at least three experiments done in quadruplicate. The K_i values (1.3 ± 0.2, 0.2 ± 0.02, and 0.039 ± 0.001, respectively) are presented as mean ± S.E. and were calculated from the formula $\alpha K_m = K_m(1+[I]/K_i)$, where αK_m represents the observed K_m in the presence of the competitive inhibitor (taurine) and [I] represents the concentration of the competitive inhibitor.

sodium was replaced by choline (Fig. 8A). As can be seen, the E61G and E61G/C93G/C94G mutants were also capable of sodium-dependent uptake (Fig. 8A). Because of the low absolute levels of [^{3}H]taurine taken up, it was not possible to determine whether the increased inhibitory potency of taurine on [^{3}H]GABA transport in the mutants was paralleled by a decreased K_m for taurine transport. However, in some batches of oocytes, it was possible to measure very small taurine-induced currents (20–40 nA at −140 mV) that were sodium-dependent and inwardly rectifying (data not shown). We were able to determine the dependence of these currents (presumably reflecting electrogenic sodium-dependent taurine translocation) on the taurine concentration for WT GAT-4 and E61G (Fig. 8B). In the case of WT, half-maximal currents (normalized to currents at 5 mM taurine) were observed at approximately 0.4 mM taurine. In the E61G mutant, the half-maximal currents were observed at approximately 8-fold lower concentrations of taurine (Fig. 8B), consistent with our observation of the 6-fold increased inhibitory potency of taurine on GABA transport in E61G (Fig. 2).

Effect of the Mutations on the Inhibitory Potency of β-Alanine and GABA. To examine whether the effect of the

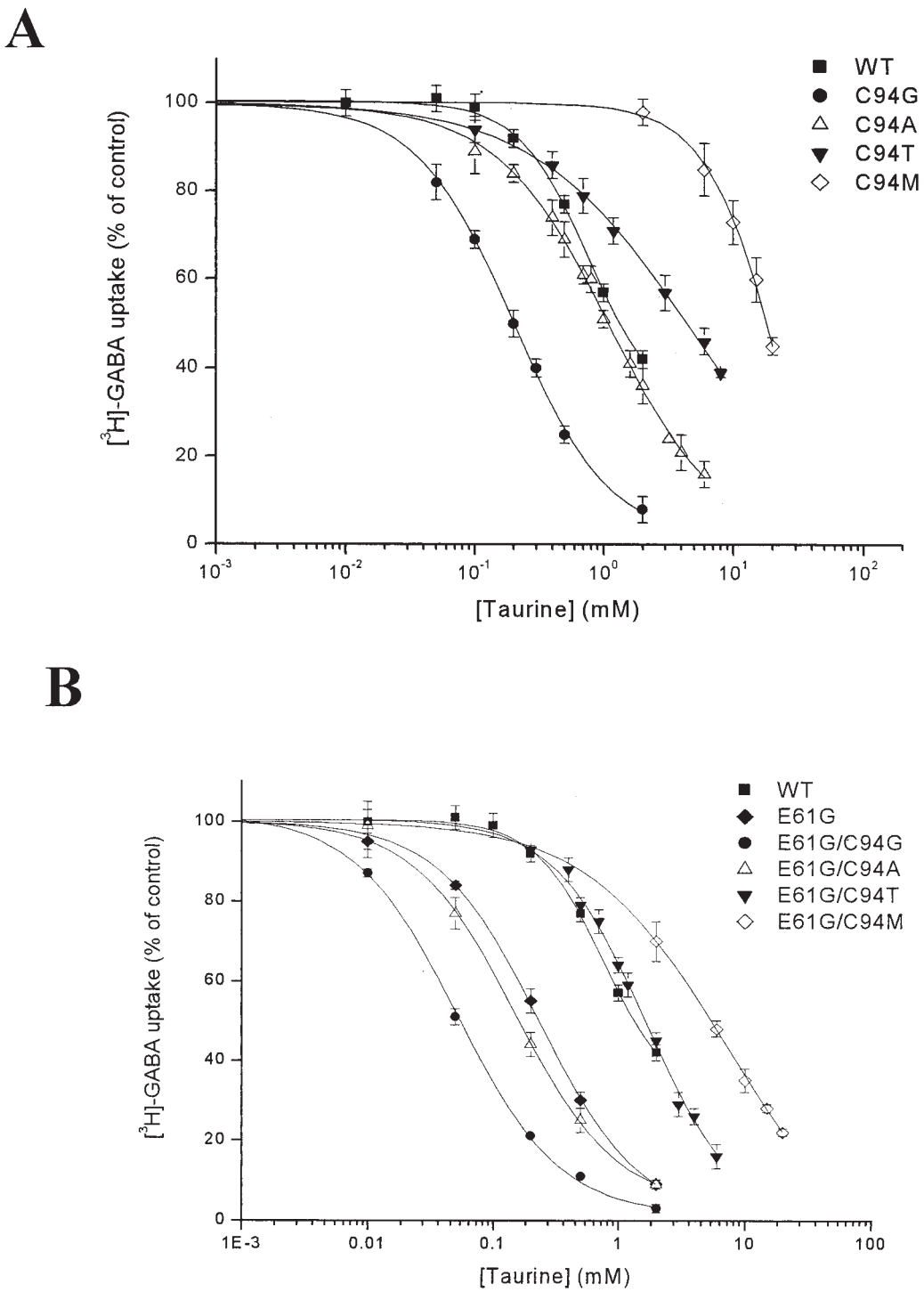

Fig. 6. Effect of the size of the residue at position 94 in TMD-II on the sensitivity of [^{3}H]GABA uptake to inhibition by taurine. A, shown is the inhibition of [^{3}H]GABA uptake by unlabeled taurine in HeLa cells transfected with the WT GAT-4 (■), C94G (●), C94A (△), C94T (▼), and C94M (◇). The respective $IC_{50(taurine)}$ values are 1.63 ± 0.06, 0.20 ± 0.02, 1.10 ± 0.06, 5.45 ± 0.53, and 17.75 ± 1.05. B, same substitution mutants at position 94 done in the E61G background: WT GAT-4 (■), E61G (◆), E61G/C94G (●), E61G/C94A (△), E61G/C94T (▼), and E61G/C94M (◇). The respective $IC_{50(taurine)}$ values are 1.63 ± 0.06, 0.26 ± 0.02, 0.05 ± 0.01, 0.16 ± 0.01, 1.71 ± 0.07, and 5.58 ± 0.58. Uptake of [^{3}H]GABA was done in NaCl-containing medium as described under *Materials and Methods* in the presence of the indicated unlabeled taurine concentrations. Values are percentage of activity of each mutant relative to that of the same mutant in the absence of unlabeled taurine and are presented as mean ± S.E. of at least three experiments done in quadruplicate.

mutations that resulted in an increased inhibitory potency of taurine was selective, the ability of β-alanine and GABA to compete with [^{3}H]GABA uptake was explored.

The E61G mutant also showed an increase in the inhibitory potency of β-alanine, but the decrease in IC_{50} (Fig. 9; 21.3 ± 1.4 versus 59.4 ± 2.1 μM, n = 4) was less than observed with taurine (Fig. 2). In the case of GABA, the IC_{50} of E61G was actually increased relative to wild-type GAT-4 (Fig. 10). In the case of the C94G mutant, an increased inhibitory potency was observed both for β-alanine (approximately 6-fold, Fig. 9) and GABA (approximately 2-fold, Fig. 10), but the change was smaller than in the case of taurine. In the case of the E61G/C94G double mutant, the increase in the inhibitory potency of β-alanine was approximately 9-fold (Fig. 9), compared with approximately 35-fold for taurine (Fig. 4). The double mutant exhibited only a small change in the inhibitory potency of unlabeled GABA (Fig. 10). As for taurine, also in the case of β-alanine and GABA, the inhibitory potency was inversely related to the size of the side chain of the substituents at position 94 (Figs. 9 and 10).

Discussion

In this article, we have identified two amino acid residues located on TMD-I and TMD-II, which influence the inhibitory potency of taurine on [^{3}H]GABA uptake by GAT-4. The inhibition of [^{3}H]GABA uptake by taurine in the wild type and in the mutants tested was competitive, and the K_i value for taurine was decreased by more than 30-fold in the E61G/C93G/C94G mutant (Fig. 5). The competitive nature of this inhibition suggests that the increased inhibitory potency of taurine observed by the mutants is a consequence of direct or indirect effects on the GABA binding pocket of GAT-4. This is further supported by the observations that the apparent affinity to β-alanine and GABA is also similarly affected by the mutations at positions 61 and 94, albeit to a lesser degree (Figs. 9 and 10).

Are glutamate-61 and cysteine-94 located at the binding pocket, or do they influence the binding pocket allosterically? In the related serotonin transporter SERT, it has been shown that when cysteine residues are introduced at positions equivalent to positions 64, 66, and 67 of GAT-4, these mutants retained activity and were accessible to impermeant sulfhydryl reagents from the external medium (Henry et al., 2003). Inhibition by these reagents could be prevented by serotonin, even at 4°C, suggesting that the protection is not caused by a large conformational change induced by the substrate (Henry et al., 2003). This suggests that these residues are near the substrate-binding pocket. A similar result has been obtained at position 64 of GAT-1, which is equivalent to position 65 of GAT-4 (Zhou et al., 2004). Because of the extremely high homology among SERT, GAT-1, GAT-4, and other members of this family in TMD-I, these residues are probably near the substrate-binding pocket in GAT-4 as well. If TMD-I is α-helical, then position 61 would also be near the binding pocket. The observation that a cysteine introduced at position 61 of GAT-4 (E61C; data not shown) and at the equivalent position in SERT (Y95C; Henry et al., 2003) does not render transport sensitive to methanethiosulfonate ethyltrimethylammonium may be explained by the selectivity of part of the binding-pocket that permits it to react with the substrate but not with the larger methanethiosulfonate ethyltrimethylammonium. It is important to mention that in the glutamate transporter EAAC-1, when a cysteine is introduced instead of arginine-447 (R447C), which seems to be directly involved in the binding of the γ-carboxyl group of glutamate, methanethiosulfonate reagents have no effect on glutamate transport (Bendahan et al., 2000). Moreover, the accessibility of position 95 of SERT, which is equivalent to position 61 of GAT-4, toward the smaller methanethiosulfon-

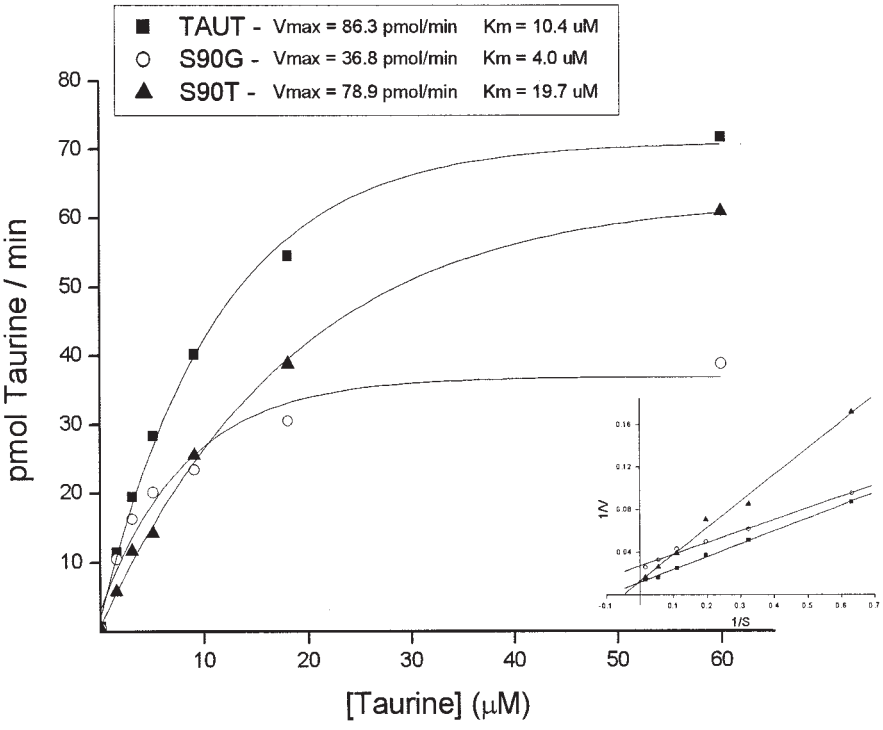

Fig. 7. Effect of substitution mutations at position 90 of TAUT on the kinetics of [^{3}H]taurine uptake. The kinetics of [^{3}H]taurine uptake in HeLa cells by TAUT (■), S90G (○, and S90T (▲) was determined as described under *Materials and Methods*. K_m and V_{max} values were determined using linear regression analysis of the Lineweaver-Burk plot (inset). The presented data are from an experiment representative of three experiments done in quadruplicate.

ate ethylammonium (Henry et al., 2003), may be attributed to the ability of this smaller reagent to reach that part of the binding pocket from the external medium and not necessarily to its ability to permeate the membrane. Thus, although the mutation's effect on taurine binding may have long-range conformational consequences, we find it likely that glutamate-61 is near the binding pocket. Further support for a direct effect comes from observations that the equivalent tyrosine-95 of SERT dictates species-selective recognition of both transport inhibitors (Barker et al., 1998) and indoleam-

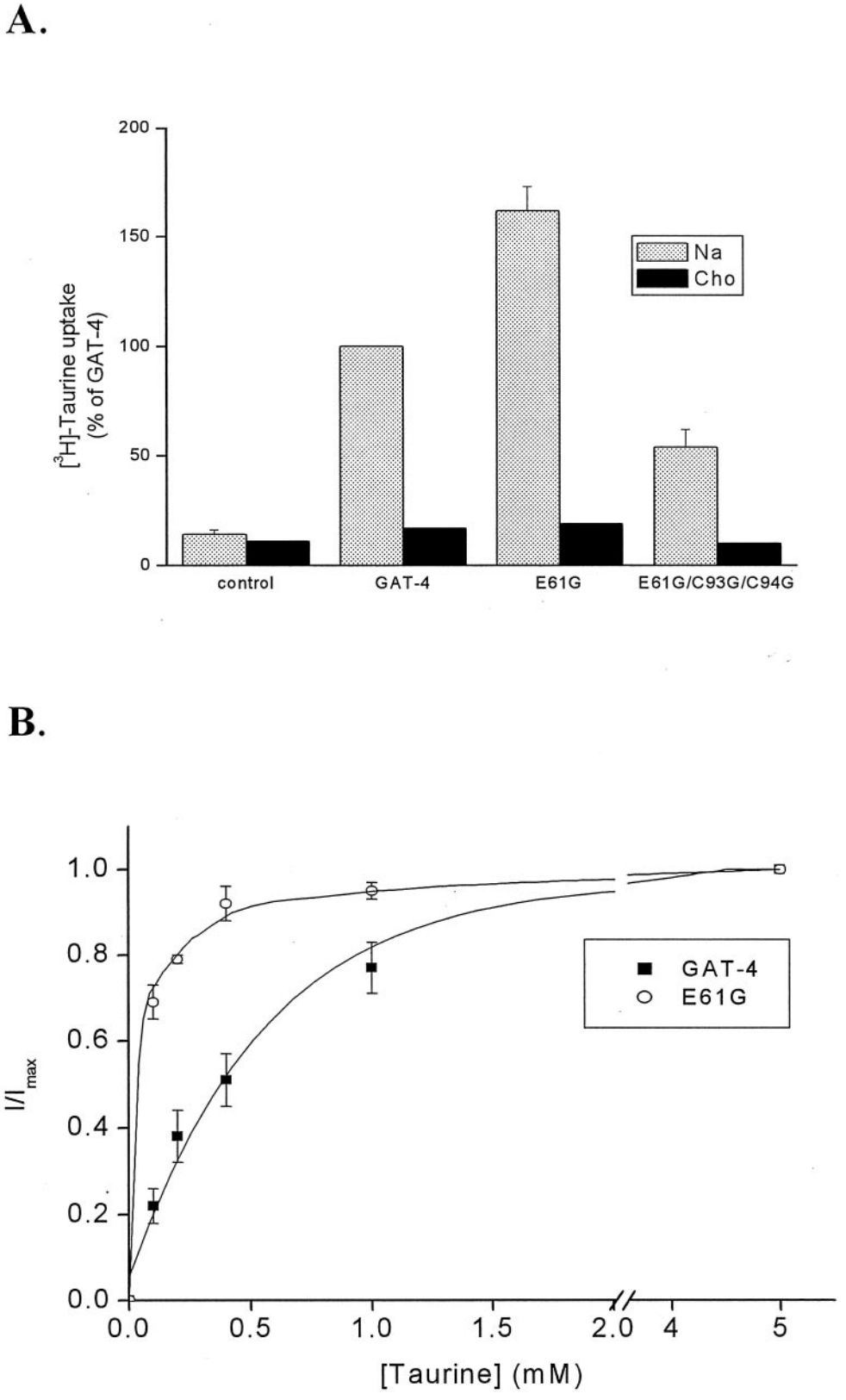

Fig. 8. Taurine uptake and taurine-induced currents. A, [^{3}H]taurine uptake in oocytes expressing GAT-4, E61G, E61G/C93G/C94G, or uninjected oocytes (control). Uptake was done in NaCl- (gray bars) or ChoCl- (dark bars) containing medium as described under *Materials and Methods*. Values are presented as mean ± S.E. of at least three experiments each done on 6 to 10 oocytes and are given as percentage of GAT-4 uptake (100% uptake reflects 390 ± 60 cpm). B, concentration dependence of taurine-induced currents in oocytes expressing GAT-4 (■) or E61G (○) was determined at −140 mV as described under *Materials and Methods*. Values are presented as mean ± S.E. of at least three oocytes and are given as percentage of I_{max} obtained at 5 mM taurine for each oocyte. The I_{max} values were similar for both GAT-4 and E61G and were in the range of 20 to 40 nA.

ine derivatives (Adkins et al., 2001). Nevertheless, in view of our observations that the sizes of the residues at positions 61 and 94 determine the inhibitory potency of taurine, it is clear that although these positions may be near the substrate binding site, they are not contact points with the substrate.

The GAT-1 counterpart of glutamate-61 of GAT-4 is tyrosine-60. This is clearly an important position of the transporter because mutation of tyrosine-60 to threonine results in a markedly reduced apparent affinity for sodium and almost completely abolishes sodium-coupled GABA flux, yet does not affect the cation leak current (Kanner, 2003). Introduction of a glutamate residue instead of tyrosine-60 of GAT-1 leads to loss of activity (Kanner, 2003), and the same is true for GAT-4 E61Y (data not shown). Thus, the precise role of tyrosine and glutamate in GAT-1 and GAT-4, respectively, remains to be clarified. One possibility is that tyrosine-60 of GAT-1 and glutamate-61 of GAT-4 limit the substrate-binding pocket through interaction with amino acid residues from other domains. In the case of GAT-4, it seems possible that replacement of glutamate by smaller residues at position 61 may enlarge the binding pocket such that the sulfonic acid group of taurine, which is larger than the carboxyl group of GABA, can now be better accommodated.

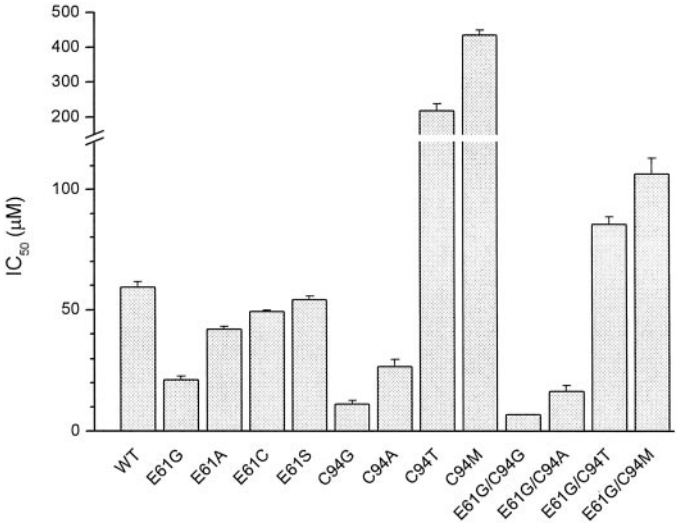

Fig. 9. Effect of substitution mutations in position 61 in TMD-I and positions 93 to 94 in TMD-II of GAT-4 on the sensitivity of [^{3}H]GABA uptake to inhibition by β-alanine. Shown are the IC_{50} values of [^{3}H]GABA uptake inhibition by unlabeled β-alanine in HeLa cells transfected with the WT GAT-4 or the indicated mutant. Uptake was done in NaCl-containing medium as described under *Materials and Methods* in the presence of increasing concentrations of unlabeled β-alanine. Values are presented as mean ± S.E. of at least three experiments done in quadruplicate.

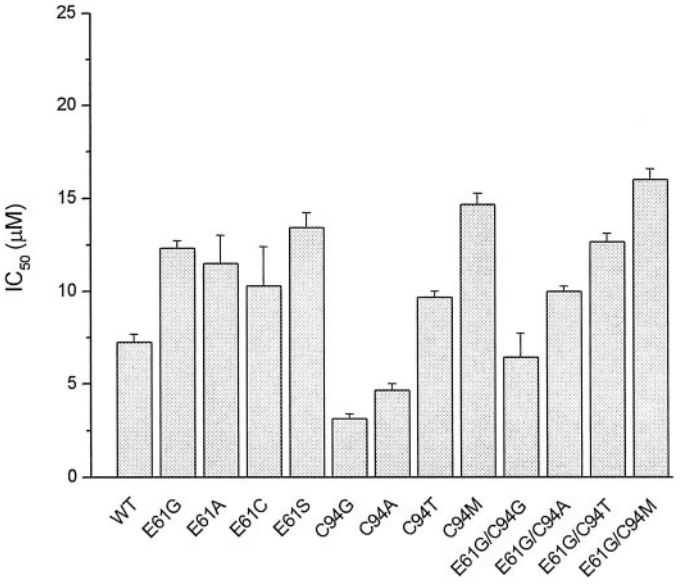

Fig. 10. Effect of substitution mutations in position 61 in TMD-I and positions 93 to 94 in TMD-II of GAT-4 on the sensitivity of [^{3}H]GABA uptake to inhibition by unlabeled GABA. Shown are the IC_{50} values of [^{3}H]GABA uptake inhibition by unlabeled GABA in HeLa cells transfected with the WT GAT-4 or the indicated mutant. Uptake was done in NaCl-containing medium as described under *Materials and Methods* in the presence of increasing concentrations of unlabeled GABA. Values are presented as mean ± S.E. of at least three experiments done in quadruplicate.

Replacement mutations at position 94 in TMD-II of GAT-4 also have an effect on the inhibitory potency of taurine, which seems to be, as for position 61, inversely related to the size of the side chain at this position (Fig. 3). This effect is potentiated when the E61G mutation is simultaneously introduced (Fig. 4). Therefore, position 94, like position 61, could be near the substrate-binding pocket. Extracellular loop I is rather short and thus it TMD-I and TMD-II may not be far from each other (Fig. 1A). If these domains are α-helical, and the helices are not tilted, positions 61 and 94 may be in fact very close (Fig. 1A). Thus, the idea that position 94 is near the substrate-binding pocket is also feasible from a structural point of view. Further support for the idea that TMD-II plays a role in substrate interactions (either direct or indirect) comes from the observation that in the taurine transporter TAUT, the K_m for taurine is also predictably influenced by the size of the side chain at this position (Fig. 7). Moreover, it has been shown recently that mutations at position 105 in TMD-II of the dopamine transporter affect the inhibitory potency of the blocker cocaine (Wu and Gu, 2003). This position is equivalent to position 91 in GAT-4. If TMD-II has an α-helical structure, this position would be near position 94, further supporting the idea that position 94 is near the substrate-binding pocket.

The impact of introducing glycines at positions 61 and 94 is specific in the sense that the effect on the inhibitory potency of taurine (Figs. 2–4 and 6) is larger than on that of β-alanine (Fig. 9) and much larger than that on GABA (Fig. 10). The explanation of these results does not seem obvious. Because taurine and β-alanine are shorter than GABA, one would intuitively expect a larger effect from increasing the size of the binding pocket on the inhibitory potency of GABA. In GAT-1, tyrosine-140 located in TMD-III is essential in GABA binding (Bismuth et al., 1997), and the analogous tyrosine-176 of SERT also seems to be crucial for serotonin binding (Chen et al., 1997). Mutation of arginine-69 in TMD-I of GAT-1 results in an impaired interaction with GABA (Kanner, 2003). Also, in the serotonin transporter, SERT, TMD-I has been implicated in substrate binding (Barker et al., 1999; Henry et al., 2003). It is also probable that in GAT-4, the residues interacting with GABA are located on TMD-I and TMD-III. The side chain of the amino acid residue at position 94 in TMD-II may force the substrate to bend around it. GABA, which is longer than β-alanine and taurine, could do this more

efficiently than the shorter substrates in wild-type GAT-4. Removal of the bulk of the side chain at position 94 would then favor binding of the shorter substrates yet would not have much effect on the ability of GABA to bind.

Even though the inhibitory potency of taurine on E61G and other GAT-4 mutants is dramatically increased, the magnitude of the currents induced by saturating taurine concentrations in E61G is similar to that in WT GAT-4 (Fig. 8B). The increased inhibitory potency of taurine on GABA transport by E61G (Fig. 2) is paralleled by the ability of lower concentrations of taurine to induce half-maximal transport currents in this mutant (Fig. 8B). The concentrations of taurine that elicited half-maximal transport currents in oocytes were 3- to 4-fold lower than the IC_{50} and K_i values for taurine on inhibition of GABA transport in HeLa cells (Figs. 2 and 5). This difference may be caused by the use of the two different expression systems. It seems that although the outward facing form of the E61G transporter is more efficient in binding taurine than the wild type, additional determinants are required to make it a more efficient taurine transporter. Such additional determinants may be required for the efficient translocation of the taurine-loaded transporter or for optimal release of taurine from the inward facing form of the transporter. Additional studies will be required to identify such determinants.

TMD-II is one of the most conserved domains in the sodium- and chloride-dependent neurotransmitter transporter family. A leucine heptad repeat in domain II has been implicated in oligomer formation during biosynthesis and subsequent targeting to the plasma membrane (Scholze et al., 2002). However, mutant transporters impaired in oligomer formation are located intracellularly but are nevertheless still functional (Scholze et al., 2002). As mentioned before, a role of TMD-II of the dopamine transporter dopamine transporter in influencing the binding pocket of the transporter has been demonstrated very recently (Wu and Gu, 2003), which is in nice agreement with our findings. This domain may be α-helical, with one face containing the heptad repeat and another face oriented toward the substrate-binding pocket. On the other hand, we cannot rule out the possibility that the effect of TMD-II on the binding pocket is via a long-range conformational effect. Further studies on the role of the amino acid residues of this domain will probably reveal additional important determinants for function of GAT-4 and other transporters of this family.

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