

# **The Glial and the Neuronal Glycine Transporters differ in their reactivity to sulfhydryl reagents.**

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## SUMMARY

The neuronal (GlyT2) and glial (GlyT1) glycine transporters, two members of the Na<sup>+</sup>/Cl<sup>-</sup> dependent neurotransmitter transporter superfamily, differ by many aspects, such as substrate specificity and Na<sup>+</sup> coupling. We have characterized under voltage-clamp their reactivity toward the membrane impermeant sulfhydryl reagent [2-(trimethylammonium)-ethyl]-methanethiosulfonate (MTSET). In *Xenopus* oocytes expressing GlyT1b, application of MTSET reduced to the same extent the Na<sup>+</sup>-dependent charge movement, the glycine-evoked current and the glycine uptake, indicating a complete inactivation of the transporters following cysteine modification. In contrast, this compound had no detectable effect on the glycine uptake and the glycine-evoked current of GlyT2a. The sensitivities to MTSET of the two transporters can be permuted by suppressing a cysteine (C62A) in the first extracellular loop (EL1) of GlyT1b and introducing one at the equivalent position in GlyT2a, either by point mutation (A223C) or by swapping the EL1 sequence (GlyT1b-EL1 and GlyT2a-EL1) resulting in AFQ ↔ CYR modification. Inactivation by MTSET was 5-times faster in GlyT2a-A223C than in GlyT2a-EL1 or GlyT1b, suggesting that the arginine in position +2 reduced the cysteine reactivity. Protection assays indicate that EL1-cysteines are less accessible in the presence of all co-transported substrates: Na<sup>+</sup>, Cl<sup>-</sup> and glycine. Application of dithioerythritol (DTE) reverses the inactivation by MTSET of the sensitive transporters. Together, these results indicate that EL1 conformation differs between GlyT1b and GlyT2a and is modified by substrate binding and translocation.

## INTRODUCTION

Na<sup>+</sup>,Cl<sup>-</sup>-coupled transporters control the extracellular and intracellular concentrations of neurotransmitters like bioamines,  $\gamma$ -aminobutyric acid (GABA) and glycine. Through the thermodynamically coupled uptake of Na<sup>+</sup>, Cl<sup>-</sup> and the transmitter, they help terminate synaptic transmission, keeping a low extracellular concentration of neurotransmitter and limiting the spill-over of neurotransmitter between neighboring synapses. At some pre-synaptic boutons, their concentrative power may also be required for an efficient supply of neurotransmitter to the low affinity vesicular transporters.

Within this superfamily, the two glycine transporters (GlyT1 and GlyT2) have distinctive properties. A well established difference concerns their substrate specificity, as sarcosine is a substrate of GlyT1b (1) but not of GlyT2a (2). Recently, other pharmacological differences have been identified: the tricyclic antidepressant amoxapine and ethanol both inhibit selectively GlyT2a (3,4). Electrophysiological characterization of the two transporters has revealed additional features differentiating GlyT2a from GlyT1b: (i) a stoichiometry of 3 Na<sup>+</sup>/ 1 Cl<sup>-</sup>/glycine compared to only 2 Na<sup>+</sup>/1 Cl<sup>-</sup>/glycine for GlyT1b (resulting in a charge to glycine flux ratio of a different charge coupling of  $\sim$ 2 for GlyT2a vs. 1 for GlyT1b) (5); (ii) a reduced capacity for reverse uptake (5); (iii) an uncoupled, outwardly-rectifying Cl<sup>-</sup> conductance (Roux M.J. and S. Supplisson, submitted); (iv) a bi-exponential transient current recorded in the absence of glycine (Roux and Supplisson, unpublished observations). The amino-acids involved in these differences between GlyT1b and GlyT2a have not been identified yet.

Sequence comparison of EL1 in the Na<sup>+</sup>, Cl<sup>-</sup> coupled transporter superfamily shows that GlyT2a together with PROT (6) and ATB<sup>0,+</sup> (7) are the only members that

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lack a conserved cysteine in EL1 that has been identified in GAT-1 (C74, (8)) and SERT (C109, (9)) as a primary target for the membrane-impermeant sulfhydryl reagent MTSET. Mutation in GlyT2a first extracellular loop (EL1) induced an increase in the outwardly rectifying leak current characteristic of this transporter (Roux M.J. and S. Supplisson, submitted), which further suggested that the conformation of this loop may differ between GlyT1b and GlyT2a. To assess this issue, we tested the reactivity to sulfhydryl reagents of GlyT1b and GlyT2a.

Transporter inhibition by MTSET required prolonged application and high concentrations of this reagent, which suggests a low accessibility of the reactive cysteine either because it is partially buried inside the protein or because it is infrequently exposed to the surface (8). In fact, the orientation of EL1 has been a matter of debate (8,10-12). The original topological model proposed for the GABA transporter GAT-1 (13) predicted cytoplasmic N- and C- termini and 12 transmembrane segments (TM1-12) linked by 6 extracellular loops (EL1-6) and 5 intracellular loops (IL1-5). In accordance to this model, most positions in EL1 were found to be accessible to extracellularly applied MTSET when mutated to cysteine in GAT-1 (8). In addition, MTSET increased dramatically the oocyte leak current (8). However, inhibition by MTSET was not detected in uptake experiments in GAT-1 transfected mammalian cells (10), while the inactivation produced by the membrane permeant (2-aminoethyl)methane thiosulfonate (MTSEA) was attributed to cysteine 399, located in IL4 (14).

We characterized under voltage-clamp the effects of MTSET and (2-sulfonatoethyl) methanethiosulfonate (MTSES) on the glycine uptake, the glycine-evoked current and the charge movement of the two wild-type glycine transporters, as well as on those of two point-mutants of the first amino acid of EL1, GlyT1b-C62A

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and GlyT2a-A223C, that exchanged the MTSET reactivity. The difference in residue number for an equivalent position in EL1 sequence is explained by the much longer N-terminal of GlyT2a (15). In addition, the GlyT2a-EL1 chimera resulting from the mutation of the first three amino acids of GlyT2a-EL1 (AFQ) to the GlyT1b sequence (CYR) was studied in detail because it influenced MTSET reactivity and had a dramatic effect on the outward Cl<sup>-</sup> current specific of GlyT2a (Roux M.J. and S. Supplisson, submitted).

## EXPERIMENTAL PROCEDURES

*Site-directed mutagenesis, RNA synthesis and expression* – Rat GlyT1b (gift from K. Smith, Synaptic Corporation (16)) and rat GlyT2a (15) cDNAs were subcloned as indicated in (1). Point mutations were performed using the method of (17). cRNAs were transcribed *in vitro* using the T7 mMessage-mMachine kit (Ambion, Austin, TX) and 10-100 ng were injected into oocytes using a nanoliter injector (World Precision Instruments, Sarasota, FL). *Xenopus laevis* oocytes were prepared and incubated as indicated in (5).

*Electrophysiology* – Two-electrode voltage-clamp was performed using a O-725A amplifier (Warner Instrument, Hamden, CT), a Digidata 1200 interface and the pClamp7 software (Axon Instruments, Foster City, CA). Glass electrodes were filled with a 3 M KCl solution and had a resistance of 0.5 – 2 M $\Omega$ . The typical extracellular recording Ringer solution contained (in mM): 100 NaCl, 1.8 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 5 HEPES, pH 7.2 adjusted with KOH. In some experiments, choline and gluconate were substituted for Na<sup>+</sup> and Cl<sup>-</sup>, respectively. Agar bridges (equilibrated in a 3 M K gluconate solution) were used when required. Salts and glycine were purchased from SIGMA (St Louis, MO). Aliquots of dithioerythritol (300 mM, SIGMA), MTSET and MTSES (100 mM, Toronto Research Chemical Co., Toronto, Canada) were

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prepared in ice-cold water and kept at -20 °C. They were thawed and diluted in the appropriate solution just prior to perfusion (< 10 seconds). Data are given as mean  $\pm$  S.E.M. with the number of experiments.

*Charge movement* - Transient currents specific to GlyT1b or GlyT2a were evoked in the absence of glycine by stepping the voltage at various potentials. These currents are Na<sup>+</sup>-dependent and capacitative in nature since their time-integrals estimated following the onset and return from the voltage step are equal in amplitude but opposite in sign. For GlyT1b, the charge movement was fitted with a single exponential function of the current decay upon repolarization. For GlyT2a and GlyT2a-EL1, the charge movement could not be fitted with a single exponential and was quantified by integration of the transient currents recorded during the membrane repolarization to the holding potential (OFF response) after subtraction of the current recorded in absence of Na<sup>+</sup>. The charge distribution as a function of membrane

potential was fitted with a Boltzmann distribution 
$$Q(V_m) = \frac{Q_{\max}}{1 + \exp\left(\frac{z\delta(V_{1/2} - V_m)F}{RT}\right)} + Cte,$$

where  $V_{1/2}$  is the half-distribution potential,  $z\delta$  the equivalent charge per transporter moving through the membrane electric field and R, T and F have their usual meanings.  $Q_{\max}$  is the total charge movement and is defined as  $Q_{\max} = z\delta F N_T$ , where  $N_T$  is the number of transporters per oocyte.

*Glycine uptake assays* – Oocytes (n = 5-15) expressing wild-type glycine transporters or various EL1 constructs were incubated for 10 minutes in 5 ml CholineCl Ringer in the presence or absence of 2 mM MTSET. Then, oocytes were incubated for 10 minutes in control NaCl Ringer or in CholineCl Ringer containing 5  $\mu$ M of [U-<sup>14</sup>C] glycine (specific activity of 103  $\mu$ Ci/ $\mu$ mole, Amersham Pharmacia Biotech, France). Oocytes were washed three times in ice-cold NaCl Ringer solution

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and placed individually in vials containing 500  $\mu$ l SDS (2%). The radioactivity was measured on a Kontron liquid scintillation counter.

*Charge coupling* - Oocytes were voltage-clamped at  $-40$ mV and perfused for 60 to 90 s with a solution containing 25  $\mu$ M of [U- $^{14}$ C] glycine and 75  $\mu$ M of cold glycine. After complete wash-out and the current back to the baseline level, oocytes were transferred in 5 ml ice-cold Ringer solution and washed twice, then lysed in vials containing 500  $\mu$ l SDS (2%) and measured for radioactivity. The charge coupling was determined as the ratio between the glycine-evoked current integral and the glycine uptake as described in (5).

## RESULTS

### *Transfer of EL1 confers MTSET sensitivity from GlyT1b to GlyT2a*

The glycine transporters GlyT1b and GlyT2a, expressed in *Xenopus* oocytes, showed differential sensitivities to MTSET. The glycine-evoked current ( $I_T$ ) (Fig. 1a) and the transient currents (Fig. 1b) recorded in the absence of glycine in GlyT1b<sup>+</sup> oocytes were strongly and irreversibly reduced following a five-minute superfusion with choline-Cl Ringer containing 1 mM MTSET. In contrast, similar applications of MTSET affected neither  $I_T$  (Fig. 1c) nor the transient currents nor the outward rectifying leak conductance (Fig. 1d) recorded in GlyT2a<sup>+</sup> oocytes. To test if these differences could result from the presence (GlyT1b) or the absence (GlyT2a) of a cysteine in the first extracellular loop (EL1), as suggested by results obtained on GAT-1 (8) and SERT (9), we applied MTSET both on the point mutant GlyT2a-A223C (Fig. 1e,f) and on the GlyT2a-EL1 chimera (Fig. 1g,h) in which the AFQ sequence of GlyT2a was replaced by the GlyT1b sequence CYR (see Table 1 for sequence comparison). With GlyT2a-A223C and GlyT2a-EL1, application of MTSET

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reduced  $I_T$  (Fig. 1e,g), the transient currents and the outwardly-rectifying leak conductance (Fig. 1f,h). However, the inhibition of GlyT2a-EL1 was partial, as for GlyT1b, while GlyT2a-A223C was fully inhibited under the same conditions.

### *MTSET and leak current*

MTSET did not change the leak current in oocytes expressing either GlyT1b (Fig. 1b) or GlyT2a (Fig. 1d) but it affected the leak current of GlyT2a-A223C and GlyT2a-EL1. Like GlyT2a, both of these constructs exhibit an outwardly-rectifying "leak" conductance at depolarized potentials, which is particularly large in the case of GlyT2a-EL1 and that is described in detail in Roux and Supplisson (submitted). While this outward "leak" conductance was not changed by MTSET in GlyT2a (Fig. 1d), it was suppressed in both GlyT2a-A223C and GlyT2a-EL1 (Fig. 1f,h). In contrast, we observed after MTSET treatment a limited increase in the inward steady-state leak current at hyperpolarized potential for these two constructs (Fig. 1f,h).

### *MTSET blocks the uptake current and the charge movement in the same proportion*

A five-minute application of MTSET reduced the charge movement of GlyT1b (Fig. 2a), GlyT2a-EL1 (Fig. 2e) and GlyT2a-A223C (Fig. 2g) but not of GlyT2a (Fig. 2c). The reduction in  $Q_{\max}$  (see table 2), which is proportional to the number of functional transporters present in the plasma membrane (see methods), indicates that the loss of transport activity observed after cysteine modification by MTSET, results from the hindrance of a kinetic step which precedes substrate binding and translocation since the charge movement of glycine transporters corresponds to  $\text{Na}^+$ - and voltage-dependent transitions that occur in the absence of glycine ((5). The  $\text{Na}^+$ -dependency of the transient currents for oocytes expressing GlyT1b, GlyT2a, GlyT2a-EL1 and GlyT2a-A223C is shown in Figure 3 with the unsubtracted OFF-currents recorded

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during the repolarization step to the holding potential in NaCl Ringer, CholineCl Ringer and after MTSET application.

In the potential range from -140 to +50 mV, the  $I_T$  – voltage relationships of GlyT1b, GlyT2a and GlyT2a-A223C (Fig. 2*b,d,g*) were quasi-linear while that of GlyT2a-EL1 was inwardly rectifying (Fig. 2*f*). For comparison,  $I_T$  values were normalized to the  $Q_{\max}$  determined before MTSET treatment. The transport currents were reduced in the same proportion at all potentials by MTSET for GlyT1b, GlyT2a-EL1 and GlyT2a-A223C transporters, as shown in Figure 2 (*b, f, h*). For each oocyte, the percentage of the remaining transport current was subsequently calculated as the mean of the experimental values between -140 and 0 mV (every 10 mV). Values obtained at voltages above 0 mV were ignored because of their small amplitudes (especially for GlyT2a-EL1). The reduction in  $I_T$  after MTSET treatment was comparable to the reduction observed in  $Q_{\max}$  as indicated in Table 2.

As expected for an inactivation process, the glycine  $EC_{50}$  was not changed by MTSET application for the two transporters tested, GlyT1b ( $22.5 \pm 1.10 \mu\text{M}$  ( $n = 7$ ) and  $19.5 \pm 1.4 \mu\text{M}$  ( $n = 3$ ) before and after MTSET application respectively) and GlyT2a-EL1 ( $24.1 \pm 3.1 \mu\text{M}$  ( $n = 3$ ) and  $23.2 \pm 1.8 \mu\text{M}$  ( $n=3$ ) before and after MTSET application respectively).

### *EL1 sequence influences the cysteine accessibility*

To compare the accessibility of the EL1 cysteine for the various sensitive constructs, we studied the time course of the MTSET inhibition. The progressive decrease in uptake current plotted as a function of the cumulative exposure to MTSET is shown in Figure 4. The decay in current had a time constant of 3.8 min and 3.9 min for GlyT1b and GlyT2a-EL1 respectively, but was 5-fold faster with GlyT2a-A223C, with a time constant of 0.67 min. The difference in the time

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constants indicates that in glycine transporters, the accessibility of the EL1-cysteine to the extracellular solution and its reactivity to MTSET are strongly influenced by the next two residues that are modified in GlyT2a (FQ vs. YR in GlyT1b).

To confirm that EL1 was fully responsible for GlyT1b sensitivity to MTSET, we constructed the reverse chimera GlyT1b-EL1. For this transporter, no change in the amplitude of glycine-evoked current was observed for period of incubation up to 20 minutes (Fig. 4).

### *MTSET have the same effect on glycine uptake and glycine-evoked current*

To verify that the reduction of the glycine-evoked current by MTSET was reflecting a comparable change in the influx rate of glycine in oocytes expressing sensitive glycine transporters, we repeated the MTSET inactivation protocol using tracer flux of glycine. A summary of these uptake experiments is shown in Figure 5A, that confirms the large reduction of the Na<sup>+</sup>-dependent glycine uptake with oocytes expressing GlyT1b, GlyT2a-EL1 and GlyT2a-A223C. No significant inhibition by MTSET are observed with GlyT2a, GlyT1b-EL1 and the point mutant GlyT1b-C62A.

Finally, we checked the charge coupling of each transporter by applying radiolabelled glycine under voltage-clamp condition as described in (5). The results shown in Figure 5B indicate that the specific charge coupling of the wild-type transporters (1 and ~2 for GlyT1b and GlyT2a respectively) are not affected by EL1 sequence modification, and that glycine-evoked current is proportional to glycine uptake.

### *Na<sup>+</sup>, Cl<sup>-</sup> and glycine limit EL1-cysteine accessibility*

MTSET applications (1 mM during 5 minutes at -40 mV) were performed on GlyT1b, GlyT2a-EL1 and GlyT2-A223C in the presence or absence of the transporter substrates to see if these substrates protected against MTSET. As described in

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Figure 6, inhibition by MTSET was reduced by the joint presence of Na<sup>+</sup> and Cl<sup>-</sup>, and was further reduced by glycine. Sarcosine, a specific substrate of GlyT1b, protected this transporter to a similar extent as glycine, but did not protect GlyT2a-EL1. In all transporters tested, Na<sup>+</sup>, Cl<sup>-</sup> or glycine alone did not induce any protection (Fig. 6). This is in good agreement with the fact that neither the uptake current nor the charge movement can be observed when one of the co-transported ions is absent ((5) and unpublished observations). The best protection was observed for GlyT2a-EL1, for which a quasi-complete protection by NaCl + glycine was found.

### *Voltage-dependence of MTSET and MTSES reactivity on EL1-cysteine*

GlyT1b oocytes were perfused with 1 mM MTSET for 5 minutes in cholineCl Ringer at holding potentials between -120 mV and 0 mV. Inhibition increased with hyperpolarization, from  $62.3 \pm 5.2\%$  (n = 4) at 0 mV to  $84.4 \pm 0.9\%$  (n = 4) at -120 mV (Fig. 7A). To test if this voltage-dependence was due to a voltage-dependent transition of the transporter in the absence of Na<sup>+</sup> or to the positive charge of MTSET, we repeated the experiments using the negatively charged MTSES. Figure 7A shows that MTSES inhibition of GlyT1b had the same sign of voltage-dependence as MTSET, with the inhibition increasing from  $51.5 \pm 1.3\%$  at 0 mV to  $79.0 \pm 0.9\%$  at -120 mV.

The inhibition of GlyT2a-EL1 by MTSET or MTSES was not voltage-dependent (Fig. 7B). In addition, the potency of MTSES on GlyT2a-EL1 and GlyT2a-A223C (Fig. 7C) was much lower than that of MTSET, as expected from their reported relative reactivity (18). This contrasts with the fact that the two reagents inhibited GlyT1b with comparable potencies (Fig. 7A).

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*Inactivation of Glycine Transporters by MTSET can be reversed with DTE.*

Covalent modification of cysteine by MTSET produced an inactivation of sensitive glycine transporters which contain a cysteine in their EL1 sequence. This inactivation persisted after complete wash-out of MTSET (Fig. 8B). We tested if the application of a reducing agent like DTE may reverse the alkylation by MTS reagents, as reported for the glucose transporter SGLT1 (19). The data, shown in Figure 8 indicate that superfusion with DTE (5 min, 10 mM in CholineCl Ringer) restored partially or totally the glycine-evoked current, with a more dramatic effect with GlyT2a-A223C (Fig. 8). In addition, the small increase in leak current observed with GlyT2a-EL1 (Fig. 1h) and GlyT2a-A223C (Fig. 1f and 8A-B) was reversed by DTE.

## DISCUSSION

The topological organization of the Na<sup>+</sup>, Cl<sup>-</sup> neurotransmitter transporter family has now received good experimental support in favor of the original model of 12 putative transmembrane segments proposed for GAT-1 (13). Nevertheless the precise organization of the first third of the protein, which contains amino acids critical for function, is still debated (8-12,14,20,21). Recent insight into the localization of the loops and the accessibility of membrane segments has been obtained (8,9,12,14) through the identification of solvent-accessible residues using the substituted-cysteine accessibility method (SCAM, see review in (18)). This method has been extensively applied to determine the residues lining the pore of ionic channels and receptors (22-25) and to map the ligand-binding crevice in G-protein coupled receptors (26).

Glycine transporters are good candidates for SCAM because one of them (GlyT2a) is totally insensitive to MTS reagents in uptake assays and voltage-clamp studies thus allowing further structure/function characterization without the need for a

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cysteine-less mutant (18). Sequence comparison reveals that GlyT2a lacks the well-conserved cysteine in EL1 that is present in GlyT1b and to which MTSET sensitivity was attributed in GAT-1 (8) and SERT (9). This cysteine substitution (A223) is the first of three consecutive differences between GlyT1b (CYR) and GlyT2a (AFQ) within a stretch of 25 otherwise conserved amino-acids in the TM1-TM2 region (table 1) which is known to be critical for function (27-30).

We decided to probe EL1 accessibility and function in glycine transporters combining glycine uptake assay and transport current recordings. We have shown previously that charge and glycine uptake are precisely correlated in oocytes expressing GlyT1b (+1 elementary charge (e)/glycine) and GlyT2a (+~2 e/glycine) (5) and that the two transporters differ notably in their pre-steady state kinetics and leak current. The interest of a dual approach was reinforced by the fact that conflicting results have been reported on GAT-1 concerning MTSET accessibility (8,10,14).

We report here that the first amino-acid of EL1 (C62) in GlyT1b which is also found in GAT-1 (C74), SERT (C109) and DAT (C90), is accessible to extracellularly applied MTSET and MTSES. All transporter-associated currents (pre-steady-state and uptake) were blocked similarly after cysteine modification by MTS reagents. In contrast, we found that none of the electrophysiological properties (glycine-evoked current, charge movement, leak current) of the wild-type GlyT2a were affected by MTSET. Introduction of a cysteine (A223C), either by point mutation or by replacing EL1 with the GlyT1b sequence, conferred MTS reagent sensitivity.

Previous results on the cysteines of Na<sup>+</sup>/Cl<sup>-</sup> coupled transporters involved high concentrations of MTS reagents ( $\geq 1$  mM) and prolonged applications (5 to 10 minutes in most cases) to achieve only partial inhibition (8-10,12,14). Similarly, the reduction of uptake current of the wild-type GlyT1b was only partial after 5-minute

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exposure to MTSET (1 mM). This partial inhibition resulted from a loss of function of the fraction of transporters which had a modified cysteine. Complete inhibition of GlyT1b and GlyT2a-EL1 required  $\geq 20$  min incubation with MTSET, while GlyT2a-A223C was fully inhibited in about 4 minutes. Rate constants estimated from the time constants of the inhibition (18) were in the order of  $4.4 \text{ M}^{-1} \text{ s}^{-1}$  for GlyT1b and GlyT2a-EL1 and of  $25 \text{ M}^{-1} \text{ s}^{-1}$  for GlyT2a-A223C. These rate constants are many orders of magnitude slower than for the reaction of MTSET with 2-Mercaptoethanol in solution (18) or for exposed cysteines near the entrance of the pore in acetylcholine receptor (31). Nevertheless, these rate constants are in the range reported for cysteines located near the cytoplasmic end of the acetylcholine receptor pore in the absence of ligand (31). This indicates that even in the more reactive GlyT2a-A223C the EL1-cysteine is exposed only transiently to the extracellular solution or is located in a diffusion-limiting crevice.

The rate of inactivation was influenced by the two other non-conserved residues of EL1. Differences in reactivity may be attributed to the presence (GlyT1b, GlyT2a-EL1) or absence (GlyT2a-A223C) of the positive charge of an arginine in position  $n+2$ . GAT-1, which has a lysine in the equivalent position, is inhibited by MTSET with a time-course (8) comparable to GlyT1b and GlyT2a-EL1. This positive charge may also be responsible for the similar rate of inhibition by MTSET and MTSES on GlyT1b, which contrasts with the  $> 10$ -fold difference in reactivity in solution between these two compounds (18). Comparable changes in reactivity ratio have been observed in the case of the CFTR channel (32), for which residues located deep inside the pore (T351, Q353) show a higher reactivity for MTSES than MTSET. In this channel, the arginine at position 352 is considered as a key residue for anion

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selectivity (32). In the wild-type GlyT1b, R64 may facilitate the access to C62 for the negatively charged but less reactive MTSES.

The orientation of the arginine, in addition to its charge, plays a role in controlling the access to EL1-cysteine, as GlyT2a-EL1 was much less sensitive to MTSES than to MTSET (by a factor of ~5, as estimated from the inhibition after 5 minutes). In addition, position 225 of GlyT2a (an arginine in GlyT2a-EL1) is not accessible to extracellularly-applied MTSET (López-Corcuera B., R. Martínez-Maza, E. Núñez, A. Geerlings and C. Aragón, submitted). Together, these results suggest that EL1 has a different conformation in GlyT1b and GlyT2a.

Another difference between the two transporters was that membrane potential influenced the MTS inhibition of GlyT1b but not of GlyT2a-EL1 in the absence of external  $\text{Na}^+$ . Because the voltage-dependence of MTSET and MTSES inhibition of GlyT1b were similar, a role of the reagent charge could be ruled out. Some voltage-dependent transition in the transporter must favor EL1 cysteine exposure at negative potentials. This transition is unlikely to correspond to extracellular  $\text{Cl}^-$  binding to the transporter, as similar inhibition levels were achieved in choline- $\text{Cl}^-$  and choline-gluconate solutions at -40 mV. Though glycine transporters capacitive currents are  $\text{Na}^+$ -dependent as are those of other  $\text{Na}^+$ -coupled transporters, around 20% of GlyT1b charge movement persist after  $\text{Na}^+$  removal (choline substitution) from the extracellular solution (Roux and Supplisson, unpublished observations). Such residual charge movement is not observed in GlyT2a or GlyT2a-EL1, consistent with the fact that no voltage-dependence was observed for GlyT2a-EL1 inhibition by MTSET or MTSES. GlyT1b probably has either endogenous charges or ions bound to intracellular sites and can undergo voltage-dependent transitions even in the absence of ions bound to the extracellular sites.

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The presence of both Na<sup>+</sup> and Cl<sup>-</sup> in the extracellular medium, which are required for the charge movement observed in response to voltage jumps in the absence of glycine, partially protected the three MTSET sensitive transporters GlyT1b, GlyT2a-A223C and GlyT2a-EL1. Na<sup>+</sup> or Cl<sup>-</sup> alone (choline or gluconate substitution) did not protect, suggesting a strong allosteric interaction between the Na<sup>+</sup> and Cl<sup>-</sup> binding sites. The addition of glycine to Na<sup>+</sup> and Cl<sup>-</sup> further protected both GlyT1b and GlyT2a-EL1. Altogether, these results indicate that the conformational changes occurring in the transport cycle modify the EL1-cysteine accessibility. In addition, the conformation of this loop appears to differ between GlyT1b and GlyT2a, may be as a consequence of the structural constraints imposed by the extra Na<sup>+</sup> binding site of GlyT2a.

## FIGURE LEGENDS

FIG.1. **Glycine-evoked and glycine-independent currents prior to and after MTSET application** for GlyT1b, GlyT2a, GlyT2a-A223C and GlyT2a-EL1. (a, c, e, g) Current evoked by 200  $\mu$ M glycine in NaCl Ringer (gray bar) at -40 mV prior to (thin line) and after (thick line) MTSET application (1 mM MTSET in choline-Cl Ringer for 5 minutes, -40 mV holding potential). The horizontal and vertical scale bars are 10 sec and 75 nA respectively. (b, d, f, h) Currents evoked by voltage-jumps to potentials between +50 mV and -130 mV from a holding potential of -40 mV in NaCl Ringer prior to and after MTSET application (same conditions as above). Horizontal and vertical scale bars are 50 ms and 250 nA respectively.

FIG.2. **Q-V<sub>m</sub> and I<sub>T</sub>-V<sub>m</sub> relationships** for GlyT1b (a, b), GlyT2a (c, d), GlyT2a-EL1 (e, f) and GlyT2a-A223C (g, h) before (●) and after (○) MTSET application (1 mM MTSET). Charge was measured in NaCl Ringer, I<sub>T</sub> in NaCl Ringer + 200  $\mu$ M glycine. For each oocyte, data were normalized to the Q<sub>max</sub> determined prior to MTSET application (n = 4 to 5).

FIG.3. **Na<sup>+</sup>-dependent charge movement.** Unsubtracted OFF currents for GlyT1b, GlyT2a, GlyT2a-EL12 and GlyT2a-A223C recorded during the repolarization step to the holding potential (-40 mV) from +50 mV to -130 mV, in the presence of NaCl, CholineCl and after exposure to MTSET (1mM).

FIG.4. **Time-course of MTSET inhibition.** Plot of the glycine-evoked current for GlyT1b (●), GlyT1b-EL1 (▼), GlyT2a-EL1 (△) and GlyT2a-A223C (○) (200  $\mu$ M

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glycine in NaCl Ringer) as the function of the time of exposure to 1 mM MTSET in cholineCl Ringer solution (-40 mV holding potential) (n = 3 to 5). Solid lines are fits to the data with single-exponential functions.

**FIG.5. Effect of MTSET on glycine uptake.** (A) Glycine uptake was performed as described in Material and Methods with oocytes expressing GlyT1b, GlyT1b-EL1, GlyT1b-C62A, GlyT2a, GlyT2a-EL1 or GlyT2a-A223C. Oocytes were pre-treated 10 min in cholineCl Ringer with or without MTSET (2 mM). Oocytes were incubated for 10 minutes in 5 ml control NaCl Ringer or cholineCl Ringer solution containing 5  $\mu$ M U-<sup>14</sup>C glycine. (B) Charge coupling was estimated as the ratio between the time-integral of the current evoked by the application (60 s to 90 s) of 25  $\mu$ M U-<sup>14</sup>C glycine with 75  $\mu$ M cold glycine and the simultaneous glycine uptake (see methods).

**FIG.6. Protection assays.** Normalized glycine-evoked current after MTSET application (5 minutes, 1mM, -40 mV) in the presence or absence of the ionic or organic substrates for GlyT1b, GlyT2a-EL1 and GlyT2a-A223C. For each transporter, all conditions that are not significantly different ( $p < 0.02$ ) from our control conditions (choline-Cl) are represented in light color (gray or white bars), whereas conditions that correspond to a significant protection compared to control are represented as black bars (n = 4 to 5). n.d. for not done.

**FIG.7. Voltage dependence of the MTSET and MTSES Inhibition.** Normalized GlyT1b (A) and GlyT2a-EL1 (B) remaining glycine-evoked currents (200  $\mu$ M, NaCl Ringer) after MTSET (○) or MTSES (▲) application as a function of the membrane potential during MTS reagents application. MTS reagents were applied for 5

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minutes at 1 mM during voltage-clamp at the indicated membrane potential in choline-Cl Ringer. **(C)** Similar results for GlyT2a-A223C for a holding potential of -40 mV (n = 4 or 5 for all conditions).

**FIG.8. Glycine transporter inactivation by MTSET is reversed by DTE.** **(A)** representative traces of glycine (100  $\mu$ M) evoked current for GlyT1b (a-c) and GlyT2a-A223C (d-f) recorded in control Ringer (a, d), after 5 minutes applications of MTSET (1 mM) in cholineCl Ringer (b, e) and after 5 minutes application of DTE (10 mM) in cholineCl Ringer (c, f). The dotted line represents the initial baseline level. **(B)** Representative experiment with GlyT2-A223C showing the baseline current ( $\circ$ ) and the steady-state glycine evoked current ( $\blacktriangle$ ) recorded every minute for a 10 s application of glycine (100  $\mu$ M). No current were recorded during the application of MTSET and DTE in cholineCl Ringer. **(C)** Normalized glycine uptake current in control NaCl Ringer (open bar), after 5 minutes application of 1 mM MTSET (closed bar) or after 5 minutes application of 10 mM DTE (gray bar).

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## **ABBREVIATIONS**

EL1: extracellular loop 1

$I_T$ : glycine-evoked current

MTSEA: (2-aminoethyl) methanethiosulfonate

MTSES: (2-sulfonatoethyl) methanethiosulfonate

MTSET: [2-(trimethylammonium)-ethyl]-methanethiosulfonate

DTE: Dithioerythritol

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We thank Philippe Ascher and Boris Barbour for critical comments and suggestions on the manuscript.

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**Table 1. Sequence alignment of the TM1-TM2 region of wild-type and mutant glycine transporters**

	TM1	EL1	TM2	
40	EFVLTSVG <sup>Y</sup> AVGLGNVWRF <sup>PYL</sup>	CYR <sup>NGG</sup>	GAFMFPYFI	<b>GlyT1b</b>
40	.....	AFQ.....		<b>GlyT1b-C62A</b>
40	.....	AYR.....		<b>GlyT1b-EL1</b>
201	D.I.SM.....	AFQ.....	LI..LM	<b>GlyT2a</b>
201	D.I.SM.....	CFQ.....	LI..LM	<b>GlyT2a-A223C</b>
201	D.I.SM.....	CYR.....	LI..LM	<b>GlyT2a-EL1</b>

All amino acids in the variable region of EL1 (box) are shown. The putative TM regions are shown in gray.

**Table 2. Percentage of reduction of Qmax and I<sub>max</sub> induced by 1mM MTSET for 5 min in oocytes expressing wild type and EL1 mutant of glycine transporters**

	GlyT1b	GlyT2a	GlyT2a-EL1	GlyT2a-A223C
<b>Charge Movement</b>				
V <sub>1/2</sub> (mV)	-19.7 ± 5.2	-35.7 ± 1.5	-41.3 ± 0.8	-22.5 ± 1.4
zδ	0.55 ± 0.04	1.48 ± 0.02	1.46 ± 0.08	1.6 ± 0.0
% reduction of Q <sub>max</sub> by MTSET	64.0 ± 2.9	9.0 ± 1.0	60.6 ± 2.4	98.0 ± 0.7
<b>Uptake current</b>				
% reduction of I <sub>max</sub> by MTSET	70.8 ± 2.0	8.5 ± 1.2	67.7 ± 1.0	97.6 ± 0.1
n	4	4	4	4

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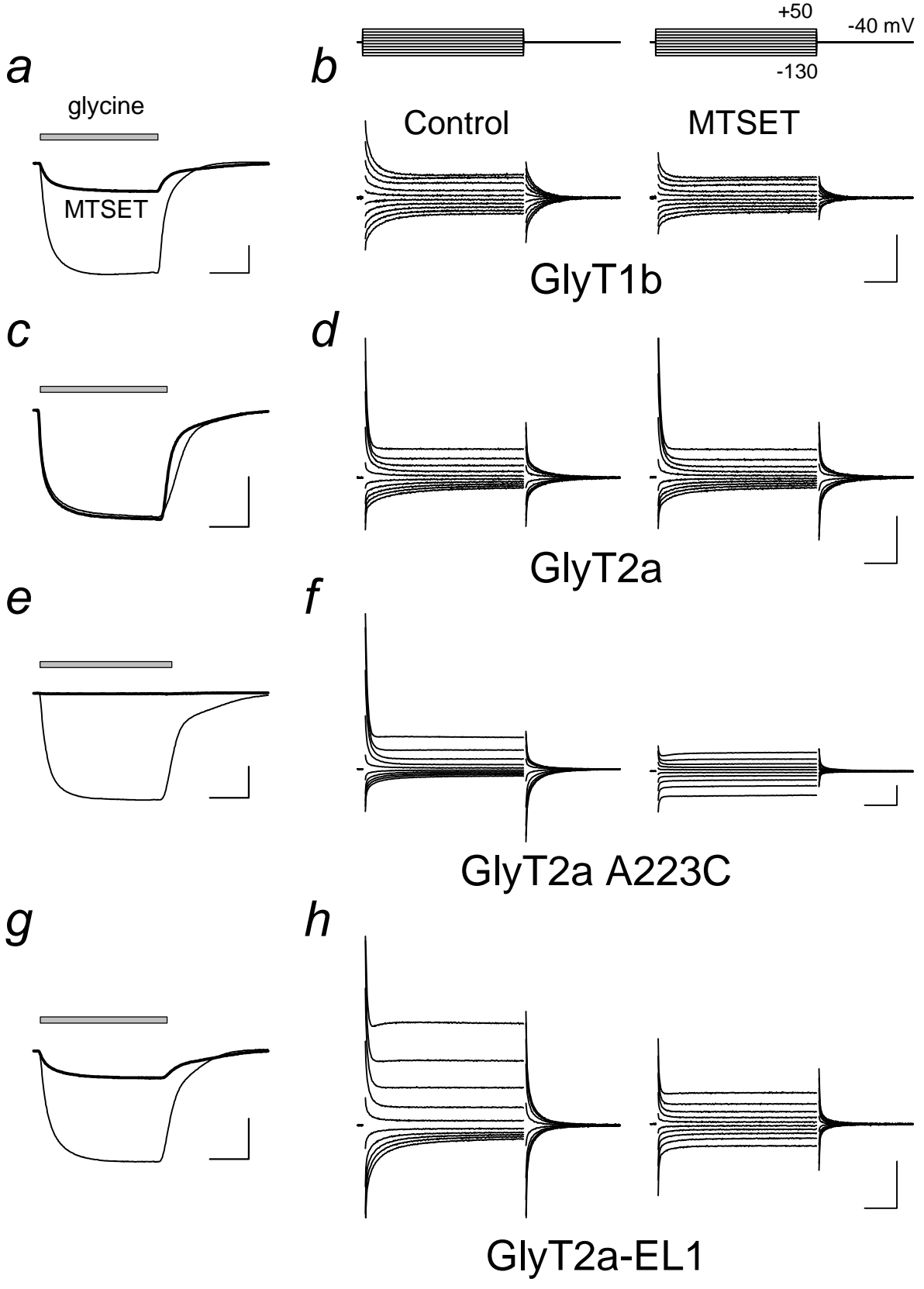


Figure 1

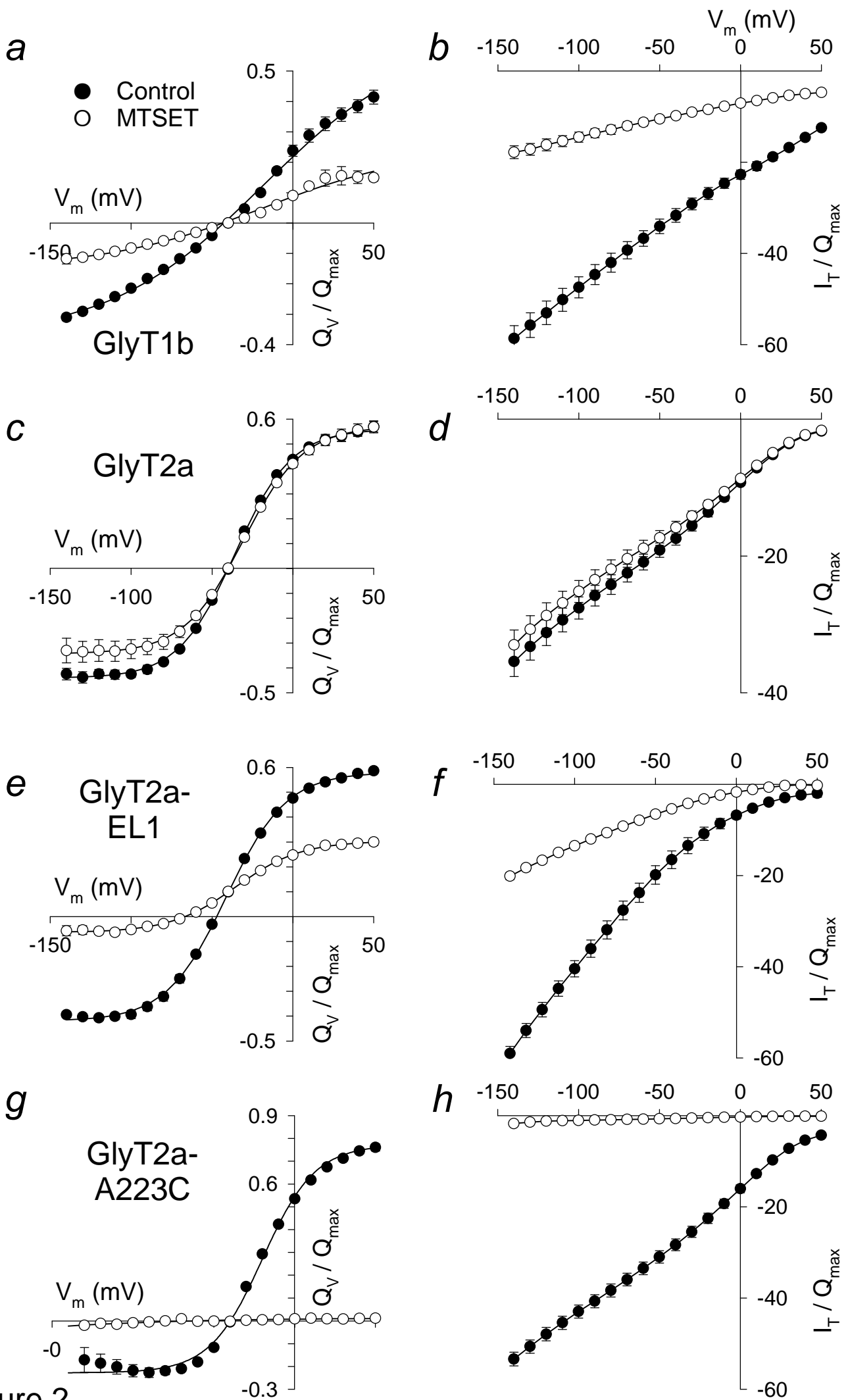


Figure 2

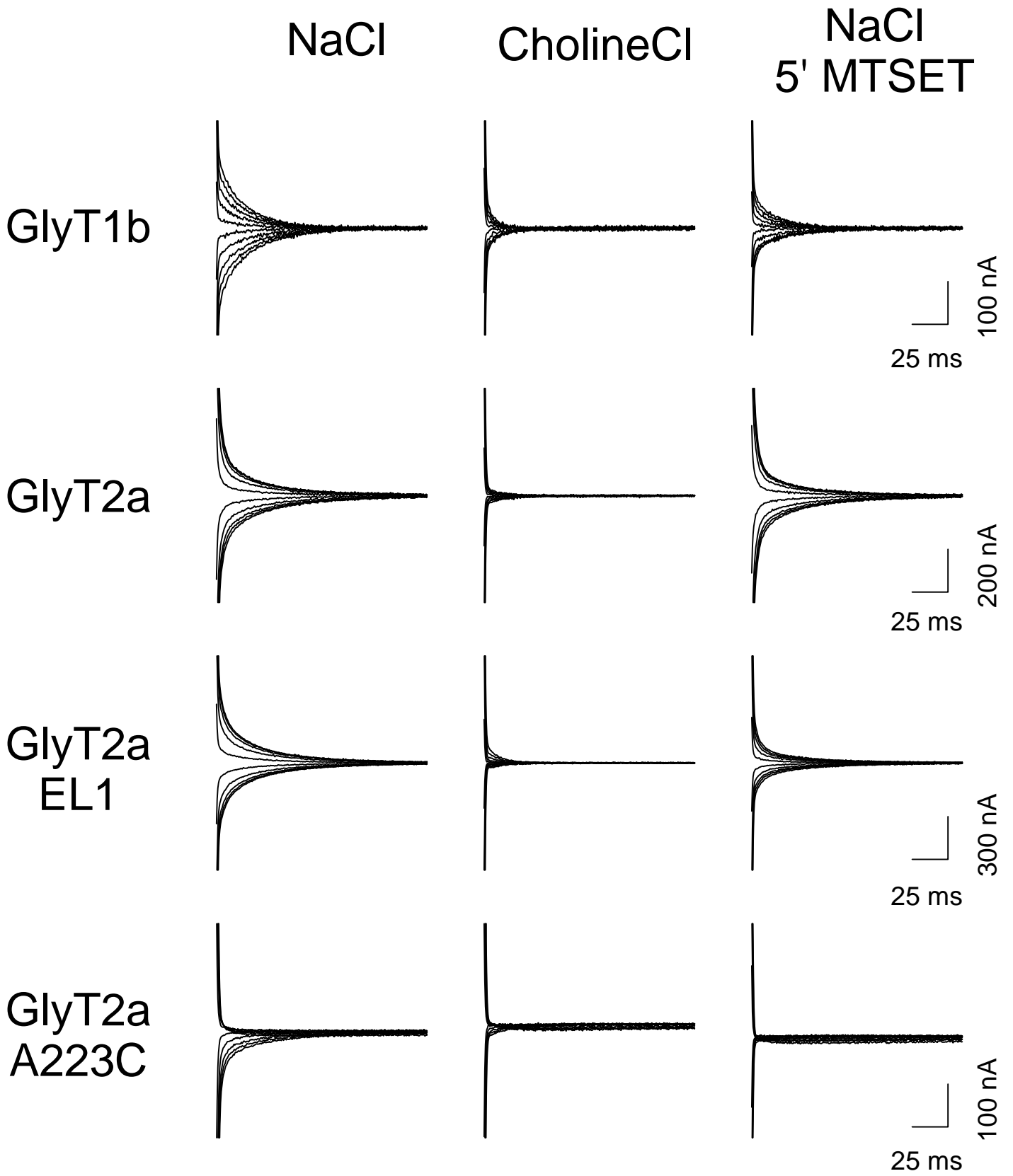


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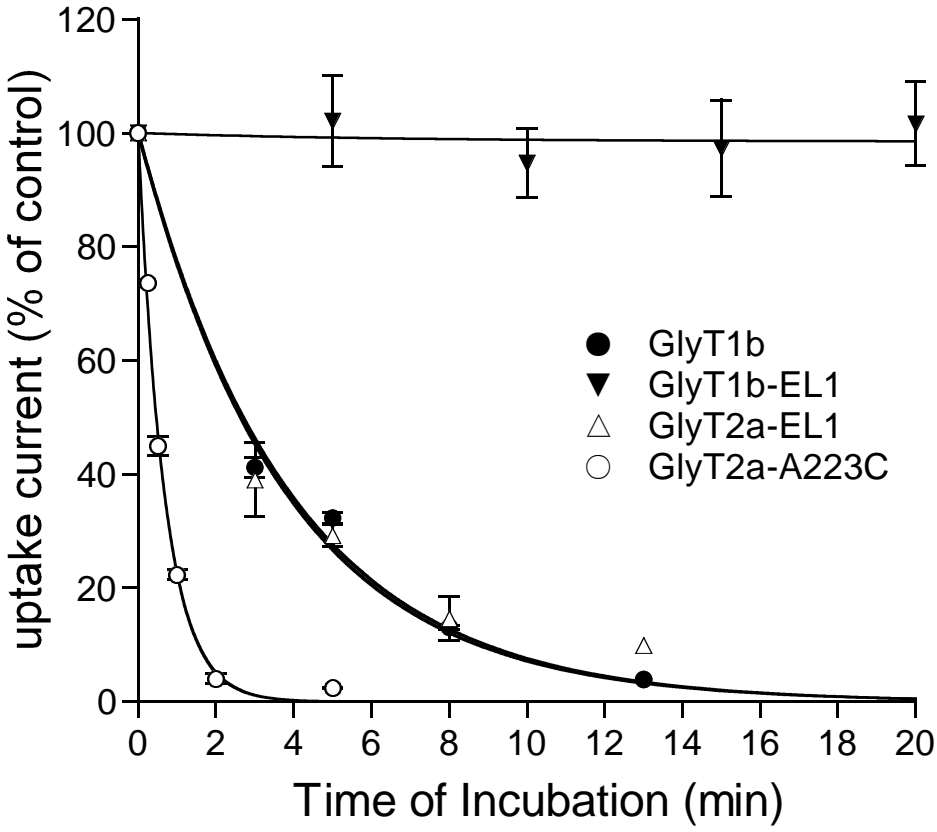


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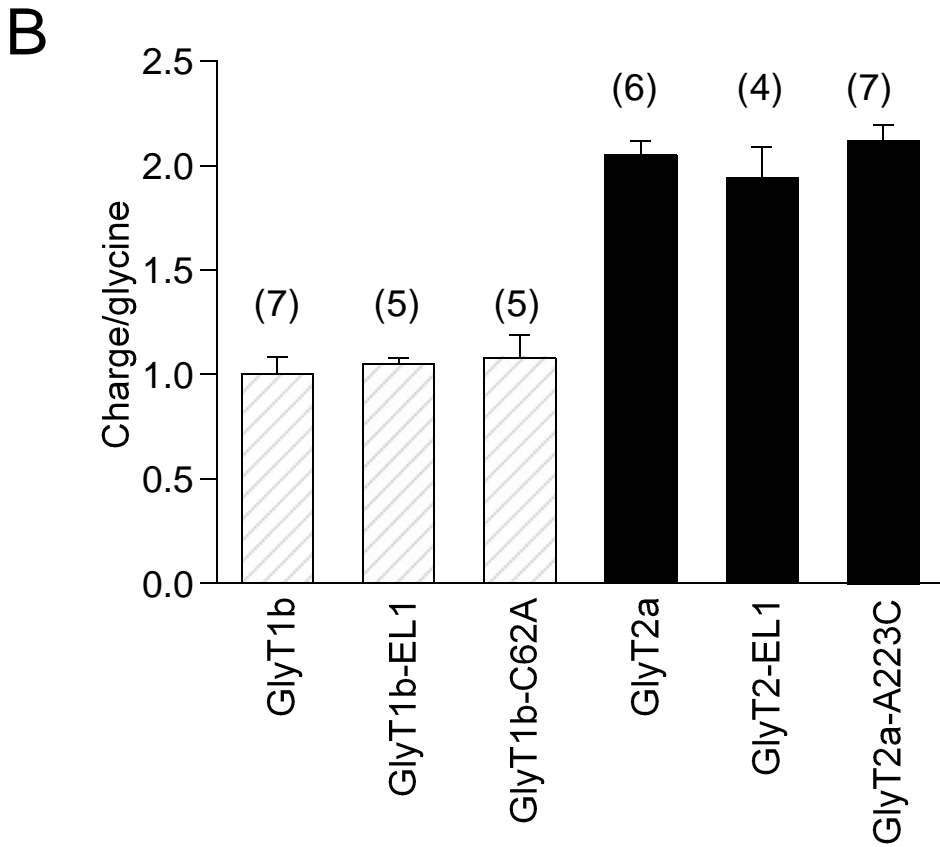
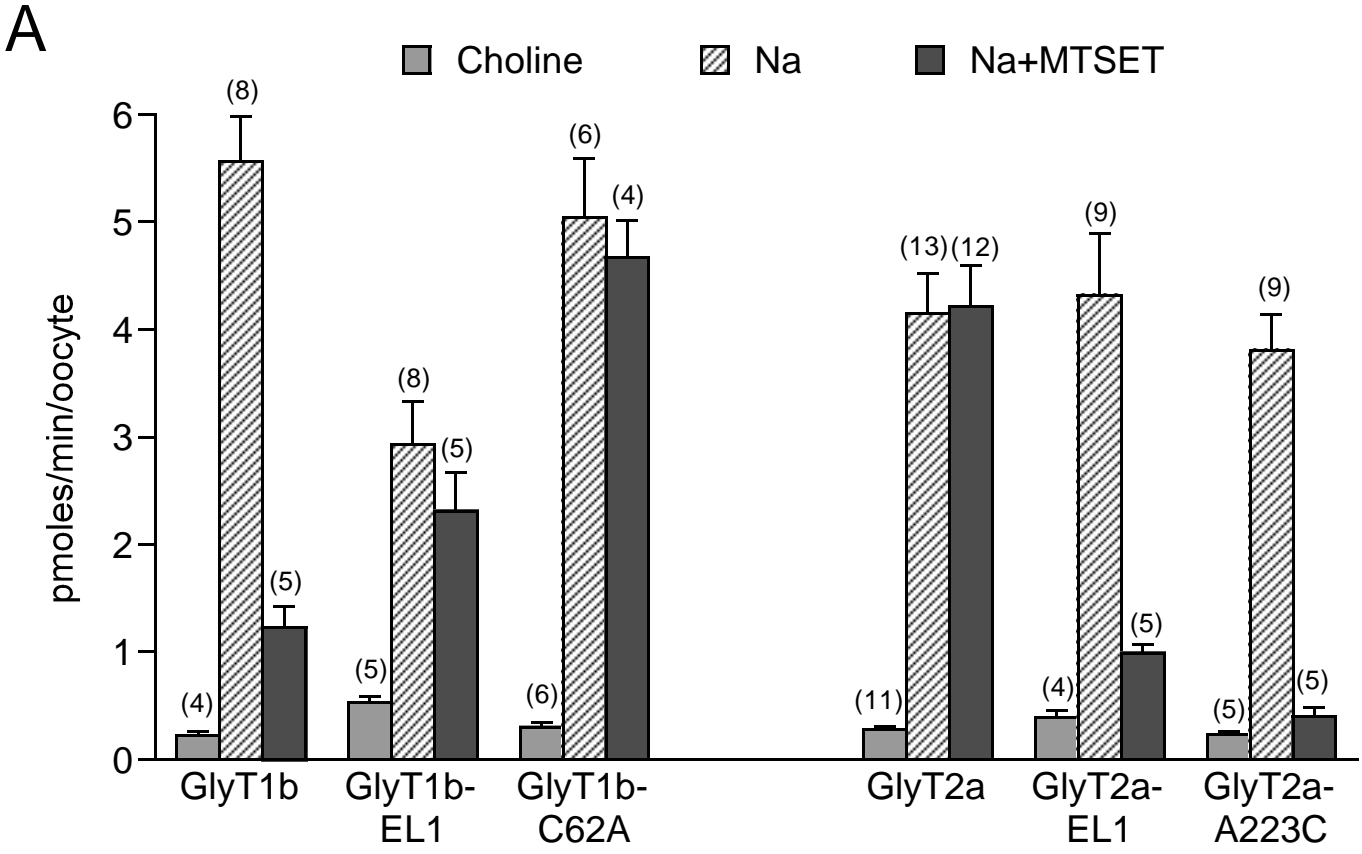


Figure 5

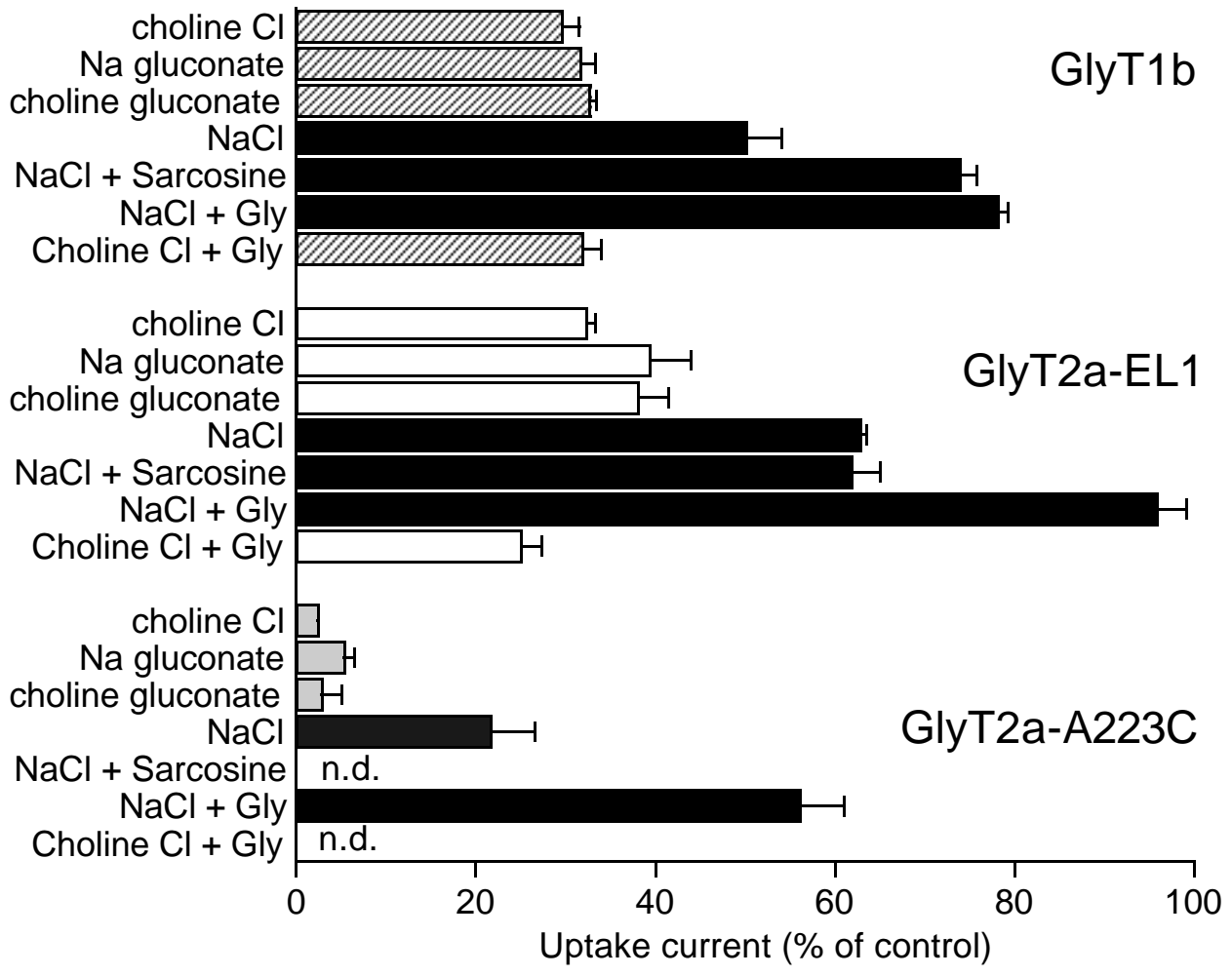


Figure 6

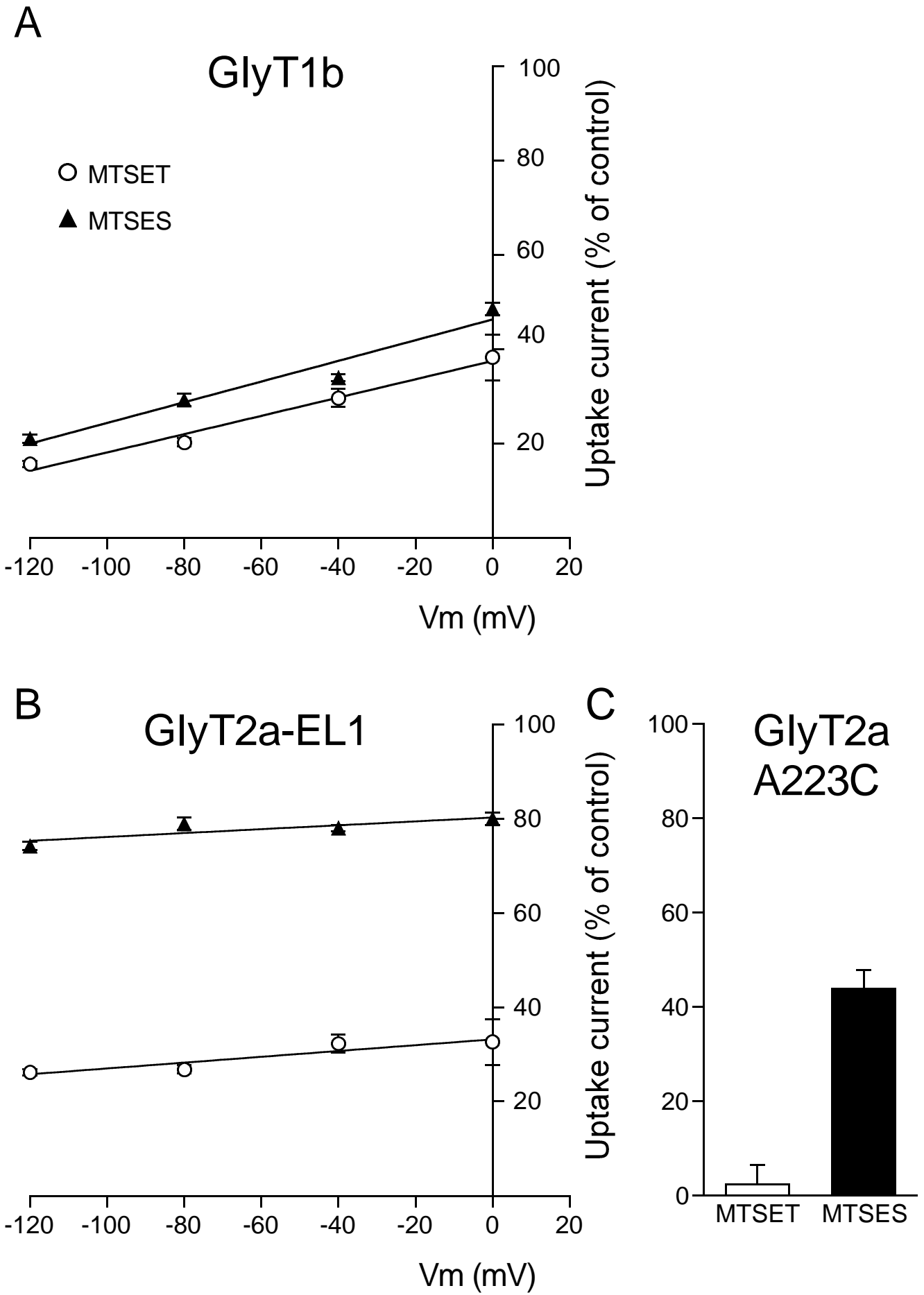


Figure 7

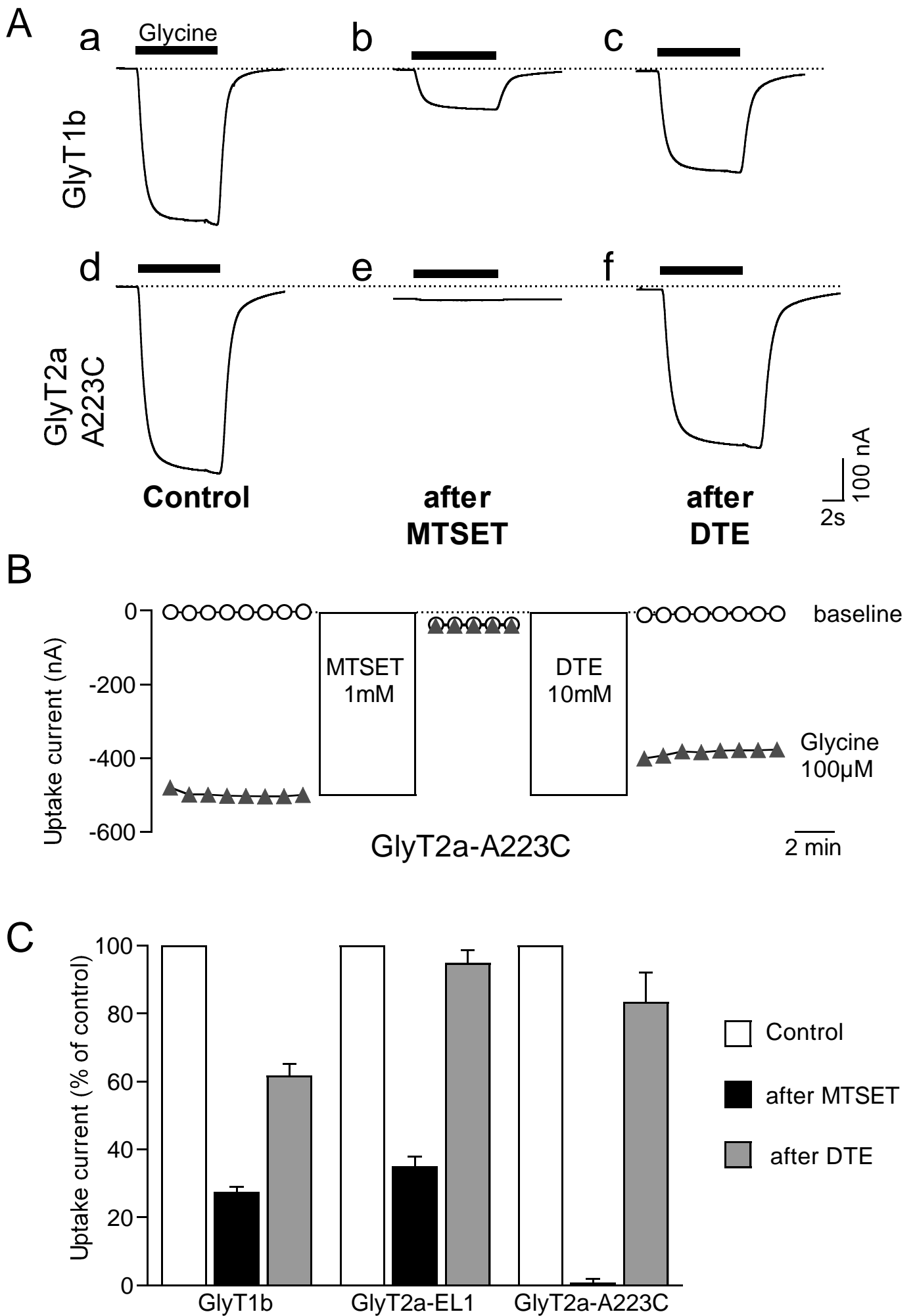


Figure 8