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A single β subunit M2 domain residue controls the picrotoxin sensitivity of αβ heteromeric glycine receptor chloride channels

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Abstract

This study investigated the residues responsible for the reduced picrotoxin sensitivity of the αβ heteromeric glycine receptor relative to the α homomeric receptor. By analogy with structurally related receptors, the β subunit M2 domain residues P278 and F282 were considered the most likely candidates for mediating this effect. These residues align with G254 and T258 of the α subunit. The T258A, T258C and T258F mutations dramatically reduced the picrotoxin sensitivity of the α homomeric receptor. Furthermore, the converse F282T mutation in the β subunit increased the picrotoxin sensitivity of the αβ heteromeric receptor. The P278G mutation in the β subunit did not affect the picrotoxin sensitivity of the αβ heteromer. Thus, a ring of five threonines at the M2 domain depth corresponding to α subunit T258 is specifically required for picrotoxin sensitivity. Mutations to α subunit T258 also profoundly influenced the apparent glycine affinity. A substituted cysteine accessibility analysis revealed that the T258C sidechain increases its pore exposure in the channel open state. This provides further evidence for an allosteric mechanism of picrotoxin inhibition, but renders it unlikely that picrotoxin (as an allosterically acting 'competitive' antagonist) binds to this residue.

Keywords: allosteric inhibition, ligand-gated ion channel, substituted cysteine accessibility method, threonine ring.

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The glycine receptor chloride channel (GlyR) is a member of the ligand-gated ion channel (LGIC) receptor family, which includes the nicotinic acetylcholine receptor (nAChR), the γ-aminobutyric acid type A and type C receptors ($GABA_AR$ and $GABA_CR$), the serotonin type 3 receptor ($5HT_3R$) and the glutamate-gated chloride channel (GluClR) (Grenningloh *et al*. 1987; Schofield *et al*. 1987; Maricq *et al*. 1991; Cully *et al*. 1994). Functional receptors of this family comprise five subunits arranged in a ring to form a central ion-conducting pore. Each subunit contains a large N-terminal extracellular domain responsible for ligand binding and four transmembrane domains (M1–M4) with a large intracellular loop between M3 and M4. The α-helical M2 segment lines the channel pore (Devillers-Thiery *et al*. 1993; Karlin and Akabas 1995; Rajendra *et al*. 1997).

Functional GlyRs can be formed as homomers comprising five α subunits or as heteromers comprising α and β subunits in the stoichiometry 3 : 2 (Langosch *et al*. 1988). The wild-type α homomeric (α/WT) GlyR is more sensitive than the wild-type αβ heteromeric (α/WT + β/WT) GlyR to inhibition by the plant alkaloid, picrotoxin (Pribilla *et al*. 1992). The molecular mechanism of picrotoxin inhibition of the GlyR has not been resolved. Replacement of M2 segment of β subunit with that of α subunit restores the picrotoxin sensitivity of the α/WT + β/WT GlyR to the level of the α/WT GlyR, implying that picrotoxin binds in the pore (Pribilla *et al*. 1992). On the other hand, picrotoxin inhibition of the α/WT GlyR is competitive and displays no use-dependence (Lynch *et al*. 1995), which is inconsistent with a classical pore-blocking action. Moreover, mutations to the arginine at position 271 in the GlyR α subunit convert picrotoxin into a non-competitive antagonist at high picrotoxin concentrations and into an allosteric potentiator at low picrotoxin concentrations (Lynch *et al*. 1995),

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Abbreviations used: DTT, dithiothreitol; $GABA_AR$, γ-aminobutyric acid type A receptor; $GABA_CR$, γ-aminobutyric acid type C receptor; GluClR, glutamate-gated chloride receptor; GlyR, glycine receptor; $5HT_3R$, serotonin type 3 receptor; LGIC, ligand-gated ion channel; MTSEA, ethylammoniummethane thiosulfonate; MTSES, sulfonatoethylmethane thiosulfonate; MTSET, 2-trimethylammoniumethylmethane thiosulfonate; nAChR, nicotinic acetylcholine receptor; n_H, Hill coefficient; pCMBS, *p*-chloromercuribenzenesulfonate; WT, wild-type.

providing further evidence for an allosteric mechanism. Thus, results to date are compatible with a model in which picrotoxin acts allosterically via a site in the pore, but does not itself physically block current flow. Its binding site could be either in the pore or in the external ligand-binding domain.

The aim of the present study is to identify the M2 residue (or residues) responsible for conferring picrotoxin sensitivity, and if possible, to determine whether or not picrotoxin acts by binding to this site. Since the GlyR β subunit M2 domain has an unusually low amino acid sequence homology with that of the α subunit (Grenningloh *et al*. 1990), a total of 13 of the 20 residues in the M2 domain are potential determinants of picrotoxin sensitivity (Pribilla *et al*. 1992). There has been a considerable research effort focussed on identifying the residues and mechanisms responsible for picrotoxin inhibition in various other members of the LGIC receptor family including the $GABA_AR$ (Newland and Cull-Candy 1992; Yoon *et al*. 1993; ffrench-Constant *et al*. 1993; Zhang *et al*. 1994; Gurley *et al*. 1995; Xu *et al*. 1995), the $GABA_CR$ (Wang *et al*. 1995; Zhang *et al*. 1995) and the GluClR (Etter *et al*. 1999). Using the terminology of Miller (1989), which assigns position 1′ to the cytoplasmic end of the M2 domain, the above studies have identified residues at positions 2′ and 6′ as major determinants of picrotoxin sensitivity. As the residues at these positions were not conserved between the GlyR α and β subunits, we hypothesized that they might be important determinants of picrotoxin sensitivity in the GlyR.

Materials and methods

Mutagenesis and expression of GlyR cDNAs

The human GlyR α1 and β subunit cDNAs were subcloned into the pCIS2 and pIRES2-EGFP plasmid vectors (Clontech, Palo Alto, CA, USA), respectively. Because the GlyR α subunit can efficiently assemble into functional GlyRs as either α homomers or α/β heteromers, green fluorescent protein expression was used as an indicator of GlyR β subunit expression. Site-directed mutagenesis was performed using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA, USA) and the successful incorporation of mutations was confirmed by sequencing the clones. Adenovirus-transformed human embryonic kidney 293 cells (ATCC CRL 1573) were passaged in a 50 : 50 mixture of minimum essential medium and Dulbecco's modified Eagle medium supplemented with 2 mM glutamate, 10% fetal calf serum and the antibiotics penicillin 50 IU/mL and streptomycin 50 μg/mL (GibcoBRL, Grand Island, NY, USA). Cells were transfected using a calcium phosphate precipitation protocol (Chen and Okayama 1987). When co-transfecting the GlyR α and β subunits, their respective cDNAs were combined in a ratio of 1 : 10 (Pribilla *et al*. 1992). After exposure to transfection solution for 24 h, cells were washed twice using the culture medium and used for recording over the following 24–72 h.

Electrophysiology

The cells were observed using a fluorescent microscope and currents were measured using the whole cell patch-clamp configuration (Hamill *et al*. 1981). Cells were perfused by a control solution that contained (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, with the pH adjusted to 7.4 with NaOH. Patch pipettes were fabricated from borosilicate hematocrit tubing (Vitrex, Modulohm, Denmark) and heat polished. Pipettes had a tip resistance of 1.5–3 MΩ when filled with the standard pipette solution which contained (in mM): 145 CsCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 EGTA, with the pH adjusted to 7.4 with NaOH. After establishment of the whole cell configuration, cells were voltage-clamped at −40 mV and membrane currents were recorded using an Axopatch 1D amplifier and pClamp7 software (Axon Instruments, Foster City, CA, USA). The cells were perfused by a parallel array of microtubular barrels through with solutions were gravity-induced.

Picrotoxin (Sigma, St Louis, MO, USA) was stored frozen as a 100-mM stock solution in dimethylsulfoxide. When dissolved into the perfusion solution, the final concentration of dimethylsulfoxide was less than 1%. The disulfide reducing agent, dithiothreitol (DTT), was prepared daily as a 1-mM solution in the standard bathing solution. Positively charged 2-trimethylammoniumethyl-methane thiosulfonate (MTSET) and negatively charged 2-sulfonatoethylmethane thiosulfonate (MTSES) were obtained from Toronto Research Chemicals (Toronto, Canada). Stock solutions of each reagent were prepared freshly each day and stored on ice until used. Once dissolved into room temperature bathing solution, they were applied to cells immediately.

The effects of MTSET and MTSES were tested using the following procedure. After establishment of the recording configuration, the glycine dose–response was measured by applying increasing glycine concentrations at 30 s intervals. Following this, two brief applications of glycine at the half-saturating (EC_{50}) concentration were followed by two brief applications at a saturating ($10 \times EC_{50}$) concentration, all at 30 s intervals. Provided current amplitude remained constant, the averaged current amplitudes were used as the control. Following application of the MTSET- or MTSES-containing solution, cells were washed in control solution for 1–3 min before the maximum current magnitudes and glycine EC_{50} values were measured again. In long-term patch recordings, both parameters were continually measured and were observed to remain constant in all mutant GlyRs for periods as long as 20 min. This strongly suggests that irreversible changes in both parameters were due to covalent modification of exposed cysteines.

Data analysis

Results are expressed as mean ± standard error of the mean (SEM) of three or more independent experiments. The empirical Hill equation, fitted by a non-linear least squares algorithm (Origin 4.0, Northampton, MA, USA), was used to calculate the IC_{50}, EC_{50} and Hill coefficient (n_H) values for glycine activation and picrotoxin inhibition. Statistical significance was determined by using either a paired or unpaired Student's *t*-test, as appropriate, with $p < 0.05$ representing significance.

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                    2′  6′
                    ↓   ↓  ↓↓  ↓  ↓
GlyR α1     253 VGLGITTVLTMTTQSSGSR
GlyR β      277 VPLGIFSVLSLASECTTLA
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Fig. 1 Sequence alignment of human GlyR α1 and β subunit M2 domains. Arrows denote those residues in the nAChR known to be exposed to the channel lumen (Akabas *et al.* 1994). The residues investigated in this study are shown in bold and labelled by their respective 2′ and 6′ positions.

Results

Identification of candidate residues

Comparison of the M2 segment amino acid sequences of the human GlyR α1 and β subunits reveals 13 pairs of non-conserved residues as potential candidates for control of picrotoxin sensitivity (Fig. 1). However, by comparison with results from other members of the LGIC superfamily, the residues corresponding to positions 2′ and 6′ were considered the most likely candidates. The 2′ residue has been identified as a determinant of picrotoxin sensitivity in several LGIC receptors including the *Drosophila* $GABA_AR$ *Rdl* subunit (ffrench-Constant *et al.* 1993), the GluClR α subunit (Etter *et al.* 1999) and a $GABA_CR$ ρ1 subunit (Wang *et al.* 1995). A $GABA_AR$ α1 subunit incorporating a cysteine-substituted mutation at this position can be protected by picrotoxin from modification by sulfhydryl-specific reagents in the $GABA_AR$ α1 subunit (Xu *et al.* 1995). The 6′ residue is also known to be a determinant of picrotoxin sensitivity in the $GABA_AR$ α1, β2 and γ2 subunits (Gurley *et al.* 1995), and a $GABA_CR$ ρ1 subunit (Zhang *et al.* 1995). However, picrotoxin could not protect the cysteine-substituted 6′ threonine residue from modification by sulfhydryl-specific reagents in the $GABA_AR$ α1 subunit (Xu *et al.* 1995). On the basis of these findings, and the fact that the 2′ and 6′ residues are not conserved between the GlyR α and β subunits, we hypothesized that picrotoxin sensitivity is controlled by either glycine 254 (corresponding to position 2′) or threonine 258 (corresponding to position 6′) of the GlyR α subunit. These residues are abbreviated as α/G254 and α/T258, respectively.

Effects of MTSES and MTSET on cysteine-substituted mutant GlyRs

The substituted cysteine accessibility method (Karlin and Akabas 1998) was used to investigate whether α/G254 and α/T258 were exposed to the channel lumen. These experiments were carried out on α homomeric GlyRs. For this procedure, it was necessary to construct the α/G254C and α/T258C mutant GlyRs. The mean glycine EC_{50} and Hill coefficient (n_H) values for the α/WT and all mutant GlyRs investigated in this study are given in Table 1. The effects of MTSES and MTSET were first investigated on the α/WT GlyR. The standard procedure was to apply glycine at both the EC_{50} value and at a saturating concentration corresponding to $10 \times EC_{50}$. The sulfhydryl-modifying reagent was then applied for 1 min either in the absence of glycine or in the presence of a saturating ($10 \times EC_{50}$) glycine concentration and the glycine current measured

Table 1 Summary of the functional properties of WT and mutant GlyRs

	Glycine			Picrotoxin			
Receptor	EC_{50} (μM)	n_H	*n*	[glycine] (μM)	IC_{50} (μM)	n_H	*n*
α/WT	26 ± 9	3.4 ± 0.3	4	30	18 ± 1	1.5 ± 0.1	5
α/G254P	147 ± 27**	2.5 ± 0.3	5	150	2.6 ± 0.6**	0.9 ± 0.05**	4
α/G254A	33 ± 1	3.5 ± 0.3	4	30	14.8 ± 7.5	1.0 ± 0.1*	4
α/G254C	282 ± 30**	2.6 ± 0.2	6	300	> 1000**		4
α/T258F	6.4 ± 1.1*	1.5 ± 0.1**	6	5	706 ± 140**	0.9 ± 0.05**	4
α/T258A	1.4 ± 0.3**	1.1 ± 0.2**	8	0.8	388 ± 73**	1.1 ± 0.3	5
α/T258C	52 ± 1*	1.6 ± 0.1**	4	50	595 ± 91**	0.8 ± 0.1**	5
α/WT + β/WT	24 ± 5	3.0 ± 0.7	4	30	259 ± 44**	1.3 ± 0.1	4
α/WT + β/P278G	24 ± 3	2.5 ± 0.2*	4	30	224 ± 86**	0.9 ± 0.1**	3
α/WT + β/F282T	32 ± 5	2.2 ± 0.2*	4	30	27 ± 13	0.7 ± 0.05**	7
α/T258F + β/F282T [a]	7.2 ± 0.7	1.5 ± 0.1	5	7	550 ± 150	0.9 ± 0.05	4
α/T258F + β/WT [a]	5.6 ± 1.2	1.4 ± 0.05	4	6	315 ± 41*	0.9 ± 0.05	5

The glycine EC_{50} and Hill coefficient (n_H) values and the picrotoxin IC_{50} and n_H values were determined as explained in Materials and methods. All errors are presented as SEM of *n* experiments. The picrotoxin IC_{50} and n_H values were measured at the glycine concentration approximating to its EC_{50} value as shown. Unless otherwise indicated, statistical significance was evaluated with respect to the α/WT GlyR values by unpaired Student's *t*-test with * and ** representing significance levels of $p < 0.05$ and $p < 0.01$, respectively.[a] Statistical significance was evaluated with respect to the α/T258F GlyR.

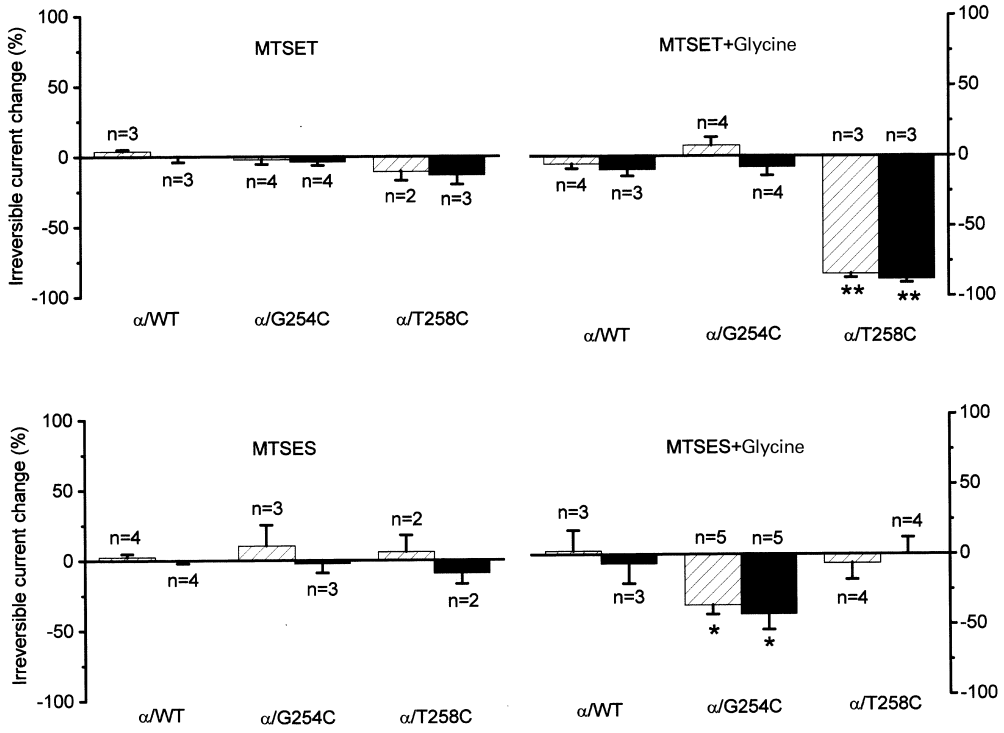

Fig. 2 Irreversible effects of MTSET and MTSES on wild-type (WT) and mutant α homomeric GlyRs. The upper left and right panels show the irreversible effect on current magnitude induced by a 1-min application of 1 mM MTSET applied in the absence and presence of glycine, respectively. Similarly, the lower left and right panels show the effect on current magnitude of a 1-min application of 10 mM MTSES applied in the absence and presence of glycine, respectively. Striped and solid bars correspond to the EC_{50} and the 10 × EC_{50} glycine concentrations, respectively. The EC_{50} glycine concentrations for the WT and each mutant GlyR are given in Table 1. The effect of the MTS reagent was taken as $(I_{gly,after}/I_{gly,before}) - 1$. Results from the mutant GlyRs were compared with those from the α/WT GlyR using an unpaired Student's *t*-test (*$p < 0.05$ and **$p < 0.01$).

again following a 1–3 min wash in control solution. MTSET and MTSES were applied at respective concentrations of 1 and 10 mM, which are in approximate inverse proportion to their reactivity rates in free solution (Stauffer and Karlin 1994). As summarized in Fig. 2, neither MTSES nor MTSET had any effect on the α/WT GlyR, regardless of whether they were applied in the presence or absence of glycine. Similarly, in the α/G254C mutant GlyR, MTSET had no effect irrespective of whether it was applied in the open or closed channel state (Fig. 2). However, although MTSES had no effect on the α/G254C mutant GlyR when applied in the channel closed state, it induced a strong irreversible inhibition when applied in the channel open state (Fig. 2). This inhibition was not dependent on the glycine concentration, implying that it may have been due to channel block. Examples of currents recorded from α/G254C mutant GlyRs in response to MTSES application in the closed and open channel states are shown in Figs 3(a and b), respectively. In Fig. 3(b), it is apparent that MTSES rapidly reduced the current activated by a saturating (2.5 mM) concentration of glycine. This inhibition was not reversed by either a 3-min wash in control solution (Fig. 3b, centre panel) or by a 1-min application of a 1-mM concentration of the reducing agent, DTT (Fig. 3b, right panel). DTT was found to have no effect on the magnitude the MTSES-induced inhibition in each of four cells in which it was examined.

MTSET had no effect on the α/T258C mutant GlyR when applied in the absence of glycine. An example of a typical experiment is shown in Fig. 4(A) and averaged results are presented in Fig. 2. However, when MTSET was applied to the α/T258C GlyR in the presence of glycine, it significantly and irreversibly inhibited the magnitude of current that could be activated by a subsequent application of glycine (Figs 2 and 4b). As shown in the example in Fig. 4(b), its mode of action was unusual. The glycine current magnitude was not affected by the application or removal of MTSET ($n = 4$). However, following the removal of glycine after MTSET exposure, the channels remained partially irreversibly activated (Fig. 4b). A subsequent glycine application reversibly activated the remaining fraction of available channels (Fig. 4b). The MTSET-induced current inhibition as shown in Fig. 2 is calculated as the magnitude of this glycine-activatable current relative to the magnitude of the

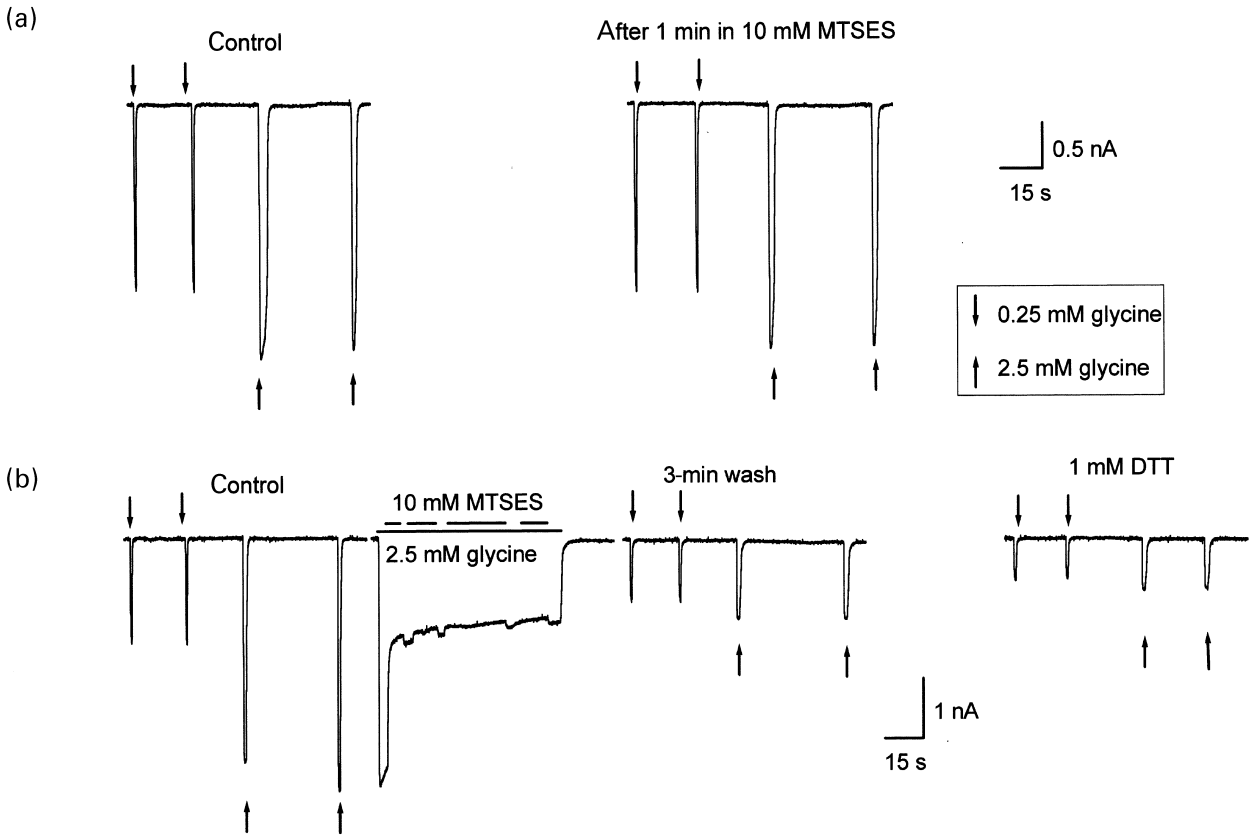

Fig. 3 Effect of MTSES on the α/G254C mutant GlyR. Currents were evoked by glycine at half-saturating concentration (0.25 mM) and saturating concentration (2.5 mM), as indicated by arrows. (a) In the absence of glycine, a 1-min application of 10 mM MTSES had no significant effect on the glycine current magnitude. (b) In the presence of 2.5 mM glycine, a 1-min application of 10 mM MTSES irreversibly inhibited the glycine current. This effect could not be reversed by a 1-min application of 1 mM DTT. Note the MTSES-induced inhibition contains a small rapidly reversible component.

original glycine-activatable current. In each of the four cells examined, the channels were returned to the closed state only after a 1-min application of the reducing agent, DTT (Fig. 4b).

Curiously, the α/T258C mutant GlyR was not irreversibly modified by MTSES in either the closed or open channel states (Fig. 2). However, when MTSES was applied in the presence of glycine it caused a strong rapid inhibition that did not recover immediately upon the removal of MTSES (Fig. 5a, centre trace). However, the current magnitude eventually returned to the original amplitude after a 3-min wash in control solution (Fig. 5a, right trace), an action which is inconsistent with the covalent modification of an exposed sulfhydryl group. It is possible that MTSES may have covalently modified α/T258C but induced no change in receptor function. This possibility was investigated by applying MTSET directly after MTSES. As shown in the example in Fig. 5(b), the prior application of MTSES did not affect the ability of MTSET to maintain the channels in the open state.

α/G254 and α/T258 as determinants of picrotoxin sensitivity

Since the GlyR β subunit confers picrotoxin insensitivity, we investigated the effect of substituting the α subunit residues with the corresponding β subunit residues at the 2′ and 6′ positions in the α/WT GlyR. These mutants corresponded to α/G254P and α/T258F, respectively. Since these mutations were not conservative, we also investigated the more conservative alanine substitution mutants, α/G254A and α/T258A. The glycine EC_{50} and n_H values for each of these mutant GlyRs is given in Table 1. Because picrotoxin is a competitive antagonist of the α/WT GlyR (Lynch *et al.* 1995), the picrotoxin IC_{50} values were measured at the glycine EC_{50} value for each mutant GlyR. The picrotoxin IC_{50} and n_H values, together with the glycine concentration at which they were determined, are shown for the WT and each mutant GlyR in Table 1. Examples of the effects of picrotoxin on the α/WT GlyR and the α/WT + β/WT GlyR are shown in Fig. 6(a) and the respective averaged picrotoxin inhibitory

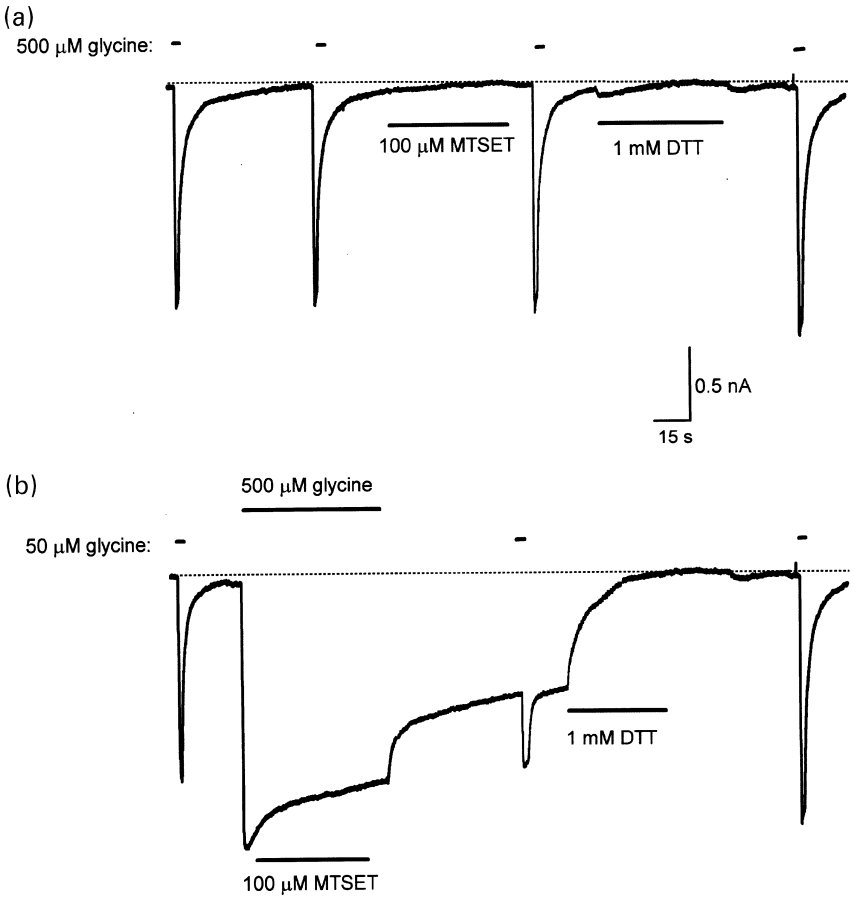

Fig. 4 Effect of MTSET on the α/T258C mutant GlyR. Currents were activated by glycine at a half-saturating concentration (50 μM) and a saturating concentration (500 μM), as indicated by bars. (a) In the absence of glycine, a 1-min application of 100 μM MTSET (and a subsequent application of 1 mM DTT) had no significant effect on the glycine current magnitude. (b) When applied in the presence of a saturating concentration (500 μM) of glycine, a 1-min application of 100 μM MTSET has no immediate effect on glycine current magnitude. However, upon the withdrawal of glycine, the channels remain activated and a 50-μM glycine application reversibly activated the remaining fraction of closed channels. A 1-min application of 1 mM DTT was required to return the channels to the closed state (right panel). Scale bars apply to all traces.

dose–responses are shown in Fig. 6(b) as unfilled squares and filled circles, respectively.

As seen in Table 1, the α/G254C mutation entirely abolished picrotoxin sensitivity, whereas α/G254A had no significant effect on picrotoxin sensitivity. Surprisingly, the α/G254P mutation increased the picrotoxin sensitivity of the α/WT GlyR by a factor of 7. Thus, although substitutions at this position can affect picrotoxin sensitivity, the α/G254P mutation is unlikely to be responsible for the reduced picrotoxin sensitivity conferred by the β/WT GlyR subunit.

The picrotoxin IC_{50} values of the α/T258F, α/T258A and α/T258C mutant GlyRs were increased by factors of 39, 22, and 21 fold, respectively, relative to the α/WT GlyR value (Table 1). Examples of the effect of picrotoxin on the α/T258F mutant GlyR are shown in Fig. 6(a) and the averaged picrotoxin inhibitory dose–response is shown in Fig. 6(b). This reduction in picrotoxin inhibitory potency compares well with the difference in picrotoxin sensitivity between the α/WT GlyR and the α/WT + β/WT GlyR (Table 1). Thus, it is possible that the 6′ residue could be responsible for the reduced picrotoxin sensitivity conferred by the β GlyR subunit. However, a limitation of this approach is that it involved a 'loss-of-function' mutation, which could conceivably have been caused by a non-specific structural disruption. A more convincing approach would be to create a picrotoxin-sensitive receptor by incorporating an α subunit residue into the corresponding position of the β subunit.

Restoration of picrotoxin sensitivity in the α/WT + β/WT heteromeric GlyR

In an attempt to restore picrotoxin sensitivity to the α/WT + β/WT heteromeric GlyR, we incorporated the corresponding α subunit residues into the β subunit at the 2′ and 6′ positions. In the β subunit, these mutations corresponded to β/P278G and β/F282T, respectively. Since the β subunit is expressed only as a heteromer with the α subunit, we examined the effects of picrotoxin on both the α/WT + β/P254G and the α/WT + β/F282T mutant GlyRs. The glycine EC_{50} and n_H values and the picrotoxin IC_{50} and n_H values for these mutant GlyRs are displayed in Table 1. Examples of the effect of picrotoxin on the α/WT + β/F282T mutant GlyR are shown in Fig. 6(a) and the averaged picrotoxin inhibitory dose–response is shown in Fig. 6(b). The picrotoxin sensitivity of the α/WT + β/P258G mutant GlyR was not significantly different to that of the α/WT + β/WT GlyR (Table 1), strengthening the earlier conclusion that the 2′ residue is not

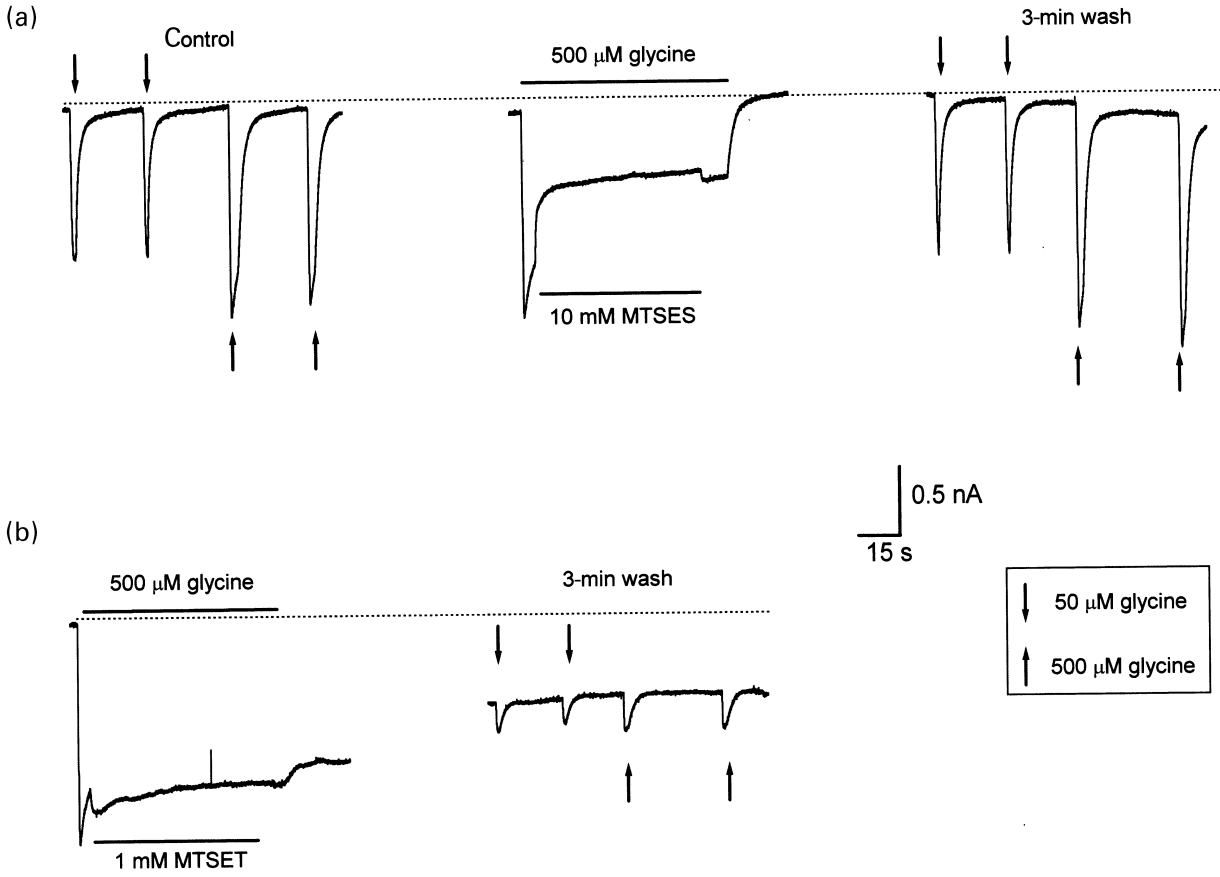

Fig. 5 Lack of effect of MTSES on the MTSET response of the α/T258C mutant GlyR. Currents were activated by half saturating (50 μM) and saturating (500 μM) concentration of glycine, as indicated by the arrows. All traces were recorded sequentially from the same cell. (a) In the presence of saturating concentration of glycine, a 1-min application of 10 mM MTSES inhibited glycine current (centre trace). This inhibition was completely reversed by a 3-min wash in control solution (right trace). (b) A subsequent 1 min application of 1 mM MTSET in presence of glycine maintained the channels in the open state (left panel). The transient downward deflection at the start of MTSET application was a solution change artefact. The channels remained open following a 3-min wash (centre trace).

a determinant of picrotoxin sensitivity. However, the picrotoxin sensitivity of the α/WT + β/F282T GlyR was increased by almost an order of magnitude to the point where it was not significantly different to that of the α/WT GlyR (Table 1 and Fig. 6b). The observation that β/F282T is a 'gain-of-function' mutation greatly strengthens the case that the 6′ residue is a major determinant of picrotoxin sensitivity.

Since the M2 domains of the α and β subunits have a low amino acid homology (Fig. 1), it remains possible that other M2 residues may also contribute to the picrotoxin resistance conferred by the β subunit. One way of investigating this is to determine whether the presence of β subunits, as distinct from the presence of phenylalanines in the 6′ position, has any effect on picrotoxin sensitivity. The results of experiments designed to investigate this are summarized in Fig. 7. It has already been established that the α/WT GlyR, which has a ring of five threonines at the 6′ position, is picrotoxin-sensitive and that the α/T258F GlyR, which has a ring of five phenylalanines at the 6′ position, is picrotoxin-insensitive. Furthermore, the α/WT + β/WT GlyR, which contains three threonines and two phenylalanines at this position, is also picrotoxin-insensitive. Thus, if the number of phenylalanines is the only crucial factor, then the α/T258F + β/F304T mutant GlyR, which has three phenylalanines, should have a similar picrotoxin sensitivity to the α/WT + β/WT GlyR. As shown in Fig. 7, this indeed turns out to be the case. As expected, the α/T258F + β/WT GlyR, which also contains five phenylalanines, is also picrotoxin-insensitive. Thus, picrotoxin insensitivity was conferred by the incorporation of two or more phenylalanines at the 6′ position of either the α or β subunit (Fig. 7). However, in the $GABA_AR$, Gurley *et al.* (1995) found that only one substituted phenylalanine at the 6′ position was sufficient to confer picrotoxin insensitivity. Due to the close structural similarities between the two receptor types, it is

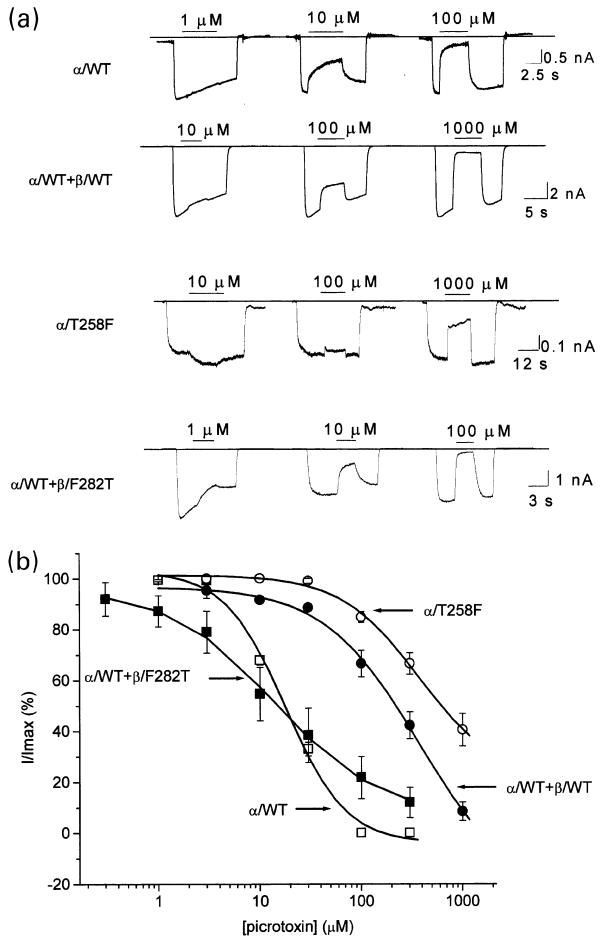

Fig. 6 Effect of picrotoxin on wild-type (WT) and mutant GlyRs. (a) Examples of picrotoxin inhibition of currents activated by a half-saturating concentration of glycine in 4 GlyR constructs as indicated. (b) Averaged picrotoxin dose–response curves for the α/WT GlyR (□), α/WT + β/WT GlyR (●), the α/T258F mutant GlyR (○) and the α/WT + β/F282T GlyR (■). Error bars indicate the SEM from at least 4 cells. Curves represent Hill equation fits to averaged data.

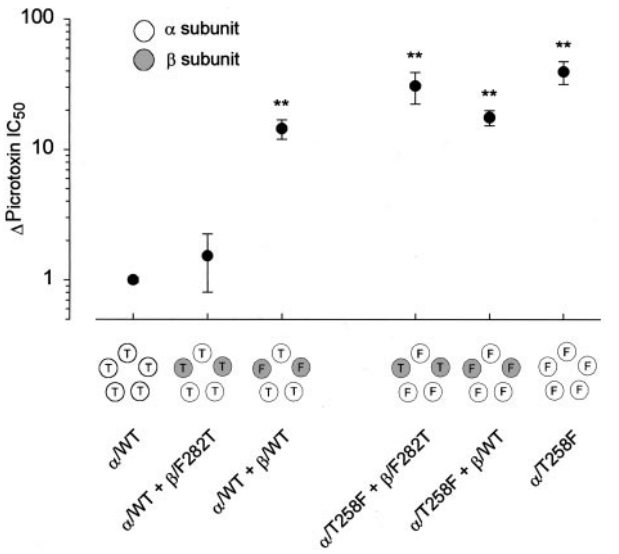

Fig. 7 Relationship between picrotoxin sensitivity and the subunit location of the 6′ phenylalanines. The picrotoxin sensitivity of the indicated GlyR constructs are displayed relative to the picrotoxin sensitivity of the α/WT GlyR. The models provide a simple representation of the subunit composition and the residue present in the 6′ position of each GlyR construct. Statistical significance was assessed by unpaired Student's *t*-test (**$p < 0.01$).

likely that only one phenylalanine would also be necessary to impart picrotoxin insensitivity in the GlyR.

Altered glycine sensitivity and cooperativity in mutant GlyRs

The glycine EC_{50} values of the mutant GlyRs examined in this study varied over a wide range. The most extreme example was the α/T258A mutant GlyR, which was 18 times more sensitive to glycine than the α/WT GlyR. Of particular note, however, is the observation that the α/T258F mutant GlyR was 4 times more sensitive to glycine than the α/WT GlyR (Table 1). This finding permitted an analysis of the subunit-specific control of glycine sensitivity. As shown in Fig. 8, in both homomeric and heteromeric GlyR constructs which contained threonines in the 6′ position of the α subunit, the glycine sensitivity was not significantly

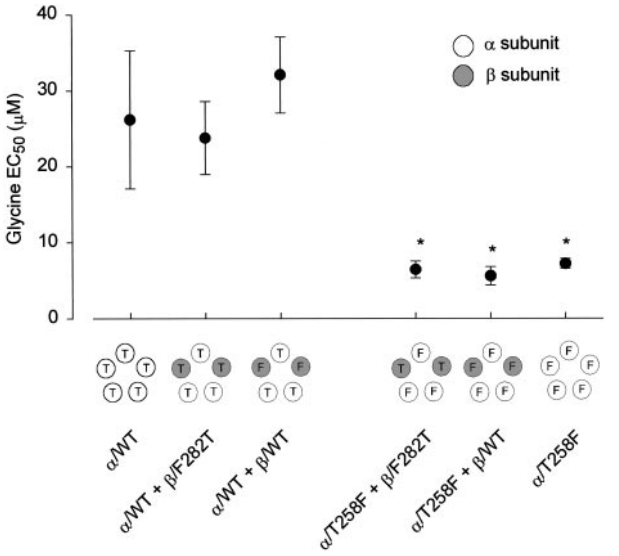

Fig. 8 Relationship between glycine sensitivity and the subunit location of the 6′ phenylalanines. The mean glycine EC_{50} values for each GlyR construct are shown. The models provide a simple representation of the subunit composition and the residue present in the 6′ position of each GlyR construct. Statistical significance was assessed by unpaired Student's *t*-test (*, $p < 0.05$).

different to that of the α/WT GlyR. However, when phenylalanines were present at the 6′ position of the α subunit, the glycine sensitivity was increased to a similar degree regardless of the identity of the residue at the 6′ position of the β subunit (Fig. 8). Thus, glycine sensitivity of the heteromeric receptors was increased by introducing phenylalanine residues at position 6′ in the α subunit, although varying the number of phenylalanines at the corresponding position of the β subunit had no effect (Fig. 8). In addition, the n_H values for glycine activation were significantly reduced in α subunits incorporating phenylalanine substitutions at the 6′ position, whereas mutations to the corresponding residue in the β subunit had no effect on this parameter (Table 1). As discussed in detail below, these observations implicate T258 in the receptor gating mechanism.

Discussion

α/G254 and α/G258 are exposed to the channel lumen in the channel open state

The positively charged MTSET had no effect on the α/G254C mutant GlyR in either the channel closed or open states (Fig. 1). However, the negatively charged MTSES was able to irreversibly modify the α/G254C mutant GlyR, but only when applied in the open state. These observations are consistent with those obtained from the nAChR (Akabas *et al*. 1994; Pascual and Karlin 1998; Wilson and Karlin 1998) and $GABA_AR$ (Xu *et al*. 1995; Xu and Akabas 1996). The state-dependence of the MTSES effect is consistent with the 2′ residue forming part of the channel gate (Wilson and Karlin 1998) and the lack of effect by MTSET is consistent with the charge selectivity filter being external to this position (Akabas *et al*. 1994; Xu *et al*. 1995; Xu and Akabas 1996). The observation that DTT could not reduce the MTSES-modified α/G254C mutant GlyR (Fig. 4b) implies that the pore at this depth is so narrow that there is insufficient space to admit DTT after the covalent modification of the exposed sulfhydryl group. This interpretation is supported by results from the nAChR which concluded on the basis of sulfhydryl reactivity rates that the 2′ residue lies in a narrow part of the pore (Pascual and Karlin 1998; Wilson and Karlin 1998).

The covalent modification of α/T258C by MTSET in the channel open state did not affect the current magnitude but maintained the channel in the open state following the withdrawal of glycine (Fig. 4b). The channels returned to the closed state only after the modification was reversed by a 1-min application of DTT. MTSET had no effect when applied in the closed state (Fig. 4a), indicating that α/T258C is exposed to the channel lumen in the open state only. Thus, covalent modification appears to sterically hinder α/T258C from resuming its closed state configuration. In addition, MTSES was unable to covalently modify α/T258C in either the closed or open states (Figs 2 and 5). These results are consistent in some respects with studies on other members of the LGIC receptor family. In the $GABA_AR$ incorporating a cysteine substitution at the 6′ position, the positively charged ethylammoniummethane thiosulfonate (MTSEA) induced a strong increase in current in both the closed and open states (Xu and Akabas 1996). However, the negatively charged *p*-chloromercuribenzenesulfonate (pCMBS) was effective in the open state only and MTSES had no effect in either state (Xu *et al*. 1995; Xu and Akabas 1996). These later findings are consistent with the present results. However, as the effects of MTSET were not investigated in the $GABA_AR$ and MTSEA was not investigated in the GlyR, a detailed comparison of results cannot be made.

In the nAChR incorporating a cysteine at the 6′ position, MTSEA strongly inhibited current but displayed very little difference in reactivity rates between the closed and open channel states (Pascual and Karlin 1998). On the other hand, MTSET and MTSES had no effect on this mutant receptor (Akabas *et al*. 1994). Apart from indicating that the 6′ residue is exposed to the pore lumen, the effects of MTSEA and MTSET are not readily reconciled with the results of the present study. The apparent discrepancies could be explained by several factors including pore selectivity differences or variations in the structural configuration, ionisation state or electrostatic potential of the 6′ substituted cysteines (Karlin and Akabas 1998).

Results obtained using other approaches support the conclusion that the 2′ and 6′ residues are exposed to the pore in the channel open state. The α/G254A mutation in the GlyR α1 subunit dramatically reduces the inhibitory potency of the channel blocker, cyanotriphenylborate (Rundstrom *et al*. 1994), strongly suggesting that this residue forms the blocker binding site. Furthermore, the 2′ and 6′ residues have been identified as determinants of ion permeation in the nAChR (Villarroel *et al*. 1991, 1992; Cohen *et al*. 1992). In addition, several studies have demonstrated that the 2′ and 6′ residues form at least part of the binding site for non-competitive nicotinic antagonists (reviewed in Devillers-Thiery *et al*. 1993).

α/T258 is crucial for picrotoxin sensitivity

The picrotoxin sensitivity of the α/WT + β/P278G GlyR was not significantly different to that of the α/WT + β/WT GlyR (Table 1). Thus, the residue at the 2′ position of the β subunit is not responsible for conferring picrotoxin resistance. However, proline and cysteine substitution mutations at the 2′ position of the GlyR α subunit (i.e. α/G254P and α/G254C) dramatically altered the picrotoxin sensitivity, although the more conservative alanine substitution mutation (α/G254A) had no effect (Table 1). Thus, it is likely that non-conservative substitutions at the 2′ position in the α

subunit can induce local structural disruptions that indirectly influence picrotoxin sensitivity of the α/WT GlyR.

Both the decreased picrotoxin sensitivity of the α/T258F mutant GlyR and the restored picrotoxin sensitivity of α/WT + β/F278T mutant GlyR indicate that β/F278 is responsible for the picrotoxin resistance of the β subunit (Fig. 7). To determine whether other non-conserved M2 residues may also have been strong determinants of picrotoxin sensitivity, we investigated the effect of the subunit composition on picrotoxin sensitivity. We found that the picrotoxin resistance is associated with at least two phenylalanines at the 6′ position, and was independent of the presence of other nonconserved β subunit residues (Fig. 7). Furthermore, since the conservative alanine substitution also induced picrotoxin insensitivity, it appears that threonines at this position are specifically required for picrotoxin sensitivity.

In agreement with these results, the 6′ residue is a determinant of picrotoxin sensitivity in the $GABA_AR$ α1, β2 and γ2 subunits (Gurley *et al*. 1995) and the $GABA_CR$ ρ1 subunit (Zhang *et al*. 1995). Indeed, both studies found that a ring of 6′ threonines was required for picrotoxin sensitivity. On the other hand, the 2′ residue has been found to be important for picrotoxin sensitivity in the $GABA_AR$ (ffrench-Constant *et al*. 1993; Xu *et al*. 1995), the $GABA_CR$ (Wang *et al*. 1995) and the GLUClR (Etter *et al*. 1999). In each of these studies, a ring of threonines was also present at the 6′ position in picrotoxin-sensitive receptors. Thus, a ring of 6′ threonines is a common requirement for picrotoxin sensitivity in members of the LGIC superfamily, whereas the identity of the residue at the 2′ position varies widely amongst picrotoxin-sensitive receptors. Therefore, if members of the LGIC superfamily share a common site of action for picrotoxin, it is likely to be formed by a ring of threonines at the 6′ position. However, as the present study has shown, mutations at the 2′ position may be able to disrupt this sensitivity. Furthermore, by analogy with the $GABA_AR$, it would appear that the incorporation of only a single phenylalanine at the 6′ position is sufficient to impart picrotoxin sensitivity (Gurley *et al*. 1995).

α/T258 is important for receptor gating

There is strong evidence that picrotoxin acts as an allosteric inhibitor of the GlyR as previous work has demonstrated that picrotoxin acts as a competitive antagonist of the α/WT GlyR and that mutations which affect channel gating cause drastic alterations to its mode of action (Lynch *et al*. 1995). By showing that α/T258C is exposed to the channel lumen in the open state only (Table 1 and Fig. 4) and that mutations to this residue cause dramatic changes to the apparent glycine affinity and cooperativity (Table 1 and Fig. 8), the present study suggests that α/T258 may contribute to the receptor gating mechanism. Such a property is expected for a residue that forms a critical component of the picrotoxin allosteric inhibition pathway. The corresponding 6′ residues of the $GABA_AR$ (Xu *et al*. 1995; Xu and Akabas 1996) and the nAChR (Devillers-Thiery *et al*. 1992; White and Cohen 1992) have also been shown to be a major determinants of receptor gating.

Although the present study does not unequivocally resolve whether picrotoxin binds to α/T258, two considerations are worthy of note. First, α/T258C is not accessible to MTSET in the channel closed state, whereas picrotoxin binds rapidly to the α/WT GlyR in the closed state (Fig. 2 in Lynch *et al*. 1995). Furthermore, since this residue increases its exposure to the channel lumen in the open state, it might be expected that the picrotoxin affinity should increase with glycine concentration if picrotoxin is binding at this site. However, in the α/WT GlyR, picrotoxin inhibitory potency diminishes as glycine concentration is increased (Lynch *et al*. 1995). Thus, the evidence presented in this study does not support the idea of picrotoxin binding to threonine 6′. Because the α/T258C mutation severely reduced the picrotoxin sensitivity (Table 1), it was not possible to use sulfhydryl modifying agents in an attempt to further investigate the possibility of picrotoxin binding to this site (Xu *et al*. 1995).

α subunit mutations influence the apparent glycine affinity

The glycine EC_{50} is affected by mutations at the 6′ position of the α subunit, whereas the mutations at the corresponding position of the β subunit have no effect (Fig. 8). Since β subunits do not contain glycine binding sites (Grenningloh *et al*. 1990), it appears that conformational changes associated with glycine binding are not symmetrically transmitted from the α to the β subunit. This appears inconsistent with results from the nAChR, where mutation of leucine 9′ in the α, β, γ and δ subunits altered the EC_{50} equally, despite the existence of ligand binding sites on the α subunit only (Filatov and White 1995; Labarca *et al*. 1995). A similar finding has also been made in αβγ heteromeric $GABA_AR$s (Chang and Weiss 1999). However, subunit-dependent differences in the effects of M2 domain mutations have been observed in the $GABA_AR$. For example, in the 12′ position of the $GABA_AR$, mutations to the β subunit dominate GABA and pentobarbitone-mediated conformational changes (Birnir *et al*. 1997). In addition, depending on the nature of the substituted residue at the 9′ position, the $GABA_AR$ can exhibit subunit-specific alterations in receptor affinity and desensitisation (Dalziel *et al*. 2000). However, further experiments are required to address the question of why glycine sensitivity is subunit-dependent whereas picrotoxin sensitivity is not.

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