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Comparative Surface Accessibility of a Pore-lining Threonine Residue (T6′) in the Glycine and $GABA_A$ Receptors*

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The substituted cysteine accessibility method was used to probe the surface exposure of a pore-lining threonine residue (T6′) common to both the glycine receptor (GlyR) and γ-aminobutyric acid, type A receptor ($GABA_AR$) chloride channels. This residue lies close to the channel activation gate, the ionic selectivity filter, and the main pore blocker binding site. Despite their high amino acid sequence homologies and common role in conducting chloride ions, recent studies have suggested that the GlyRs and $GABA_ARs$ have divergent open state pore structures at the 6′ position. When both the human $\alpha1_{T6'C}$ homomeric GlyR and the rat $\alpha1_{T6'C}\beta1_{T6'C}$ heteromeric $GABA_AR$ were expressed in human embryonic kidney 293 cells, their 6′ residue surface accessibilities differed significantly in the closed state. However, when a soluble cysteine-modifying compound was applied in the presence of saturating agonist concentrations, both receptors were locked into the open state. This action was not induced by oxidizing agents in either receptor. These results provide evidence for a conserved pore opening mechanism in anion-selective members of the ligand-gated ion channel family. The results also indicate that the $GABA_AR$ pore structure at the 6′ level may vary between different expression systems.

The ligand-gated ion channel (LGIC)[1] superfamily includes the nicotinic acetylcholine receptor (nAChR), serotonin type 3 receptor ($5HT_3R$), $GABA_A$ receptor ($GABA_AR$), and glycine receptor (GlyR), as well as invertebrate glutamate and histidine receptors (1). Functional receptors of this family comprise five homologous subunits arranged in a ring to form a central ion-conducting pore. Each subunit is composed of a large extracellular ligand-binding N-terminal domain, four membrane-spanning segments (M1–M4), and a large intracellular domain between M3 and M4.

The pore-lining, second transmembrane (M2) domain has an α-helical secondary structure that undergoes a conformational change as the channel is opened (2). To investigate this process in detail, state-dependent differences in the surface exposure of M2 domain residues can be assayed using the substituted cysteine accessibility method (3). In this technique, residues are mutated individually to cysteines, and changes in their reactivity rates with soluble cysteine-reactive reagents can identify structural changes between different functional states. As expected for receptors belonging to the same family, this technique has generally yielded a good correlation between the open state M2 domain secondary structures of the nAChR (4–7), $GABA_AR$ (8), and $5HT_3R$ (9, 10).

The M2 domain 6′ residue, which is a threonine in the GlyR α1 subunit and the $GABA_AR$ α1 and β1 subunits (see Fig. 1*A*), lines a critical part of the pore. It is close to the activation gate (6, 11, 12) and the ionic selectivity filter (13–15) and forms the main pore blocker binding site (reviewed in Ref. 16). Therefore, structural differences at this level may be expected to have significant functional consequences. In the homomeric $\alpha1_{T6'C}$ GlyR expressed in a mammalian HEK293 cell line, Shan *et al.* (17) concluded that the surface exposure of introduced 6′ cysteines was increased in the channel open state. In contrast, in the $\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_AR$ expressed in *Xenopus* oocytes, the 6′ cysteines were found to be exposed in the closed state and rotated to face the adjacent subunits in the open state (18). Thus, despite having a high M2 domain amino acid sequence homology (see Fig. 1*A*) and a common function in conducting chloride ions, the GlyR and $GABA_AR$ appear to be structurally divergent at this position.

The aim of this study was to conduct a detailed comparative study into the surface accessibility of the 6′ cysteines in the GlyR and $GABA_AR$ when both are expressed recombinantly in a common (HEK293 cell) expression system. The main findings are that the respective pore structures at the 6′ positions are significantly different in the closed states but that there appear to be similarities in the mechanisms of channel opening. The results also reveal distinct differences in the structural and functional properties of $GABA_ARs$ depending on whether they are expressed in *Xenopus* oocytes or HEK293 cells.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression of GlyR and $GABA_AR$ cDNAs—The human GlyR α1 subunit cDNA was subcloned into the pCIS2 plasmid vector, and the rat $GABA_AR$ α1 and β1 subunit cDNAs were subcloned into the pIRES2-EGFP plasmid vector (Clontech, Palo Alto, CA). Site-directed mutagenesis was performed using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA), and the successful incorporation of mutations was confirmed by sequencing the clones. Adenovirus-transformed HEK293 cells (ATCC CRL 1573) were passaged in a 50:50

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[1] The abbreviations used are: LGIC, ligand-gated ion channel; nAChR, nicotinic acetylcholine receptor; GABA, γ-aminobutyric acid; $GABA_AR$, GABA, type A receptor; GlyR, glycine receptor; MTSET, methanethiosulfonate ethyltrimethylammonium; MTSEA, methanethiosulfonate ethylammonium; Cu:phen, copper-*O*-phenanthroline; DTT, dithiothreitol; EC_{50}, half-saturating concentration; n_H, Hill coefficient; I_{max}, maximum (saturating) current magnitude; MTSES, methanethiosulfonate ethylsulfonate; MTS, methanethiosulfonate; HEK, human embryonic kidney; WT, wild-type.

mixture of minimal essential medium and Dulbecco's modified Eagle's medium supplemented with 2 mM glutamate, 10% fetal calf serum and the antibiotics, penicillin (at 50 IU/ml), and streptomycin (at 50 μg/ml). Cells were transfected using a calcium phosphate precipitation protocol (19). When co-transfecting the $GABA_AR$ α1 and β1 subunits, their respective cDNAs were combined in a ratio of 1:1. 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. Cells were perfused by a control solution that contained the following (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 megohms when filled with the standard pipette solution, which contained the following (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, Union City, CA). The cells were perfused by a parallel array of microtubular barrels through which solutions were gravity-induced. All experiments were conducted at room temperature (19–22 °C).

Methanethiosulfonate ethyltrimethylammonium (MTSET) and methanethiosulfonate ethylammonium (MTSEA) were obtained from Toronto Research Chemicals (Toronto, Ontario, Canada), whereas all other reagents were obtained from Sigma. MTSET and MTSEA were dissolved directly into the bath solution at the final concentrations of 1 and 2.5 mM, respectively, unless indicated otherwise. The oxidizing reagent, copper-*O*-phenanthroline (Cu:phen) was prepared by mixing $CuSO_4$ (stored as 100 mM stock solution in H_20 at −10 °C) and 1,10-phenanthroline (stored as 400 mM stock solution in ethanol at −10 °C). The final concentrations of copper and 1,10-phenanthroline in the control bathing solution were 100 and 400 μM, respectively. H_2O_2, maintained as a 30% stock solution, was diluted to 0.3% in the control bathing solution. MTSET, MTSEA, Cu:phen, and H_2O_2 were used for no longer than 10 min after being dissolved into the bathing solution at room temperature. The disulfide-reducing reagent, dithiothreitol (DTT), was prepared daily as a 1 or 10 mM solution in control bathing solution.

The effects of all sulfhydryl-specific reagents were tested using the following procedure. After establishment of the recording configuration, two brief applications of agonist at the half-saturating (EC_{50}) concentration were followed by two brief applications at a saturating (10–20 × 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 sulfhydryl-specific reagents, cells were washed in control solution for at 1–3 min before the EC_{50} and EC_{100} agonist-activated currents were measured again.

Data Analysis—All data were analyzed using Origin 4.0 (Northampton, MA) or Sigmastat 1.0 (Jandel Scientific). Results are expressed as means ± S.E. of three or more independent experiments. The empirical Hill equation, fitted by a non-linear least squares algorithm, was used to calculate the EC_{50} and Hill coefficient (n_H) values for glycine and GABA activation. Statistical significance was determined by either linear regression or by one-way analysis of variance using the Student's-Newmans-Keul *post hoc* test for unpaired data, with $p < 0.05$ representing significance.

RESULTS

Sulfhydryl Modification of the $\alpha 1_{T6'C}$ GlyR—This study investigated the surface accessibility of the 6′ residues of the GlyR α1 subunit and the $GABA_AR$ α1 and β1 subunits. As shown in Fig. 1, each of the WT receptor subunits contains a threonine at this position. In this study the threonines were mutated to cysteines to enable cysteine-specific reagents to be used as probes of 6′ surface accessibility (3). The GlyR α1 subunit also contained the C41A mutation, which eliminated the only uncross-linked external cysteine. The $GABA_AR$ α1 and β1 subunits contained no uncross-linked external cysteines.

The mean EC_{50}, n_H, and I_{max} values for glycine-activated currents in the $\alpha 1_{WT}$ and $\alpha 1_{T6'C}$ GlyRs are summarized in Table I. In the absence of glycine, there was no significant difference in the resting conductance of cells expressing $\alpha 1_{WT}$ and $\alpha 1_{T6'C}$ GlyRs, implying that the T6′C mutation did not induce a steady-state leak conductance through the channels.

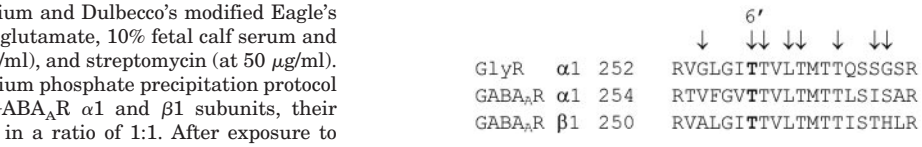

FIG. 1. **Amino acid sequence alignment of the M2 transmembrane segments of human GlyR α1 subunit and the rat $GABA_AR$ α1 and β1 subunits.** The residues mutated to cysteine in this study are indicated in *bold* and numbered 6′ according to the system of Miller (26), which assigns 1′ to the most intracellular M2 domain residue and 20′ to the most extracellular residue. *Arrows* denote those residues in the $GABA_AR$ α1 subunit that are exposed to the channel lumen (8).

We demonstrated previously that a 1-min application of 1 mM MTSET had no significant effect on the $\alpha 1_{WT}$ GlyR regardless of whether it was applied in the closed or channel open states (17, 20). Similarly, MTSET had no effect on the $\alpha 1_{T6'C}$ GlyR when applied in the closed channel state (17). However, when MTSET was applied to the $\alpha 1_{T6'C}$ GlyR in the presence of a saturating (0.5 mM) concentration of glycine, the channels remained partially activated following the removal of glycine and MTSET (17). Following the removal of glycine, the currents declined to 86 ± 2.4% ($n = 6$) of the control current magnitude and remained stable at this level until closed by a 1-min application of 10 mM DTT (*e.g.* Fig. 2*A*). When 0.5 mM glycine was applied to the MTSET-modified GlyRs, it reversibly activated an additional current component (Fig. 2*A*). At any given time after the completion of the MTSET treatment, the total magnitude of the locked-open plus glycine-gated current was larger than that which could be activated in the same cell by a continuous application of 0.5 mM glycine alone. This point is illustrated in Fig 2, *A–C*. Fig. 2*B* shows the effect of a long application of 0.5 mM glycine to the same cell as in Fig. 2*A*, and both traces are shown superimposed in Fig. 2*C*. This experiment was repeated in five cells, and the relative current magnitudes were quantitated at a common time point 2 min after the initial application of glycine. It was found that an application of 0.5 mM glycine to the MTSET-modified GlyRs resulted in a net current magnitude that was 167 ± 6% ($n = 5$) larger than that activated in the same cell by a continuous application of 0.5 mM glycine alone. Together, these observations indicate that MTSET locked the channels into the open state but did not lock significant numbers of channels into either the closed or desensitized states. The MTSET-induced increase in net current magnitude at late times was most likely because of a reduced transition rate from the open to the desensitized state.

Because MTSET induced no current change in the presence of a saturating glycine concentration, its reaction rate in the fully activated state could not be measured. However, in the presence of an EC_{50} (30 μM) concentration of glycine, the reaction proceeded with a time constant of 1.2 ± 0.1 s ($n = 4$), indicating a reaction rate of around 830 M^{-1} s^{-1}. This is about 250 times smaller than the rate constant for the reaction of MTSET with 2-mercaptoethanol in free solution, the decrease because of electrostatic repulsion, steric hindrance, or suppressed ionization of the cysteine thiol (3). The possible contributions of these factors to the reactivity of T6′C are considered further below.

When applied at a concentration of 10 mM for 60 s, MTSES had no significant effect on either the $\alpha 1_{WT}$ or $\alpha 1_{T6'C}$ GlyRs regardless of whether it was applied in the absence or presence of a saturating concentration of glycine (17). In addition, a prior MTSES application in either the closed or open state did not significantly attenuate the ability of MTSET to lock the $\alpha 1_{T6'C}$

TABLE I
Glycine and picrotoxin effects at α1 GlyRs incorporating the indicated amino acid substitutions at the 6′ position

6′ residue	Glycine				Picrotoxin			
	EC_{50}	n_H	I_{max}	n	[Glycine]	IC_{50}	n_H	n
	μM		nA		μM	μM		
Thr (WT)[a]	26 ± 9	3.4 ± 0.3	2.2 ± 1.1	4	30	18 ± 1	1.5 ± 0.1	5
Phe	6.4 ± 1.1[b]	1.5 ± 0.1	1.1 ± 0.28	6	5	706 ± 140[b]	0.9 ± 0.05	4
Ala	1.4 ± 0.3[b]	1.1 ± 0.2	1.2 ± 0.28	8	0.8	388 ± 73[b]	1.1 ± 0.3	5
Cys	52 ± 1[b]	1.6 ± 0.1	1.4 ± 0.29	4	50	595 ± 91[b]	0.8 ± 0.1	5
Gly	279 ± 97[b]	1.9 ± 0.2	2.6 ± 0.40	4	280	332 ± 55[b]	0.7 ± 0.02	3
Leu	68 ± 12[b]	2.1 ± 0.3	2.1 ± 0.80	4	70	339 ± 39[b]	1.5 ± 0.3	4
Tyr[c]	1.1 ± 0.05[b]	ND[d]	0.46 ± 0.07	4	ND	ND	ND	ND
	(0.9 ± 0.11)[b]	(1.8 ± 0.12)	(0.99 ± 0.04)	(3)				
Ser	ND	ND	<0.1[b]	15	ND	ND	ND	ND
Glu	ND	ND	<0.2[b]	20	ND	ND	ND	ND
Lys	ND	ND	<0.1[b]	21	ND	ND	ND	ND
Gln	ND	ND	<0.1[b]	17	ND	ND	ND	ND

[a] Results for the WT and Phe, Ala, and Cys mutants are reproduced from Shan *et al.* (17).
[b] The data are significantly different from WT GlyR ($p < 0.05$).
[c] The glycine n_H was not given, because trace glycine in the control solution distorted the current magnitude at lower glycine concentrations. As a correction, data shown in parentheses were recorded from cells that were switched from 1 μM strychnine immediately into glycine-containing solutions.
[d] ND, not determined.

GlyR into the open state (17). Thus, MTSES did not react with T6′C.

A 60-s application of 2.5 mM MTSEA also had no significant effect on the $\alpha1_{WT}$ GlyR regardless of whether it was applied in the closed or open states (Table II). Similarly, when applied in the closed state to the $\alpha1_{T6'C}$ GlyR, 2.5 mM MTSEA had no significant effect on the magnitude of currents activated by an EC_{50} (30 μM) or a saturating (500 μM) concentration of glycine (Table II). In addition, prior exposure of the $\alpha1_{T6'C}$ GlyR to MTSEA in the closed state did not significantly affect the ability of a subsequent application of 100 μM MTSET plus 500 μM glycine to lock the channels open (Fig. 2*D*). A 60-s application of MTSEA plus 500 μM glycine also had no effect on the magnitude of currents activated by either 20 or 500 μM glycine (Table II), although it dramatically attenuated the effect of a subsequent application of MTSET (Fig. 2*E*). As shown in Table II, MTSET plus 500 μM glycine caused 85 ± 3% of channels to be locked into the open state, while simultaneously reducing the magnitude of the glycine-activable current by 88 ± 2% (both $n = 3$). Following MTSEA exposure, MTSET plus 500 μM glycine caused only 16 ± 5% of channels to be locked into the open state while reducing the magnitude of the glycine-activable current by 17 ± 8% (both $n = 4$). Both of these values are significantly different from those obtained without MTSEA pre-treatment. Taken together, these results provide strong evidence that MTSEA modifies T6′C in the channel open state but not in the closed state.

The effects of MTSEA were likely to have been caused by the covalent attachment of an ethylammonium group to the 6′ cysteine in the open state. The inability of MTSEA modification to lock the channels open may have been because of the smaller size of MTSEA relative to MTSET. On the other hand, the effects of MTSET may have been because of one of two mechanisms. One possibility is that it directly modified the 6′ cysteines by covalently attaching an ethyltrimethylammonium group. In this case the reaction would have proceeded only in the open state, and the resulting cysteine modification would have maintained the pore in the open state. However, because the methanethiosulfonate (MTS) group contains a disulfide bond that could directly catalyze the formation of other disulfide bonds, it is also possible that MTSET may have behaved as an oxidizing agent; MTSET can add thioethyltrimethylammonium to one cysteine, and a second cysteine can displace this group in a sulfhydryl-disulfide interchange to generate a cystine-cysteine disulfide. MTSET could thereby induce the formation of disulfide bonds between subunits, preventing the channels from closing.

To discriminate between these two possibilities, we tested the effects of oxidizing reagents on the GlyR. We examined the effects of 1-min applications of 0.3% H_2O_2 and 100:400 μM Cu:phen on the $\alpha1_{WT}$ and $\alpha1_{T6'C}$ GlyRs. As summarized in Table II, neither reagent had any effect on either the half-maximal or maximal current magnitudes of the $\alpha1_{WT}$ or the $\alpha1_{T6'C}$ GlyRs. Furthermore, neither reagent was able to mimic the effect of MTSET in maintaining the $\alpha1_{T6'C}$ GlyR in the open state ($n = 3$ for each reagent). An example of such an experiment on the $\alpha1_{T6'C}$ GlyR is shown in Fig. 3. Although Cu:phen induced a weak transient inhibition, it had no irreversible effects (Fig. 3*B*). The H109A mutation, which eliminates zinc inhibition (21), had no effect on this transient inhibitory action of copper (data not shown).

Cysteine reactivity with thiol-containing compounds is determined by the local electrostatic potential, the sulfhydryl ionization state, and steric accessibility of the MTS reagent to the sulfhydryl group (3). Unfortunately, it was not possible to determine the contribution of electrostatic potential changes as the only available soluble, negatively charged MTS derivative, MTSES, had no measurable effect (17). However, it is unlikely that electrostatic potential changes alone would have been able to account for the infinitely large observed reaction rate difference (see Ref. 5). Thus, the reaction rate was likely to have been dominated by the sulfhydryl ionization state or steric accessibility. Because the MTS reaction rate increases dramatically with thiol ionization (22), and thiol ionization is suppressed in a hydrophobic environment, one possibility is that the 6′ cysteines exist in a hydrophobic environment in the closed state (perhaps by facing the protein interior) and increase their exposure to the aqueous environment in the open state. An equally plausible alternative is that the 6′ cysteines remain in an aqueous environment in the closed state but that access of the externally applied MTS reagents in the closed state is precluded by either an electrostatic impediment or pore constriction external to the 6′ position. In either scenario, the access of MTSET to the 6′ cysteines is increased in the open state, and MTSET holds the channel open by covalently attaching a positively charged ethyltrimethylammonium group to T6′C.

Sulfhydryl Modification of the $\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_AR$—Both of the above models contrast dramatically with results obtained recently on the structurally and functionally homologous $GABA_AR$ by Horenstein *et al.* (18). That study investigated the

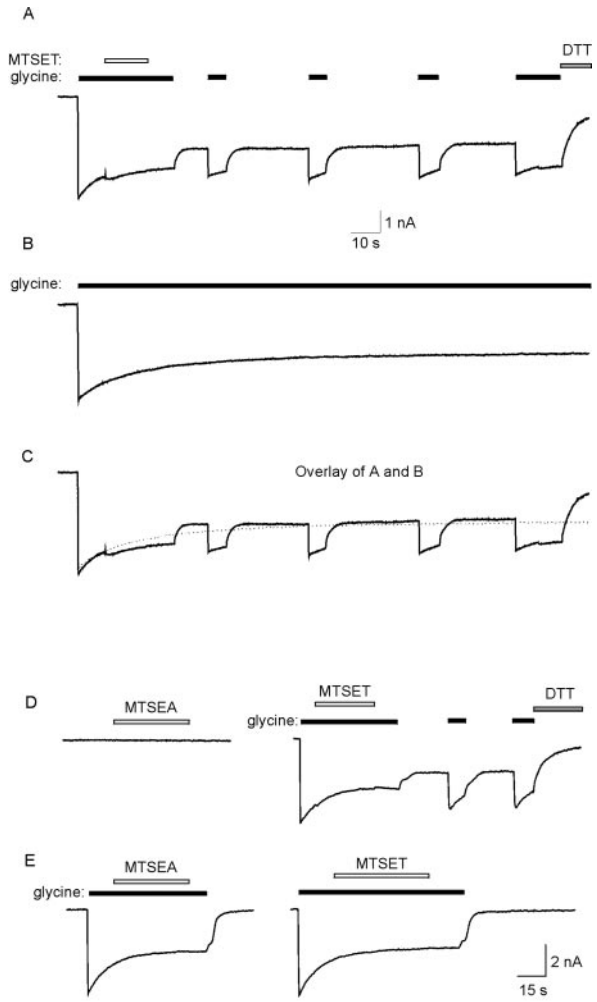

FIG. 2. **Effects of MTSET and MTSEA on the $\alpha 1_{T6'C}$ GlyR.** *A*, currents were activated by 0.5 mM glycine, and MTSET was applied at a concentration of 100 μM for the period indicated by the *unfilled bar*. The channels remained partially activated upon the withdrawal of glycine, and a subsequent glycine application reversibly activated an additional current. Channels were closed only by the application of 10 mM DTT. *Scale bars* apply to all traces in *A–C*. *B*, effect of a long application of 0.5 mM glycine to the same cell as in *A*. *C*, superposition of the traces in *A* and *B* reveals the increase in net magnitude of glycine-gated currents following MTSET modification. *D*, both traces were recorded sequentially from the same cell. The *left* trace shows the effect of 2.5 mM MTSEA in the closed state. The *right panel* shows that MTSEA pre-treatment does not affect the ability of 100 μM MTSET plus 0.5 mM glycine to lock the channels in the open state. *E*, both traces were recorded sequentially from the same cell. The *left trace* shows the lack of effect of 2.5 mM MTSEA when co-applied with 0.5 mM glycine. The *right panel* shows that MTSEA pre-treatment abolishes the ability of 100 μM MTSET plus 0.5 mM glycine to lock the channels in the open state. All displayed currents in *D* and *E* were recorded from the same cell, and *scale bars* apply to all traces.

state-dependent reactivity changes of the T6′C residues in the rat $\alpha 1_{T6'C}\beta 1_{T6'C}$ $GABA_AR$ expressed recombinantly in *Xenopus* oocytes. They concluded that the T6′C residues are exposed to the external aqueous environment in the closed state and rotate to face the adjacent subunit when the channel is opened. Furthermore, when applied in the open state, Cu:phen promotes the formation of an intersubunit disulfide bond between adjacent $\beta 1$ subunits that locks the channel in the open state (18). We examined the effects of cysteine-reactive reagents on the rat $\alpha 1_{WT}\beta 1_{WT}$ and $\alpha 1_{T6'C}\beta 1_{T6'C}$ $GABA_ARs$ expressed recombinantly in mammalian HEK293 cells.

The mean EC_{50}, n_H, and I_{max} values for GABA-activated currents in the WT and mutant $GABA_ARs$ are summarized in Table III. We were surprised to find that incorporation of the T6′C mutations into both the $\alpha 1$ and $\beta 1$ subunits resulted in a dramatic increase in the rate of desensitization (*e.g.* Fig. 4*A*). In the presence of a saturating 20 μM (10 × EC_{50}) GABA concentration, the $\alpha 1_{WT}\beta 1_{WT}$ $GABA_AR$ desensitized with a time constant of 1370 ± 280 ms (n = 4) whereas in the presence of 100 μM (20 × EC_{50}) GABA, the $\alpha 1_{T6'C}\beta 1_{T6'C}$ $GABA_AR$ desensitized with a time constant of 87 ± 2 ms (n = 4). This rapid desensitization rate made it difficult to apply cysteine-modifying reagents with a high degree of confidence to the channel open state. In the absence of GABA, there was no significant difference in the resting conductance of cells expressing $\alpha 1_{WT\beta}1_{WT}$ and $\alpha 1_{T6'C}\beta 1_{T6'C}$ $GABA_ARs$, implying that the mutations did not induce a steady-state leak conductance through the receptors.

When activated by 20 μM GABA, the $\alpha 1_{WT}\beta 1_{WT}$ $GABA_AR$ was weakly but significantly potentiated by a 2-min application of 10 mM DTT (see Fig. 4*B* and Table II). Upon removal of DTT, currents gradually returned to the control magnitude over the following 3–5 min. This effect is similar to that observed when the same receptors are expressed in *Xenopus* oocytes (18). In contrast to this relatively modest effect, a 10 mM application of DTT caused a dramatic potentiation of the $\alpha 1_{T6'C}\beta 1_{T6'C}$ $GABA_AR$ when activated by 100 μM GABA (see Fig. 4*C* and Table II). It appears that the T6′C residues of both the $\alpha 1$ and $\beta 1$ subunits contributed to this effect as DTT had a similar effect on the $\alpha 1_{WT}\beta 1_{T6'C}$ $GABA_AR$ and the $\alpha 1_{T6'C}\beta 1_{WT}$ $GABA_AR$ (Table II). The DTT-potentiated currents in the $\alpha 1_{T6'C}\beta 1_{T6'C}$ $GABA_AR$ declined progressively when the cell was perfused in DTT-free bathing solution (Fig. 4*C*). The potentation observed in both the WT and mutant receptors may have been because of either the reduction of endogenous disulfide bonds or a pharmacological effect of DTT at the alcohol or anesthetic binding site (23). To discriminate between these two possible modes of action, we investigated the effect of 200 mM ethanol in the presence of a saturating (100 μM) GABA concentration on both the $\alpha 1_{WT\beta}1_{WT}$ $GABA_AR$ and the $\alpha 1_{T6'C}\beta 1_{T6'C}$ $GABA_AR$. As summarized in Table II, ethanol had no significant effect on either receptor, indicating that DTT was acting by reducing endogenous disulfide bonds.

When applied in the closed channel state, Cu:phen had no effect on the $\alpha 1_{WT\beta}1_{WT}$ $GABA_AR$ (Table II, Fig. 5*A*, *left panel*). However, in the $\alpha 1_{T6'C}\beta 1_{T6'C}$ $GABA_AR$, the rate of current reduction upon removal of DTT was accelerated dramatically by Cu:phen (Fig. 5*B*). Following the removal of DTT, the GABA-activated current reduced to 76 ± 3% (n = 3) after 20 s in the standard bathing solution. However, in the presence of Cu:phen, the GABA-activated current magnitude reduced to 3.3 ± 2% (n = 3) of control magnitude after 20 s. When combined with the results obtained using DTT, these results indicate that disulfide bonds form spontaneously, but relatively slowly, in the closed state in the $\alpha 1_{T6'C}\beta 1_{T6'C}$ $GABA_AR$. Because this slow rate of disulfide bond formation complicated investigations into the reactivity of the 6′ cysteines, all subsequent experiments on $\alpha 1_{T6'C}\beta 1_{T6'C}$ $GABA_ARs$ in the closed state were performed immediately following a 2-min exposure to 10 mM DTT to ensure that all 6′ cysteines were in the reduced state. Then, the effects of subsequent pharmacological manipulations were compared with the effects of spontaneous disulfide formation in the same cell.

When applied in the presence of 20 μM GABA, Cu:phen had no effect on the $\alpha 1_{WT}\beta 1_{WT}$ $GABA_AR$ (Table II, Fig. 5*A*, *right panel*). However, when Cu:phen was applied to the $\alpha 1_{T6'C}\beta 1_{T6'C}$ $GABA_AR$ in the presence of 100 μM GABA, it had two distinct effects. First, it reversibly reopened the channel from the desensitized state (Fig. 5*C*). Second, following the removal of Cu:phen,

TABLE II

Effects of cysteine-modifying reagents on WT and mutant GlyRs and $GABA_A$Rs

*, significant relative to the corresponding WT response ($p < 0.05$); **, highly significant relative to the corresponding WT response ($p < 0.01$); ND, not determined.

Chemical treatment	Receptor	Applied without agonist				Applied with saturating concentration of agonist			
		% change in base line[a]	% change in EC_{50} current	% change in saturating current	n	% change in base line[a]	% change in EC_{50} current	% change in saturating current	n
DTT (10 mM)	$\alpha1_{WT}$ GlyR	ND	ND	−1.0 ± 2.0	3	ND	ND	ND	ND
	$\alpha1_{T6'C}$ GlyR	ND	ND	8.0 ± 3.8	3	ND	ND	ND	ND
	$\alpha1_{WT}\beta_{WT}$ $GABA_A$R	ND	ND	27 ± 11	11	ND	ND	ND	ND
	$\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_A$R	ND	ND	460 ± 110**	13	ND	ND	ND	ND
	$\alpha1_{T6'C}\beta1_{WT}$ $GABA_A$R	ND	ND	158 ± 59*	6	ND	ND	ND	ND
	$\alpha1_{WT}\beta1_{T6'C}$ $GABA_A$R	ND	ND	200 ± 69**	5	ND	ND	ND	ND
Ethanol (200 mM)	$\alpha1_{WT}\beta1_{WT}$ $GABA_A$R	ND	ND	2.7 ± 4.9	3	ND	ND	ND	ND
	$\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_A$R	ND	ND	−1 ± 9	3	ND	ND	ND	ND
MTSET (1 mM)	$\alpha1_{WT}$GlyR	0.7 ± 0.7	2.7 ± 1.2	−1.0 ± 2.9	3	3.3 ± 1.8	−5.5 ± 3.5	−9.7 ± 4.7	3
	$\alpha1_{T6'C}$ GlyR	0.7 ± 0.7	−11 ± 6	−13 ± 6	3	85 ± 3**	−84 ± 3**	−88 ± 2**	3
	$\alpha1_{WT}\beta1_{WT}$ $GABA_A$R	−0.2 ± 0.7	ND	−12 ± 7	5	1.4 ± 0.7	ND	−28 ± 6	7
	$\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_A$R	0 ± 0	ND	−94 ± 4**	3	100 ± 0**	ND	−100 ± 0**	3
MTSEA (2.5 mM)	$\alpha1_{WT}$ GlyR	−0.5 ± 1.0	2.0 ± 8.0	−8.8 ± 3.9	4	−0.7 ± 0.3	−8.3 ± 8.9	−28 ± 5.5	3
	$\alpha1_{T6'C}$ GlyR	−3 ± 1.8	16 ± 6.8	1.8 ± 3.3	5	6.5 ± 4.5	−9.7 ± 14	−32 ± 6.7	6
	$\alpha1_{WT}\beta1_{WT}$ $GABA_A$R	−2.3 ± 0.9	ND	−7.3 ± 2.7	3	−3.3 ± 2.8	ND	−15 ± 7.6	3
	$\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_A$R	30 ± 5**	ND	−81 ± 3.8**	3	100 ± 0**	ND	−99 ± 0.7**	3
Cu:phen (0.1:0.4 mM)	$\alpha1_{WT}$ GlyR	−1.0 ± 1.2	3.0 ± 5.1	1.3 ± 6.4	3	−1.3 ± 0.9	−4.7 ± 6.8	3.7 ± 8.1	3
	$\alpha1_{T6'C}$ GlyR	−1.0 ± 1.0	−5.7 ± 2.3	−9.0 ± 2.5	3	−4.0 ± 2.5	3.7 ± 9.8	−14 ± 6.6	3
	$\alpha1_{WT}\beta1_{WT}$ $GABA_A$R	−0.2 ± 0.2	ND	−16 ± 2.2	5	0 ± 0	ND	−7.8 ± 8.2	4
	$\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_A$R	0.7 ± 0.7	ND	−98 ± 2.3**	3	3.3 ± 3.3	ND	−91 ± 2**	4
H_2O_2 (0.3%)	$\alpha1_{WT}$ GlyR	−6.0 ± 3.5	12 ± 13	−2.7 ± 5.4	3	−7.3 ± 1.2	15 ± 7.7	−5.3 ± 8.1	3
	$\alpha1_{T6'C}$ GlyR	−0.7 ± 0.7	7.3 ± 4.7	−8.0 ± 3.5	3	−1.3 ± 9.0	−8.3 ± 7.8	−11 ± 7.2	3

[a] Changes in base line are expressed as a percentage of the saturating agonist-activated current. An irreversible increase in inward current following treatment is represented as a positive percentage.

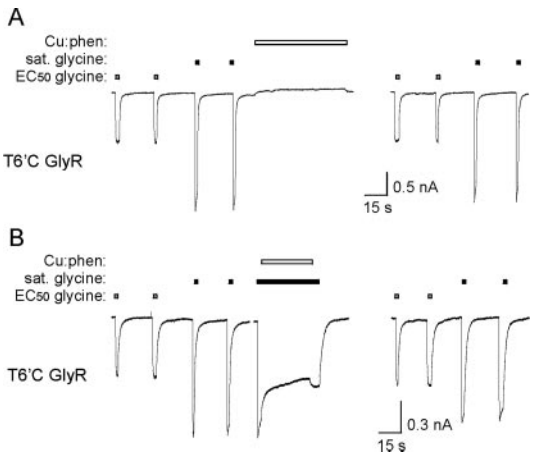

FIG. 3. **Effect of Cu:phen on the $\alpha1_{T6'C}$ GlyR.** Cu:phen had no irreversible effect on the $\alpha1_{T6'C}$ GlyR, regardless of whether it was applied in the channel closed state (*A*) or open state (*B*). Glycine was applied at concentrations of 50 μM (EC_{50}) and 0.5 mM (saturating; *sat.*), as indicated.

the peak magnitude of GABA-activated currents was decreased dramatically (see Table II and Fig. 5*C*). This reduction in current was not spontaneously reversible but was reversed by a 30–60-s application of 10 mM DTT (see Table II and Fig. 5*C*).

We were surprised by the ability of GABA + Cu:phen to reopen the channels and investigated this phenomenon further. The reopening effect was found to require the simultaneous presence of GABA and Cu:phen. If either reagent was removed, the receptor immediately resumed a non-conducting configuration ($n = 5$ for each condition). Application of 100 μM $CuSO_4$ in the presence of GABA caused no detectable current activation ($n = 3$ cells), thus eliminating a putative pharmacological action of copper. Furthermore, in the continuous presence of GABA, a second application of Cu:phen elicited a current of similar magnitude to the first ($n = 3$ cells). This last observation eliminated the possibility that the formation of disulfide bonds following the first application of Cu:phen may have closed the channels and prevented Cu:phen from subsequently reopening them. Finally, H_2O_2 also caused a dramatic 87 ± 3% ($n = 4$) reduction in the magnitude of the GABA-activable current that was reversed by 10 mM DTT (Fig. 5*D*). However, H_2O_2 did not activate the receptors convincingly. Although Cu:phen activated a current with a magnitude of 28 ± 3% ($n = 3$) of the saturating GABA-activated current magnitude, H_2O_2 activated a current of only 7 ± 2% ($n = 4$) of the saturating GABA current magnitude. This difference was significant ($p < 0.05$) using a one-way analysis of variance.

MTSET was used to further investigate the state-dependent surface accessibility of the 6′ cysteines. MTSET had no signif-

TABLE III
EC_{50}, n_H, and I_{max} values for GABA-activated currents in WT and mutant $GABA_A$Rs

$GABA_A$R	GABA EC_{50}	n_H	I_{max}	n
	μM		nA	
$\alpha1_{WT}\beta1_{WT}$ $GABA_A$R	1.7 ± 0.3	2.2 ± 0.6	2.7 ± 1.7	3
$\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_A$R	5.4 ± 1.8	1.0 ± 0.1	0.35 ± 0.06	3
$\alpha1_{T6'C}\beta1_{WT}$ $GABA_A$R	3.1 ± 0.8	1.4 ± 0.1	0.86 ± 0.28	3
$\alpha1_{WT}\beta1_{T6'C}$ $GABA_A$R	0.43 ± 0.07	1.4 ± 0.4	1.7 ± 0.8	3

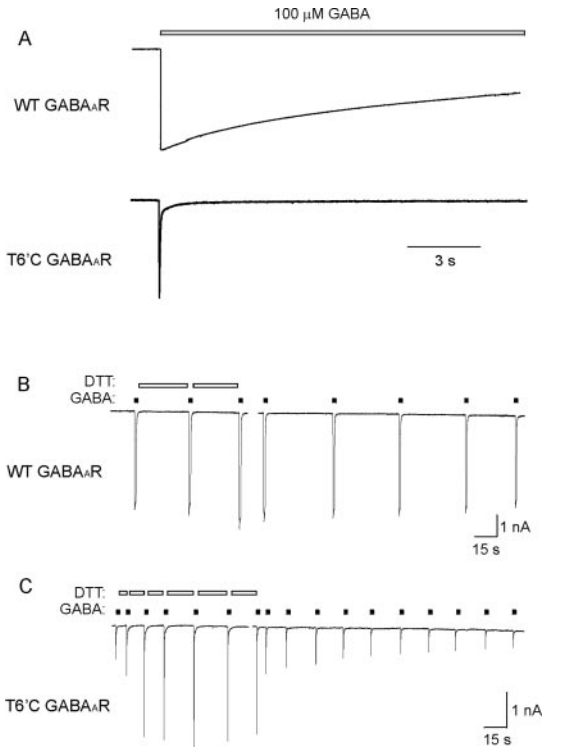

FIG. 4. **Effect of 10 mM DTT on $GABA_A$R current magnitude.** *A*, comparative responses of the $\alpha1_{WT}\beta1_{WT}$ $GABA_A$R and the $\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_A$R to a long application of 100 μM GABA. *B*, in the $\alpha1_{WT}\beta1_{WT}$ $GABA_A$R, DTT has a weak effect on the magnitude of currents activated by a saturating (20 μM) GABA concentration. *C*, In the $\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_A$R, DTT induces a dramatic increase in the magnitude of currents activated by a saturating (100 μM) GABA concentration.

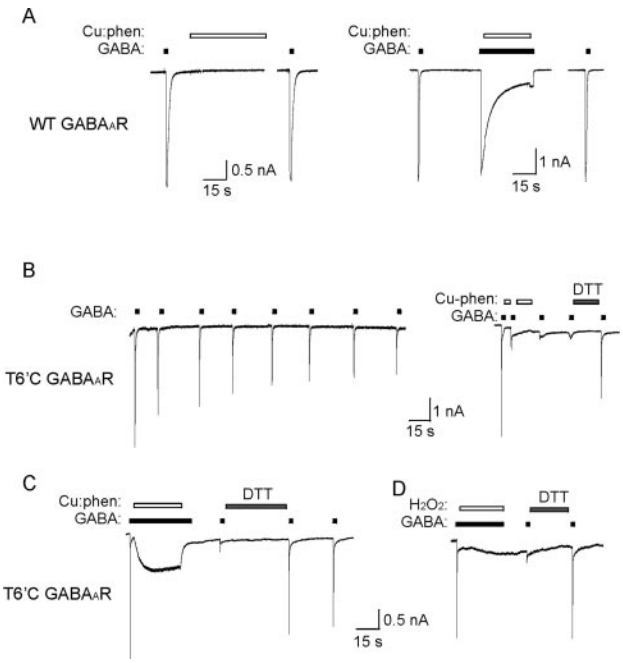

FIG. 5. **Effects of 100:400 μM Cu:phen and 0.3% H_2O_2 on currents activated by saturating GABA concentrations in WT and mutant $GABA_A$Rs.** All recordings shown in this figure were commenced immediately after the completion of a 1-min cell exposure to 10 mM DTT. *A*, in the $\alpha1_{WT}\beta1_{WT}$ $GABA_A$R, Cu:phen has no effect on the saturating (20 μM) GABA-activated current magnitude, regardless of whether it was applied in the closed or open states. *B*, both traces were recorded from the same cell expressing $\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_A$Rs. In the *left panel*, a gradual reduction in the magnitude of currents activated by 100 μM GABA is observed upon switching the bath solution to the standard control solution. In the *right panel*, the current reduction rate was greatly accelerated by 100:400 μM Cu:phen and reversed by a subsequent application of 10 mM DTT. *C*, when applied together with 100 μM GABA in the desensitized state, Cu:phen reversibly reopens the channels. However, a subsequent GABA application reveals a dramatic current reduction that is reversed by 10 mM DTT, implying the formation of disulfide bonds in the closed or desensitized states. *D*, results of a similar experiment to *C*, but using 0.3% H_2O_2 in place of Cu:phen.

icant effect on the $\alpha1_{WT}\beta1_{WT}$ $GABA_A$R regardless of whether it was applied in the absence or presence of GABA (see Fig. 6*A* and Table II). However, when MTSET was applied to the $\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_A$R in the closed channel state, its effects closely resembled those of Cu:phen. Following the removal of DTT, the GABA-activated current reduced to 74 ± 6% (n = 4) after 20 s in the standard bathing solution (*e.g.* Fig. 6*B*, *left panel*). However, in the presence of MTSET, the GABA-activated current reduced to 14 ± 3% (n = 4) of control magnitude after 20 s.

The effect of MTSET on the $\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_A$R was also examined in the desensitized state. In this experiment, GABA was applied 2 s before MTSET to ensure that >90% of receptors were in the desensitized state. MTSET was found to re-open the channels from this state (Fig. 6*C*). This reaction proceeded with an average time constant of 35 ± 9 s (n = 4), indicating a mean reaction rate of 29 $M^{-1}s^{-1}$. Upon removal of both MTSET and GABA, the current magnitude reduced to a steady-state level of 31 ± 5% (n = 4) of the peak MTSET-induced current magnitude (Fig. 6*C*), indicating that around one-third of the channels were held in the open state. MTSET modification also strongly reduced the magnitude of the current that was available for activation by GABA (Fig. 6*C*), indicating that the remainder of the channels were returned to the closed desensitized state. The MTSET-modified receptors were returned efficiently to the closed state by DTT, and a subsequent application of GABA activated the currents with a peak magnitude similar to the original control (Fig. 6*C*). Similar results were observed in each of four cells.

These results indicate that the effects of MTSET on the $\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_A$R depend on whether it is applied in the closed or desensitized states. Because it is unlikely that both actions could have been mediated by covalent attachment of the same ethyltrimethylammonium group, it is possible that at least one of the actions may have been mediated by MTSET acting as an oxidizing reagent or by reacting with a non-identical set of subunits.

MTSEA, applied in either the closed and open states, has been shown previously to irreversibly reduce the magnitude of currents in *Xenopus* oocyte-expressed $\alpha1_{T6'C}\beta1\gamma2$ $GABA_A$Rs (8). In this study, we investigated the effect of 2.5 mM MTSEA on the $\alpha1_{WT}\beta1_{WT}$ $GABA_A$R and the $\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_A$R ex-

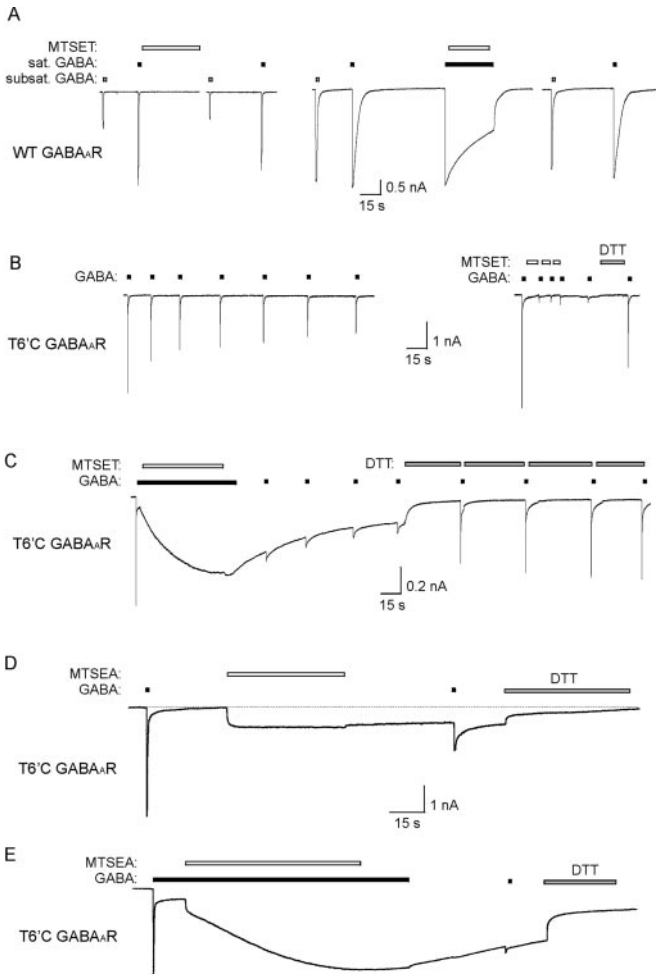

FIG. 6. **Effect of 1 mM MTSET and 2.5 mM MTSEA on $GABA_A$Rs.** All recordings shown in this figure were commenced immediately after the completion of a 1-min cell exposure to 10 mM DTT. *A*, MTSET had no significant effect on the $\alpha1_{WT}\beta1_{WT}$ $GABA_A$R regardless of whether applied in the absence (*left panel*) or presence (*right panel*) of a saturating (20 μM) GABA concentration. *B*, both traces were recorded from the same cell expressing $\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_A$Rs. GABA was applied at a saturating (100 μM) concentration throughout. The *left panel* shows the effect of exposure to standard bathing solution immediately following removal of DTT. In the *right panel*, the current reduction rate was greatly accelerated by MTSET and reversed by a subsequent application of 10 mM DTT. *C*, when applied to together with 100 μM GABA in the desensitized state, MTSET reopens the channels and locks them in the open state after the removal of GABA. This effect is reversed by 10 mM DTT, and a subsequent GABA application activates the original control current magnitude. *D*, application of 2.5 mM MTSEA in the closed state induces partial irreversible activation of the $\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_A$Rs and a decrease in magnitude of a subsequent application of 100 μM GABA. The channels were returned to the closed state by 10 mM DTT. *E*, when applied together with 100 μM GABA in the desensitized state, MTSET reopens the channels and locks them in the open state after the removal of GABA. This effect is reversed by 10 mM DTT.

pressed in HEK293 cells. As summarized in Table II, MTSEA had no effect on the $\alpha1_{WT}\beta1_{WT}$ $GABA_A$R in either the absence or presence of a saturating GABA concentration. However, when applied in the closed state to the $\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_A$R, it irreversibly activated the channels to 30 ± 5% (n = 3) of the peak current magnitude while simultaneously reducing the magnitude of the current activated by a saturating (20 μM) concentration of GABA (see Fig. 6*D* and Table II). A 10 mM DTT application efficiently closed the channels and restored the original magnitude of the GABA-activated current. When applied together with 20 μM GABA in the channel-desensitized state, MTSEA mimicked the effect of MTSET in returning the channels to the open state (see Fig. 6*E* and Table II).

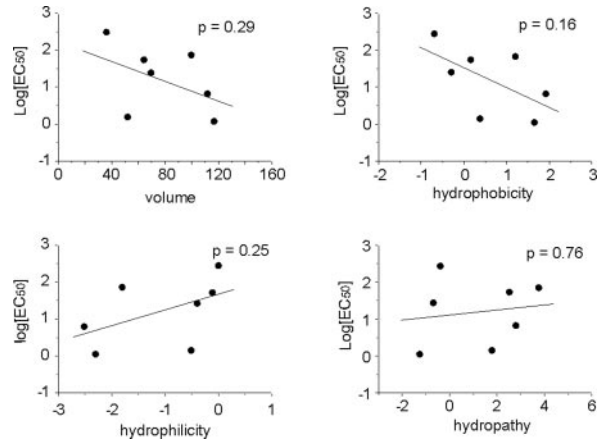

FIG. 7. **Correlation between the mean glycine EC_{50} and the physicochemical properties of the introduced amino acids at the GlyR 6′ position.** The log (EC_{50}) for glycine was plotted against the amino acid volume (27), hydrophobicity (28), hydrophilicity (29), and hydropathy (30). The p value refers to the probability that the linear coefficient R value was zero.

Effect of 6′ Mutagenesis on GlyR Function—To further probe the relationship between the physicochemical properties of the 6′ residue and the function of the receptor, we introduced a series of mutations at the 6′ position of the GlyR α1 subunit. The identity of these mutations and their effects on I_{max}, EC_{50}, and n_H values of glycine-gated currents in α1 homomeric receptors are summarized in Table I. This table also shows the effect of each mutation on the picrotoxin sensitivity of currents activated by the EC_{50} glycine concentrations as indicated. Note that GlyRs incorporating serine, glutamine, glutamic acid, and lysine mutations did not yield measurable currents. Interestingly, glutamine, glutamic acid, and lysine were the most polar amino acids tested.

The EC_{50} is a measure of the free energy input required to activate the receptor. If channel opening is accompanied by a movement of the 6′ residue toward an increasingly hydrophilic environment, it might be expected that the ease of activating the receptor should be a function of the hydropathy of the introduced amino acid. This was investigated by plotting the glycine EC_{50} values against some properties of the substituted amino acids (Fig. 7). This figure reveals that there was no significant correlation between glycine EC_{50} and side-chain volume, hydrophilicity, hydrophobicity, or hydropathy. We conclude that the relationship between the channel gating energy and the physicochemical properties of the introduced residues is complex.

DISCUSSION

GlyR in the Closed and Open States—When applied in the absence of glycine, MTSET has no effect, but when applied in the presence of glycine, MTSET locks the $\alpha1_{T6'C}$ GlyR in the open state (17). Because this action is not mimicked by oxidizing reagents, MTSET must act by adding a polar quaternary ammonium group to one or more 6′ cysteines in the open state only. This attached group prevents the channel from closing either by steric hindrance because of its size or by biasing the conformational equilibrium toward the open state because of its affinity with the aqueous pore environment. The smaller hydrophilic cysteine-specific reagent, MTSEA, also modified T6′C in the open state only. However, MTSEA-modified GlyRs closed readily upon removal of glycine. Together, these observations indicate that GlyR channel opening is accompanied by an increase in the exposure of the 6′ cysteines to the external aqueous environment. This may arise because of either 1) an increase in the ionization state of the cysteines because of a transition

from a hydrophobic (protein interior) to a hydrophilic (pore-lining) environment or 2) the removal of a barrier impeding the accessibility of the cysteines to externally applied MTS reagents.

Limited support for the former alternative is provided by the mutagenesis experiments summarized in Fig. 7 and Table I. In particular, the three most polar substitutions, glutamic acid, glutamine, and lysine, did not yield functional receptors. It is possible that these residues could not tolerate being buried in a hydrophobic environment in the closed state and induced a conformational change that disrupted receptor function. Apart from these three residues, there was a poor correlation between amino acid physicochemical properties and glycine EC_{50} values, implying a complex effect of the T6′ substitutions on GlyR activation energetics.

The $GABA_AR$ in the Closed State—When expressed in HEK293 cells, DTT induced a large (~ 400%), reversible current increase in the $\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_AR$. Conversely, Cu:phen or MTSET caused a dramatic decrease in current magnitude. This current reduction was not spontaneously reversible but was reversed by a further application of DTT. The most likely explanation is that Cu:phen promoted the formation of disulfide bonds in the channel closed state, thereby preventing the channels from opening. The ambient dissolved oxygen in the control bathing solution may have been sufficient to catalyze the formation of these disulfides at a slow rate. By reducing these bonds, DTT would have increased the number of receptors available for activation. MTSET appeared to be acting as an oxidizing reagent as its effect in the channel closed state mimicked that of Cu:phen but differed drastically from its effect in the channel-desensitized state. As discussed below, MTSET directly modified the 6′ cysteines in the desensitized state. In the closed state it is likely that MTSET either modified the 6′ cysteines on the other (non-identical) subunit or indeed behaved as an oxidizing agent.

When applied in the closed state, MTSEA had two effects on the DTT-reduced $\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_ARs$. First, it locked the receptors into a partially open state, and second, it reduced the magnitude of the GABA-activated current (Fig. 6*D*). Both effects were reversed by DTT. Because MTSEA had virtually identical effects when applied in the desensitized state (Fig. 6*E*), both effects were most likely to have been the result of direct MTSEA modification of the 6′ cysteines. These results agree in part with those of Xu and Akabas (8). They found that the 6′ cysteines of *Xenopus* oocyte-expressed $\alpha1_{T6'C}\beta1_{WT}$ $GABA_ARs$ also reacted with MTSEA in the closed and open states. However, they found that MTSEA reduced current flux but did not lock the receptors into the partially open state.

Biochemical cross-linking experiments on the same $GABA_AR$ subunits expressed in HEK293 cells show that intersubunit dimers do not form in the presence of Cu:phen in the closed state (18). Because both the $\alpha1$ and $\beta1$ subunits contain endogenous cysteines in membrane-spanning domains, the disulfide bond formation is therefore likely to occur between the 6′ and endogenous cysteines within a single subunit. Following their reduction by DTT in the closed state, the 6′ cysteines remain inaccessible to direct covalent modification by MTSET but accessible to modification by the smaller MTSEA.

The $GABA_AR$ in the Open and Desensitized States—In *Xenopus* oocyte-expressed $\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_ARs$, the co-application of Cu-phen with a saturating concentration of GABA locked the channels in the open state (18). In contrast, when the same $\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_ARs$ were expressed in HEK293 cells, the GABA-gated currents desensitized too rapidly to reliably apply cysteine-reactive reagents in the open state. Following the application of H_2O_2 or Cu:phen with GABA in the channel-desensitized state, the current magnitude was reduced dramatically. Because this effect was reversed by DTT, it is concluded that disulfide bond formation locked the channels in the desensitized state. However, it is important to note that desensitization is not necessarily accompanied by disulfide bond formation.

Biochemical cross-linking experiments on the same $GABA_ARs$ expressed in HEK293 cells show that $\beta1$ subunits dimerized only in the presence of both GABA and Cu:phen (18). When taken in isolation, this experiment does not resolve whether the dimerization occurred in the open or desensitized states. However, when taken together with the electrophysiological data presented here, the results strongly suggest that $\beta1$ subunit dimerization occurs in the desensitized state.

We were surprised to find that the co-application of GABA and Cu:phen reopened the channels from the desensitized state. Even more surprising was the observation that a second application of Cu:phen activated a current with similar magnitude to the first, as this implies that Cu:phen can open dimerized channels. Although we do not understand the mechanism by which this occurred, it was unlikely to have been an effect of oxidation as it was not replicated by H_2O_2, and it was not a pharmacological effect of copper.

When MTSET or MTSEA were applied in the desensitized state, they locked around 30% of the channels into the open state with the remainder being returned to the desensitized state. Because this effect was not mimicked by Cu:phen or H_2O_2 but was reversed by DTT, it must have been because of the direct covalent modification of the 6′ cysteines. The extremely slow reaction rate implies that access to the 6′ cysteines in the desensitized state was limited by steric hindrance, a non-polar environment, electrostatic repulsion, or a combination of these factors. One possibility is that the reaction could occur only during rare spontaneous transitions from the desensitized to the open state (24). In this case, the MTSET or MTSEA modification may have sterically prevented the channel from re-closing. Alternatively, the reaction may have proceeded slowly in the desensitized state. In this case, the increased hydrophilicity of the attached group may have opened the channels by favoring a conformation where the 6′ side chain had increased exposure to the aqueous pore. The difference in 6′ cysteine reactivity with MTSET between the closed and desensitized states provides strong evidence for a pore structural difference between these configurations. This is consistent with a recent study on the nAChR that also showed a different pore structure between the closed and desensitized states (25). Interestingly, the nAChR 6′ cysteine was accessible to MTSEA in the closed state but not in the desensitized state (25), implying that the structural basis of desensitization is not identical to that observed here for the $\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_AR$.

CONCLUSIONS

The closed state reactivity of 6′ cysteines in the GlyR and the $GABA_AR$ differ in two respects. First, the $GABA_AR$ 6′ cysteines spontaneously form disulfide bonds in the closed state, whereas those of the GlyR do not. Second, the $GABA_AR$ 6′ cysteines are accessible to externally applied MTSEA whereas the GlyR 6′ cysteines are not. Although it is not possible to define the structural basis for these differences, these results provide evidence for divergent pore structures in the closed channel state. Closed state structural differences have been identified previously in cationic members of the LGIC family. Although the nAChR pore was shown to admit externally applied MTSEA and MTSET as far as the 2′ residue (4–6), access of the same compounds in the $5HT_3R$ pore was impeded near the 14′ residue (10). Thus, closed state pore structures show considerable variation in both anionic and cationic members of the LGIC family.

On the other hand, substituted cysteine accessibility studies reveal that cationic LGIC family members have remarkably similar patterns of M2 domain residue exposure in the channel open state (4–7, 9, 10). Of particular relevance to the present study, MTSET modification of 6′ cysteines irreversibly inhibited current in both the nAChR and $5HT_3R$, whereas MTSES had no effect on either receptor (4–7, 9, 10). The present study could not directly compare 6′ cysteine accessibility in the open states of the $\alpha1_{T6'C}$ GlyR and $\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_AR$ because of the fast desensitization rate of the $\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_AR$. The observation that MTSET locked both receptors into the partially open state provides strong evidence for a common activation mechanism in this part of the pore. However, the pore structures are unlikely to be identical as MTSEA also locked the $\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_AR$ in the open state but had no such effect on the $\alpha1_{T6'C}$ GlyR.

The present study reveals distinct differences in the properties of $GABA_ARs$ expressed in *Xenopus* oocytes and HEK293 cells. When expressed in HEK293 cells, the 6′ cysteines can form disulfide bonds in the closed state. However, this does not occur when the same receptors are expressed in *Xenopus* oocytes (18). Furthermore, when expressed in HEK293 cells, the $GABA_AR$ is locked in the desensitized state by Cu:phen, but when expressed in *Xenopus* oocytes, it is locked in the open state by Cu:phen (18). Together, these results indicate the surface orientation of the $GABA_AR$ 6′ cysteines varies dramatically depending on the expression system. Moreover, $\alpha1_{T6'C}\beta1_{T6'C}$ $GABA_ARs$ expressed in HEK293 cells desensitize at a much faster rate than they do when expressed in *Xenopus* oocytes. These structural and functional differences could be because of expression system-specific differences in subunit folding and assembly, post-translational modifications, or membrane lipid composition. Regardless of their origin, the results indicate that caution should be applied when comparing results obtained using the two expression systems.

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