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Agonist-specific conformational changes in the structure of the α1-γ2 subunit interface of the $GABA_A$ receptor

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Running title: SCAM analysis of γ2(S195C)

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ABSTRACT

The $GABA_A$ receptor undergoes conformational changes upon the binding of agonist that lead to the opening of the channel gate and a flow of small anions across the cell membrane. Besides the transmitter GABA, allosteric ligands such as the general anesthetics pentobarbital and etomidate can activate the receptor. Here, we have investigated the agonist-specificity of structural changes in the extracellular domain of the receptor. We employed the substituted cysteine accessibility method and focused on the γ2(S195C) site (Loop F). We show that modification of the site with (2-sulfonatoethyl)methanethiosulfonate (MTSES) results in an enhanced response to GABA, indicating accessibility of the resting receptor to the modifying agent. Coapplication of GABA or muscimol, but not gabazine, with MTSES prevented the effect suggesting that GABA and muscimol elicit a conformational change that reduces access to the γ2(S195C) site. Exposure of the receptors to MTSES in the presence of the allosteric activators pentobarbital and etomidate resulted in enhanced current response indicating accessibility and labeling of the γ2(S195C) site. However, comparison of the rates of modification indicated that labeling in the presence of etomidate was significantly faster than in the presence of pentobarbital, gabazine or in resting receptors. We infer from the data that the structure of the α1-γ2 subunit interface undergoes agonist-specific conformational changes.

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INTRODUCTION

Cys-loop transmitter-gated ion channels are membrane proteins that convert the chemical signal of the ligand to an electrical signal, i.e., current flow across the membrane. The $GABA_A$ receptor, a major contributor to fast synaptic inhibition in the mammalian central nervous system, responds to the binding of GABA with a conformational change that leads to the opening of the gate allowing the movement of small anions through the channel. The transmitter binding sites are located in the extracellular domain at the interfaces between the β and α subunits whereas the channel gate is in the membrane-spanning region, approximately 40 Å away, so the conformational changes associated with channel activation involve a significant portion of the protein.

The structural elements forming the transduction path are best known for the closely-related nicotinic acetylcholine receptor where rate-equilibrium linear free energy relationship and computational studies have shed light onto the sequence of structural events during gating (Auerbach, 2010; Zheng and Auerbach, 2011). The available information for the $GABA_A$ receptor suggests that the receptor functions analogously. The gating of the $GABA_A$ receptor follows the long-axis of the protein involving residues in the loop-based binding site for GABA, the pre-M1 segment in the α and β subunits, and the electrostatic link between extracellular loops 2 and 7 and the M2-M3 linker in the α subunit (Kash et al., 2003; Keramidas et al., 2006; Tran et al., 2011; Williams et al., 2010). Recent work has indicated that channel activation may also elicit structural changes in the fifth, non-transmitter binding subunit. Using voltage-clamp fluorometry, Wang and coworkers (Wang et al., 2010) showed that exposure to GABA but not the competitive antagonist gabazine elicits a fluorescence change (interpreted as a conformational change) at the γ2(S195C) site. A positive correlation between agonist efficacy and magnitude of fluorescence change was interpreted as implying that this region underwent an activation-associated conformational change.

A number of allosteric ligands are capable of activating the $GABA_A$ receptor. These include barbiturates (e.g., pentobarbital), neuroactive steroids (e.g., allopregnanolone), and general anesthetics (e.g., etomidate). The allosteric ligands interact with their own individual binding sites which are distinct from the transmitter binding site (Amin and Weiss, 1993; Hosie et al., 2006; Li et al., 2006; Serafini et

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al., 2000; Ueno et al., 1997). Single-channel recordings have shown that allosteric ligands and GABA elicit channel activity with the same single-channel conductance suggesting that the structure of the open pore is similar (Akk and Steinbach, 2000). However, the conformational changes that lead to channel opening can be different. In the pre-M1 region of the α1 subunit, the movements triggered by GABA are distinct from those observed in the presence of pentobarbital as evidenced by effects on modification of introduced cysteines by methanethiosulfonate reagents (Mercado and Czajkowski, 2008). Similarly, access of the sulfhydryl reagent *p*-chloromercuribenzene sulfonate to the residues in the M3 domain of the α1 subunit demonstrates dissimilar patterns when the receptor is activated by propofol instead of GABA (Williams and Akabas, 2002). Finally, voltage-clamp fluorometry has revealed that the movements near the transmitter binding site in the α1 subunit, and at the extracellular end of the M2 domain of the β2 subunit differ for GABA, pentobarbital and etomidate (Akk et al., 2011; Muroi et al., 2009).

Here, we have examined the nature of conformational changes in the extracellular domain of the receptor. We focused on the Loop F residue γ2S195 and employed the substituted cysteine accessibility method (Karlin and Akabas, 1998) to probe the agonist-specificity of movements. Our data indicate that channel activation by orthosteric and allosteric ligands differently affect accessibility of the γ2(S195C) site to (2-sulfonatoethyl)methanethiosulfonate. We infer that the α1-γ2 subunit interface structure undergoes agonist-specific conformational changes.

METHODS

Mutagenesis and expression in oocytes.

The experiments were conducted on wild-type α1β2γ2L and mutated α1β2γ2(S195C) rat $GABA_A$ receptors. The γ2(S195C) and β2(I180C) mutations were generated using the QuikChange site-directed mutagenesis kit (Stratagene, San Diego, CA). The α1 and γ2 subunits contained the FLAG epitope in the aminoterminus of the subunits (Ueno et al., 1996). The mutant clones were fully sequenced to verify that only the desired mutation had been produced. The cDNAs for the receptor subunits were subcloned into the pcDNA3 expression vector in the T7 orientation. The cDNA was

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linearized by Xba I (NEB Labs, Ipswich, MA) digestion, and cRNA was produced using mMessage mMachine (Ambion, Austin, TX). The *Xenopus laevis* oocytes were injected with 7-14 ng cRNA per construct in a final volume of 20-60 nl, and incubated in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 2.5 mM Na pyruvate, 5 mM HEPES; pH 7.4) at 16 ^oC for 1-4 days before recording.

Electrophysiological recordings.

Standard two-electrode voltage clamp was used to record the currents. Both voltage and current electrodes were patch-clamp electrodes filled with 3 M KCl and had resistances of 0.5 to 1.5 MΩ. The oocytes were typically clamped at -60 mV. The chamber (RC-1Z, Warner Instruments, Hamden, CT) was perfused continuously at approximately 5 ml min^{-1}. Bath solution (92.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES; pH 7.4) was perfused between all test applications. Solutions were applied from glass reservoirs via metal or Teflon tubing to reduce adsorption. Solutions were switched by pClamp using a Warner Instruments VC-8T valve controller. The current responses were amplified with an Axoclamp 900A amplifier (Molecular Devices, Sunnyvale, CA), digitized with a Digidata 1320 series digitizer (Molecular Devices) at a 100 Hz sampling rate, and stored using pClamp (Molecular Devices). The traces were analyzed with Clampfit (Molecular Devices).

Protocols used in labeling experiments.

We employed sodium (2-sulfonatoethyl)methanethiosulfonate (MTSES; CAS Number 184644-83-5; Figure 3D), obtained from Toronto Research Chemicals (Toronto, Canada) as the main sulfhydryl-modifying agent. Stock solutions of MTSES were made at 50 mM in bath solution and stored at -80^oC. Dilutions to the working concentrations were made immediately before the experiment.

Each oocyte was initially exposed to 100 µM GABA in the absence and presence of 100 µM $ZnCl_2$, to determine the maximal response from the cell and to verify the incorporation of the γ2 subunit in surface receptor-complexes (Krishek et al., 1998). In the next step, the oocyte was stabilized by applying a low concentration (0.1-0.5 µM) of GABA eliciting an EC_7 (range: EC_{5-10}) response. The 5 sec applications were separated by 115 s washouts in bath and repeated until the peak GABA-activated

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currents varied by less than 5 %. In most cells, it took 5 to 10 applications of GABA for the responses to stabilize. The oocyte was then treated with 1 mM MTSES for 1 min, followed by a washout in bath for 1 min, and several additional applications of EC_7 GABA. The effect of MTSES is expressed in % of control and is calculated as ($I_{GABA\text{-}post}/I_{GABA\text{-}pre}$), where $I_{GABA\text{-}pre}$ and $I_{GABA\text{-}post}$ are the peak currents elicited by EC_7 GABA before and after exposure to MTSES, respectively. In some experiments, an agonist (GABA, muscimol, etomidate, or pentobarbital) or antagonist (gabazine) was coapplied with MTSES. In these experiments, the duration of washout following exposure to MTSES was 3 min. In some experiments, [2-(trimethylammonium) ethyl]methanethiosulfonate bromide (MTSET; Toronto Research Chemicals, CAS Number 91774-25-3) was used as the sulfhydryl-modifying agent. The protocols were the same for both reagents.

The rate of reaction for MTSES modification of the γ2(S195C) residue was determined by measuring the cumulative outcome of sequential applications of low-concentration MTSES on the peak current. For that, following the stabilization of responses to GABA (as described above), MTSES was applied for 20 sec followed by a 1 min washout and a test application of EC_7 GABA. The procedure was repeated until the peak GABA response was no longer changing. To determine the effect of a drug on the rate of reaction, MTSES was coapplied with an agonist or antagonist of the receptor. The concentration of MTSES used in these experiments was 20 μM for MTSES + etomidate, and 100 μM for resting receptors, MTSES + pentobarbital, and MTSES + gabazine.

The statistical significance of the consequences of labeling was assessed in two ways. In the first, the effect ratio (peak response after treatment relative to peak response before treatment) was compared to a value of 1 (no effect) using a paired t-test. This test was designed to indicate whether there was a significant effect of the treatment on responsiveness to GABA. The ratio was used to control for differences in the responsiveness of individual oocytes (i.e. the control values differed). The second test was to compare the effect ratio in a given condition (e.g. labeling in the presence of GABA) to the effect in resting receptors (no additional drugs present during labeling) using an unpaired t-test with equal variance. This test was designed to indicate whether the consequences of labeling in a condition differed from labeling of resting receptors.

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To determine the rate of reaction, the change in current amplitude was plotted versus cumulative time of MTSES exposure. Peak current at each time point was normalized to the peak current determined before the first application of MTSES (at t = 0), and the pseudo first-order rate constant (k_1) was determined by fitting a curve describing the data to a single-exponential growth function. The second-order rate constant (k_2) was calculated by dividing the fitted k_1 with the concentration of MTSES used in the experiment. To verify that the expected changes in the pseudo first-order rate constant do not significantly modify the calculated second-order rate constant we varied the concentration of MTSES several-fold (1-20 μM). Further details on the procedures can be found in previous publications describing the approach (Kloda and Czajkowski, 2007; Pascual and Karlin, 1998).

Structures.

Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR001081).

RESULTS

Effect of the γ2(S195C) mutation on receptor activation.

The serine residue at position 195 (Loop F; Figure 1) in the rat γ2 subunit was mutated to a cysteine, and the mutated γ2 subunit was coexpressed with wild-type rat α1 and β2 subunit in *Xenopus laevis* oocytes. The presence of the mutation was generally well tolerated. To gain insight into the effect of the mutation on surface expression levels, we examined the peak responses to saturating GABA. In five cells expressing α1β2γ2(S195C) receptors, the average peak response to 100 μM GABA was 0.8 μA (range: 0.7 to 1 μA). For comparison, the average peak response to saturating GABA in cells expressing wild-type α1β2γ2L receptors was 0.9 μA (range: 0.7 to 1.2 μA, 4 cells). We conclude that peak GABA responses are similar in receptors containing the wild-type or mutated γ2 subunits, suggesting that the γ2(S195C) mutation does not strongly interfere with receptor assembly.

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The concentration-response curve for GABA in the mutant receptor was right-shifted compared to the wild-type receptor. The EC_{50} for the α1β2γ2(S195C) receptor was 11.5 ± 0.7 μM and the Hill coefficient was 1.0 ± 0.04 (5 cells). For comparison, we estimate that the wild-type α1β2γ2L receptor has an EC_{50} of 1.8 ± 0.2 μM and a Hill coefficient of 0.9 ± 0.1 (4 cells). The concentration-response curves for the mutant and wild-type receptors are shown in Figure 2A. The incorporation of the γ2 subunit in receptor-complexes was verified by lack of inhibition by Zn^{++} (Figure 2B).

To gain insight into the effect of the γ2(S195C) mutation on channel gating efficacy, we examined potentiation of receptors exposed to a high concentration of GABA by pentobarbital. The effects of potentiators are typically best observed at low agonist concentrations whereas at high agonist concentrations potentiation is reduced or becomes insignificant. This is due to the open probability of the α1β2γ2 receptor approaching 1 in the presence of high concentrations of GABA (Steinbach and Akk, 2001). However, under conditions where the maximal open probability of the receptor is reduced, by a mutation or the use of a low-efficacy agonist, potentiation can be observed even when the receptor is activated by a saturating concentration of agonist (Bianchi and Macdonald, 2003; Bracamontes and Steinbach, 2009; O'Shea et al., 2000). Accordingly, we reasoned that if the γ2(S195C) mutation significantly reduces the gating efficacy for GABA then the mutant receptors will retain their ability to be potentiated even when activated by a high concentration of GABA. The data show that α1β2γ2(S195C) receptors are potentiated to 113 ± 4 % (mean ± S.D., 5 cells; $p < 0.001$, paired t-test vs. no effect) of control when 50 μM pentobarbital is coapplied with 100 μM GABA. For comparison, α1β2γ2 wild-type cells demonstrate potentiation to 109 ± 2 % (5 cells; $p < 0.001$) of control under identical conditions. Sample traces are shown in Figure 2C. While statistically significant, the magnitude of potentiation is negligible. We conclude that the γ2(S195C) mutation does not drastically reduce gating efficacy by GABA.

MTSES modification following occupation of the orthosteric binding site.

To determine the accessibility of the introduced cysteine at the 195 position in the γ2 subunit, oocytes expressing α1β2γ2(S195C) receptors were exposed to 1 mM MTSES for 1 min. Comparison of

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peak currents elicited by GABA producing an EC_7 response recorded before and after exposure to MTSES showed that exposure to MTSES increased the peak response to 141 ± 7 % of control (mean ± S.D., 6 cells; $p < 0.001$). A sample recording is shown in Figure 3A. The positive functional effect observed in α1β2γ2(S195C) receptors following exposure to MTSES can be explained by covalent modification of the introduced cysteine. The potentiating effect of labeling was best observed when testing with low concentrations of GABA. In experiments employing a higher test concentration of GABA (>EC_{40}), the effect of labeling was diminished or not observed (not shown). This indicates that the maximal response to GABA is not affected by MTSES.

As a control, we exposed wild-type α1β2γ2L receptors to MTSES. In 3 cells, the response to an EC_7 concentration of GABA following a 1 min exposure to 1 mM MTSES was 93 ± 8 % of control ($p > 0.26$). As an additional control, each cell was also tested with 100 μM $ZnCl_2$ to verify the expression and incorporation of the γ2L subunit (Figure 2B). We infer from the data that the γ2(S195C) site is accessible to MTSES, and that the modification results in an enhanced functional response to GABA.

To determine whether channel activation induces structural rearrangements in the vicinity of the γ2(S195C) site, we coapplied a high concentration (100 μM) of GABA with MTSES. Comparison of EC_7 GABA responses recorded before and after exposure to MTSES+GABA showed no change in peak amplitude (94 ± 11 % of control, n = 6 cells; $p > 0.22$ vs. no effect; $p < 0.001$ for difference to resting receptors). A sample recording is shown in Figure 3B. A control experiment demonstrated that a 1 min application of 100 μM GABA alone, followed by a 3 min wash did not affect the subsequent response to EC_7 GABA (97 ± 7 % of control, 3 cells). We propose that channel activation by GABA elicits a structural rearrangement at the interface between the α1 and γ2 subunits that leads to inaccessibility of the γ2(S195C) residue to MTSES.

To gain insight into the concentration-dependence of GABA-mediated protection against labeling, we examined MTSES labeling in the presence of lower concentrations of GABA. We tested 0.1-0.3 μM GABA and 4 μM GABA that produced 5 ± 1 % and 44 ± 4 % of the maximal response, respectively. Our data demonstrate that in the presence of 0.1-0.3 μM GABA the application of 1 mM MTSES results in productive labeling at the γ2(S195C) site. The response to EC_7 GABA after application of 0.1-0.3 μM

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GABA + MTSES was 119 ± 11 % of control (6 cells; $p < 0.01$). In contrast, when the cells were exposed to MTSES in the presence of 4 μM GABA no labeling was evident. The response to EC_7 GABA after MTSES + 4 μM GABA application was 90 ± 5 % of control (3 cells), similar to the value obtained with 100 μM (>EC_{90}) GABA (94 %, above).

For further implication of the transmitter binding site in structural changes in the α1-γ2 subunit interface, we probed the effect of MTSES in the presence of muscimol (a GABA-site agonist) or gabazine (SR-95531, a competitive antagonist of the GABA-site). Application of 100 μM muscimol resulted in peak currents that were similar in magnitude (89 ± 9 %, 5 cells) to those observed in the presence of 100 μM GABA. Coapplication of 100 μM muscimol with MTSES, similarly to GABA, protected modification of the γ2(S195C) residue. The response to EC_7 GABA following exposure to MTSES+muscimol was 93 ± 22 % (5 cells; $p > 0.5$ vs. no effect; $p < 0.001$ vs. resting receptors) of the peak response to GABA before the application of MTSES. As expected, the application of gabazine did not elicit functional responses from α1β2γ2(S195C) receptors. The responses to low concentrations of GABA were enhanced (150 ± 10 % of control, 3 cells; $p < 0.05$) following coapplication of MTSES and 50 μM gabazine. Thus, exposure to GABA or muscimol, but not gabazine, protects the γ2(S195C) site from labeling. In our interpretation, channel activation mediated by occupation of the transmitter binding site initiates the conformational change in the extracellular domain of the receptor that renders the γ2(S195C) residue inaccessible to MTSES. The data are summarized in Figure 3C.

As a control, we tested MTSES labeling during coapplication of GABA and gabazine. We reasoned that if the competitive antagonist gabazine displaces GABA from the transmitter binding site then MTSES should be able to label the receptors, resulting in an enhanced response to low GABA. We exposed the cells to 1 mM MTSES in the presence of GABA eliciting an approximately 45 % of the maximal response (4-10 μM) and 50 μM gabazine. The application of 50 μM gabazine fully suppressed the electrophysiological response to GABA. Comparison of peak GABA currents before and after MTSES exposure demonstrated productive labeling. The peak GABA currents after application of MTSES + GABA + gabazine were 136 ± 18 % (4 cells; $p < 0.05$) of control.

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The findings suggest that the conformational changes in the vicinity of the γ2(S195C) site take place following activation of the receptor initiated at the transmitter binding site. The data are in agreement with the previous study which employed voltage-clamp fluorometry and suggested that conformational changes in Loop F of the γ2 subunit are associated with channel gating (Wang et al., 2010).

MTSES modification following occupation of allosteric binding sites.

The $GABA_A$ receptor can be activated by a number of allosteric ligands including barbiturates and etomidate. These drugs interact with their own individual binding sites, distinct from the orthosteric, neurotransmitter binding site. In the next set of experiments, we tested whether channel activation by allosteric ligands induces a conformational change similar to that observed in the presence of GABA.

We tested etomidate and pentobarbital. Etomidate, at 5 μM, was a strong agonist of the α1β2γ2(S195C) receptor, with peak responses of 64 ± 9 % (3 cells) of saturating GABA. Cells exposed to 100 μM pentobarbital similarly showed large functional responses (39 ± 6 % of saturating GABA, 3 cells). When etomidate or pentobarbital were coapplied with MTSES, the peak amplitudes of responses to low GABA showed significant enhancement. In the presence of etomidate, the peak response was 141 ± 8 of control (3 cells), and in the presence of pentobarbital, the peak response was 164 ± 6 % of control (3 cells). The functional effect of modification is similar to that observed for resting receptors, but different from what is observed when MTSES is applied in the presence of GABA. The data thus suggest that receptors activated by etomidate or pentobarbital are static or undergo different conformational changes at the α1-γ2 interface compared to receptors activated by GABA. Sample traces and a summary of the data are given in Figure 4.

In control experiments, we verified that applications of pentobarbital and etomidate did not cause long-lasting effects that may have underlain or modified the responses observed when the drugs were coapplied with MTSES. Comparison of responses to a low concentration (<EC_{10}) of GABA showed that a 1 min application of 100 μM pentobarbital followed by a 3 min wash in bath was fully reversible and did not affect receptor activation. The peak GABA response after application of pentobarbital was 101 ±

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7 % (5 cells) of the response observed before exposure. In 4 cells exposed to 5 μM etomidate, the response to a low concentration of GABA was 101 ± 20 % of control.

These findings demonstrate that channel activation by the allosteric activators etomidate and pentobarbital does not elicit a conformational change similar to GABA. We propose that channel activation by an agonist interacting with the GABA-site, not gating per se, is required to render the γ2(S195C) site inaccessible to a cysteine-modifying reagent.

Rates of reaction for MTSES modification.

In the experiments described above, we employed long (1 min) applications of a high concentration (1 mM) of MTSES to demonstrate that modification of resting receptors as well as those exposed to gabazine, etomidate or pentobarbital results in enhanced response to GABA. As the modification reaction is essentially irreversible such applications are expected to result in a maximal, steady-state effect when the cysteine residue is accessible. In the next set of experiments, we examined the rates of modification to gain further insight into the structure of the interface. The rate of modification by MTSES depends, in part, on the access pathway to the thiol group of the cysteine residue. Thus, a difference in rates can be interpreted as a difference in structures that are achieved under various conditions. This approach is capable of revealing more subtle differences in accessibility of the introduced cysteine residue and conformation.

We applied short (20 s) pulses of a lower concentration (20 to 100 μM) of MTSES, alone, or in the presence of 50 μM gabazine, 5 μM etomidate or 100 μM pentobarbital. Each pulse was followed by a washout period and a test application of EC_7 GABA. The changes in responses to EC_7 GABA were plotted as a function of cumulative exposure to MTSES. These data were used to estimate the pseudo-first order and second order rate constants for MTSES modification (more details in Methods). Data from individual cells are summarized in Figure 5.

We estimate that the second-order rate constant for resting receptors is 115 ± 25 $M^{-1}s^{-1}$ (5 cells). When MTSES is applied in the presence of gabazine or pentobarbital, the rate constant is not significantly different from that for resting receptors (Table 1). In contrast, in the presence of etomidate,

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the rate constant increases by approximately twenty-fold to 2484 ± 1314 $M^{-1}s^{-1}$ (3 cells). We take this as an indication that the structure of the α1-γ2 interface in receptors activated by etomidate is different from that in resting receptors or those exposed to gabazine or pentobarbital. Control experiments (data not shown) were conducted in the presence of 1-10 μM MTSES + etomidate to verify the presence of expected changes in the pseudo first-order rate constant.

Overall, we infer from these data that channel activation by etomidate and GABA, but not pentobarbital nor occupation by gabazine, causes conformational changes in the vicinity of the γ2(S195C) site. Given the drastically different rates of reaction (2484 $M^{-1}s^{-1}$ for etomidate, ~0 for GABA), the structures of receptors activated by GABA and etomidate are different from each other.

Mechanism of functional changes following MTS modification.

The data suggest that under certain conditions (resting receptors, in the presence of pentobarbital, gabazine or etomidate), MTSES reacts with the γ2(S195C) residue. This leads to enhancement of the functional response to GABA, observed by comparing peak responses to low concentrations of GABA before and after exposure to MTSES. What causes the enhanced functional response? We conducted several experiments aimed at bettering our understanding of the mechanism.

First, we tested the possibility that an altered charge interaction at the α1-γ2 subunit interface plays a role. For that we used, instead of the negatively-charged MTSES, a positively-charged modifying reagent [2-(trimethylammonium)ethyl] methanethiosulfonate (MTSET). We reasoned that if charge interaction underlies the enhanced response to GABA then a charge switch, from negative to positive, would likely alter the effect.

The data indicate that MTSET behaves similarly to MTSES. The application of 1 mM MTSET resulted in an enhanced response to low concentrations of GABA (120 ± 8 % of control, n = 4 cells; $p < 0.05$). When MTSET was applied in the presence of 100 μM GABA, the effect was not observed (95 ± 3 % of control, n = 3 cells, $p > 0.05$). These data show that MTSET and MTSES behave qualitatively similarly, and suggest that the functional effect of MTSES is not due to introduction of a negative charge at the α1-γ2 subunit interface. A potentiating effect has also been observed following labeling of

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this residue by MTSEA-biotin (Hanson and Czajkowski, 2008). We propose that the sulfhydryl-modifying agents act through a steric effect in the subunit interface.

Benzodiazepines potentiate the $GABA_A$ receptor through interactions with the α-γ subunit interface where several Loop F residues have been shown to affect the efficacy of several benzodiazepine ligands (Morlock and Czajkowski, 2011; Padgett and Lummis, 2008). We tested a possibility that the MTSES molecule occupies the benzodiazepine binding cavity thereby imitating the effect of a bound benzodiazepine and underlying the enhanced current response following exposure to MTSES. We hypothesized that if bound MTSES occupies the benzodiazepine binding cavity then potentiation by a benzodiazepine such as diazepam would be reduced following labeling. For that, potentiation by diazepam before and after labeling with MTSES was compared. We found that exposure to MTSES does not significantly reduce potentiation by diazepam. Mutant α1β2γ2(S195C) receptors activated by 0.5 μM GABA (EC_5) and exposed to 1 μM diazepam showed 4.3 ± 1.8-fold (n = 5 cells) potentiation prior to exposure to MTSES, and 4.3 ± 1.0-fold (5 cells) potentiation after exposure to MTSES. Thus, the data do not confirm the hypothesis that occupation of the benzodiazepine site underlies current enhancement observed following labeling with MTSES.

Finally, we probed whether labeling with MTSES results in a general, non-specific enhancement of channel function or whether the effect is specific to activation by the transmitter. For that, we tested whether activation by a low concentration of etomidate is enhanced following exposure to MTSES. The experimental protocol was analogous to that for GABA as described above. A cell was exposed to 0.5 μM etomidate until a stable response was obtained. This concentration of etomidate elicited a peak response that was 5 ± 0.3 % of that in the presence of maximal GABA. The cell was then exposed to 1 mM MTSES for 1 min, and, following a washout, responses to 0.5 μM etomidate recorded again. We found that responses to etomidate were not enhanced following exposure to MTSES (100 ± 2 % of control, n = 3 cells; $p > 0.7$). This finding indicates that MTSES labeling at the α1-γ2 subunit interface specifically enhances subsequent responses to low GABA.

DISCUSSION

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The data presented here demonstrate that labeling with the cysteine-modifying agent MTSES results in an enhanced current response from α1β2γ2(S195C) receptors. When labeling is carried out in the presence of a high (>EC_{40}) concentration of GABA the subsequent functional effect is not observed. We interpret these findings as an indication of a conformational change that is associated with channel activation via occupation of the transmitter binding site at the β2-α1 subunit interface and transmitted to the α1-γ2 interface.

The results are consistent with the idea that the conformational change at the α1-γ2 interface is associated with global rearrangements during channel activation rather than resulting as a consequence of ligand binding. Another GABA-site agonist, muscimol, acts like GABA producing a conformational change that protects the γ2(S195C) residue from labeling. In contrast, a competitive antagonist of GABA, gabazine (SR-95531) is unable to elicit such a rearrangement. Our work further demonstrates that allosteric activators, such as pentobarbital and etomidate have distinct effects on the structure in the vicinity of the γ2(S195C) site. The application of pentobarbital appears to cause no structural rearrangements whereas etomidate elicits a structural change that is different from that during activation by GABA. The second-order rate constant for modification (k_2) is significantly higher in the presence of etomidate, suggesting that channel activation by etomidate exposes the γ2(S195C) residue to the aqueous phase. Thus, distinct conformational changes in the extracellular domain accompany channel activation by the transmitter GABA and allosteric agonists. Etomidate is thought to have a binding site in the intersubunit cavity formed by the αM1 and βM3 membrane-spanning domains (Li et al., 2006), while the interaction site of pentobarbital is unknown but likely involves the membrane-spanning domains (Serafini et al., 2000). Interestingly, modification of the α1S195C residue can affect activation mediated by occupation of the GABA-binding site but not activation mediated by the allosteric activator etomidate. It is not clear whether the γ2 subunit itself undergoes conformational changes in this region, or whether its structure affects the energetics of movements of the α1 subunit.

Previous work has demonstrated the involvement of the fifth subunit in rearrangements occurring during channel activation. Wang and coworkers (Wang et al., 2010), by employing voltage-clamp fluorometry, showed that the environment around residues in Loop F of the γ2 subunit undergoes

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ligand-specific structural changes during channel activation. GABA and β-alanine were equally effective at producing the current response whereas the fluorescence change produced in the presence of β-alanine was significantly smaller than in the presence of GABA. The application of the competitive inhibitor gabazine was without effect on the fluorescence signal. These data are in agreement with our findings demonstrating that the rate of modification at the γ2(S195C) site is unchanged in resting receptors vs. in the presence of gabazine (Table 1).

The high-affinity benzodiazepine binding site is located at the α-γ interface (Boileau et al., 1998; Sigel, 2002). Mutations to this interface can have large effects on binding affinity and/or efficacy of benzodiazepine ligands (Hanson and Czajkowski, 2008; Hanson et al., 2008; Morlock and Czajkowski, 2011; Padgett and Lummis, 2008). Specifically, residues of Loop F of the γ2 subunit appear to play an important role in defining the efficacy of benzodiazepine ligands (Hanson and Czajkowski, 2008; Padgett and Lummis, 2008). A study examining the effect of benzodiazepines on the rate of reaction of modification of a nearby residue γ2(R197C) by MTSET found that the positive benzodiazepine site modulators flurazepam and zolpidem slow the reaction rate (Hanson and Czajkowski, 2008). This indicates that flurazepam and zolpidem induce a conformational change at the γ2(R197C) site or directly impede access of the modifying agent. Interestingly, the same study also found that the application of GABA or pentobarbital did not influence the rate of modification for MTSET at the γ2(R197C) site. This differs from our data which demonstrate that modification at the γ2(S195C) site is strongly affected by GABA (and another GABA-site agonist, muscimol) but not pentobarbital. The overall implications of this difference are unclear. We note that an unchanged rate of reaction does not strictly imply lack of activation-associated movement. Different structures but with a similar access path for the MTS reagent would be expected to result in similar nominal rates of reaction.

Recent fluorescence work on homomeric ρ1 $GABA_A$ and α1 glycine receptors has suggested that Loop F moves in response to ligand binding but is not directly involved in coupling ligand binding to activation (Khatri et al., 2009; Pless and Lynch, 2009; Zhang et al., 2009). This notion is based on the findings that partial and full agonists as well as competitive antagonists elicit similar conformational changes within Loop F. These data are in agreement with voltage-clamp fluorometry conducted on

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α1β2γ2 $GABA_A$ receptors suggesting lack of correlation of the ability of the GABA-site ligand to elicit functional response and movements in Loop F of the transmitter-binding α1 subunit (Wang et al., 2010).

Overall, we propose that the extracellular interface between the α1 and γ2 subunits of the $GABA_A$ receptor undergoes structural rearrangements during channel activation, and that the movements are specific to the agonist used to activate the receptor. In future work it will be interesting to probe other regions in the fifth subunit to generate a map of regions involved in the structural rearrangements in the presence of different activators.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Eaton, Steinbach and Akk.

Conducted experiments: Eaton, Lim, Bracamontes and Akk.

Contributed new reagents or analytic tools: N/A

Performed data analysis: Eaton, Lim and Akk

Wrote or contributed to the writing of the manuscript: Eaton, Steinbach and Akk.

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REFERENCES

Akk G, Li P, Bracamontes J, Wang M and Steinbach JH (2011) Pharmacology of structural changes at the GABA(A) receptor transmitter binding site. *Br J Pharmacol* **162**(4):840-850.

Akk G and Steinbach JH (2000) Activation and block of recombinant GABA(A) receptors by pentobarbitone: a single-channel study. *Br J Pharmacol* **130**(2):249-258.

Amin J and Weiss DS (1993) GABAA receptor needs two homologous domains of the beta-subunit for activation by GABA but not by pentobarbital. *Nature* **366**(6455):565-569.

Auerbach A (2010) The gating isomerization of neuromuscular acetylcholine receptors. *J Physiol* **588**(Pt 4):573-586.

Bianchi MT and Macdonald RL (2003) Neurosteroids shift partial agonist activation of GABA(A) receptor channels from low- to high-efficacy gating patterns. *J Neurosci* **23**(34):10934-10943.

Boileau AJ, Kucken AM, Evers AR and Czajkowski C (1998) Molecular dissection of benzodiazepine binding and allosteric coupling using chimeric gamma-aminobutyric acidA receptor subunits. *Mol Pharmacol* **53**(2):295-303.

Bracamontes JR and Steinbach JH (2009) Steroid interaction with a single potentiating site is sufficient to modulate GABA-A receptor function. *Mol Pharmacol* **75**(4):973-981.

Hanson SM and Czajkowski C (2008) Structural mechanisms underlying benzodiazepine modulation of the GABA(A) receptor. *J Neurosci* **28**(13):3490-3499.

Hanson SM, Morlock EV, Satyshur KA and Czajkowski C (2008) Structural requirements for eszopiclone and zolpidem binding to the gamma-aminobutyric acid type-A (GABAA) receptor are different. *J Med Chem* **51**(22):7243-7252.

Hosie AM, Wilkins ME, da Silva HM and Smart TG (2006) Endogenous neurosteroids regulate GABAA receptors through two discrete transmembrane sites. *Nature* **444**(7118):486-489.

Karlin A and Akabas MH (1998) Substituted-cysteine accessibility method. *Methods Enzymol* **293**:123-145.

Kash TL, Jenkins A, Kelley JC, Trudell JR and Harrison NL (2003) Coupling of agonist binding to channel gating in the GABA(A) receptor. *Nature* **421**(6920):272-275.

Keramidas A, Kash TL and Harrison NL (2006) The pre-M1 segment of the alpha1 subunit is a transduction element in the activation of the GABAA receptor. *J Physiol* **575**(Pt 1):11-22.

Khatri A, Sedelnikova A and Weiss DS (2009) Structural rearrangements in loop F of the GABA receptor signal ligand binding, not channel activation. *Biophys J* **96**(1):45-55.

Kloda JH and Czajkowski C (2007) Agonist-, antagonist-, and benzodiazepine-induced structural changes in the alpha1 Met113-Leu132 region of the GABAA receptor. *Mol Pharmacol* **71**(2):483-493.

Krishek BJ, Moss SJ and Smart TG (1998) Interaction of H+ and Zn2+ on recombinant and native rat neuronal GABAA receptors. *J Physiol* **507 (Pt 3)**:639-652.

Li GD, Chiara DC, Sawyer GW, Husain SS, Olsen RW and Cohen JB (2006) Identification of a GABAA receptor anesthetic binding site at subunit interfaces by photolabeling with an etomidate analog. *J Neurosci* **26**(45):11599-11605.

Mercado J and Czajkowski C (2008) Gamma-aminobutyric acid (GABA) and pentobarbital induce different conformational rearrangements in the GABA A receptor alpha1 and beta2 pre-M1 regions. *J Biol Chem* **283**(22):15250-15257.

Morlock EV and Czajkowski C (2011) Different Residues in the GABAA Receptor Benzodiazepine Binding Pocket Mediate Benzodiazepine Efficacy and Binding. *Mol Pharmacol* **80**(1):14-22.

Muroi Y, Theusch CM, Czajkowski C and Jackson MB (2009) Distinct structural changes in the GABAA receptor elicited by pentobarbital and GABA. *Biophys J* **96**(2):499-509.

O'Shea SM, Wong LC and Harrison NL (2000) Propofol increases agonist efficacy at the GABA(A) receptor. *Brain Res* **852**(2):344-348.

Padgett CL and Lummis SC (2008) The F-loop of the GABA A receptor gamma2 subunit contributes to benzodiazepine modulation. *J Biol Chem* **283**(5):2702-2708.

Pascual JM and Karlin A (1998) State-dependent accessibility and electrostatic potential in the channel of the acetylcholine receptor. Inferences from rates of reaction of thiosulfonates with substituted cysteines in the M2 segment of the alpha subunit. *J Gen Physiol* **111**(6):717-739.

Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC and Ferrin TE (2004) UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem* **25**(13):1605-1612.

Pless SA and Lynch JW (2009) Ligand-specific conformational changes in the alpha1 glycine receptor ligand-binding domain. *J Biol Chem* **284**(23):15847-15856.

Serafini R, Bracamontes J and Steinbach JH (2000) Structural domains of the human GABAA receptor 3 subunit involved in the actions of pentobarbital. *J Physiol* **524 Pt 3**:649-676.

Sigel E (2002) Mapping of the benzodiazepine recognition site on GABA(A) receptors. *Curr Top Med Chem* **2**(8):833-839.

Steinbach JH and Akk G (2001) Modulation of GABA(A) receptor channel gating by pentobarbital. *J Physiol* **537**(Pt 3):715-733.

Tran PN, Laha KT and Wagner DA (2011) A tight coupling between betaY97 and betaF200 of the GABA(A) receptor mediates GABA binding. *J Neurochem* **119**(2):283-293.

Ueno S, Bracamontes J, Zorumski C, Weiss DS and Steinbach JH (1997) Bicuculline and gabazine are allosteric inhibitors of channel opening of the GABAA receptor. *J Neurosci* **17**(2):625-634.

Ueno S, Zorumski C, Bracamontes J and Steinbach JH (1996) Endogenous subunits can cause ambiguities in the pharmacology of exogenous gamma-aminobutyric acidA receptors expressed in human embryonic kidney 293 cells. *Mol Pharmacol* **50**(4):931-938.

Unwin N (2005) Refined structure of the nicotinic acetylcholine receptor at 4A resolution. *J Mol Biol* **346**(4):967-989.

Wang Q, Pless SA and Lynch JW (2010) Ligand- and subunit-specific conformational changes in the ligand-binding domain and the TM2-TM3 linker of {alpha}1 {beta}2 {gamma}2 GABAA receptors. *J Biol Chem* **285**(51):40373-40386.

Williams CA, Bell SV and Jenkins A (2010) A residue in loop 9 of the beta2-subunit stabilizes the closed state of the GABAA receptor. *J Biol Chem* **285**(10):7281-7287.

Williams DB and Akabas MH (2002) Structural evidence that propofol stabilizes different GABA(A) receptor states at potentiating and activating concentrations. *J Neurosci* **22**(17):7417-7424.

Zhang J, Xue F and Chang Y (2009) Agonist- and antagonist-induced conformational changes of loop F and their contributions to the rho1 GABA receptor function. *J Physiol* **587**(Pt 1):139-153.

Zheng W and Auerbach A (2011) Decrypting the sequence of structural events during the gating transition of pentameric ligand-gated ion channels based on an interpolated elastic network model. *PLoS Comput Biol* **7**(1):e1001046.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Side-view of the $GABA_A$ receptor subunit interface. (**A**) Structure of the α1-γ2 subunit interface and the membrane-spanning regions. The α1 subunit is shown in yellow, the γ2 subunit is shown in blue. Loops A, B and C in the α1 subunit, and Loops D and E in the γ2 subunit are shown in black. Loop F in the γ2 subunit is shown in red. The γ2(S195C) residue is shown as red spheres. (**B**) Expanded view of the loop region of the extracellular domain. The figure was made using UCSF Chimera (Pettersen et al., 2004) by threading the $GABA_A$ receptor subunits onto the structures of the *Torpedo* nicotinic receptor (Unwin, 2005).

Figure 2. Properties of the wild-type and mutant receptors. (**A**) GABA concentration-response curves for the wild-type α1β2γ2L and mutant α1β2γ2(S195C) receptors. The data were normalized to the responses obtained at 100 μM (wild-type) or 1 mM GABA (mutant). The data points show mean ± s.e.m. from 4-5 cells. The curves were fitted to the Hill equation. The best fit parameters for the wild-type receptor are: I_{max} = 1.0 ± 0.03, EC_{50} = 1.8 ± 0.2 μM, n_H = 0.9 ± 0.1. The best fit parameters for the mutant receptor are: I_{max} = 1.0 ± 0.02, EC_{50} = 11.5 ± 0.7 μM, n_H = 1.0 ± 0.04. (**B**) The presence of the γ2 subunit in receptor-complexes was demonstrated by resistance to inhibition by zinc. Cells expressing α1β2γ2 or α1β2γ2(S195C) receptors (top and middle traces) were activated by 100 μM GABA in the absence and presence of 100 μM $ZnCl_2$. The presence of $ZnCl_2$ had a minimal effect on the current responses, as expected for receptors containing the γ subunit (Krishek et al., 1998). For comparison, the lower trace shows the effect of $ZnCl_2$ on a cell expressing α1β2 receptors. (**C**) Comparison of the effect of 50 μM pentobarbital on currents elicited by 100 μM GABA indicates that the γ2(S195C) mutation does not significantly reduce maximal open probability. Reduced maximal open probability would be expected to result in an ability to be potentiated even when the receptors are activated by saturating GABA. All three receptor types were potentiated by pentobarbital when low concentrations of GABA were used to activate the receptors (not shown).

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Figure 3. Modification of the γ2(S195C) site by MTSES in the presence of transmitter-site ligands. (**A**) Cells expressing α1β2γ2(S195C) receptors were exposed to 5 s pulses of 0.3 μM GABA (~EC_7), separated by 115 s washout periods. It typically took 5 to 10 applications of GABA for the responses to stabilize. The cell was then exposed to 1 mM MTSES for 1 min, followed by a 1 min washout in bath and further applications of 0.3 μM GABA. Each panel shows the last two control responses (left traces) and two responses after exposure to MTSES ± GABA, to demonstrate stability of responses. From this experiment, we calculate the ratio of the peak response after MTSES exposure ($I_{post\text{-}MTSES}$) to the peak response before MTSES exposure ($I_{pre\text{-}MTSES}$). The experiment demonstrates an increase in the peak response following exposure to MTSES. (**B**) When MTSES was coapplied with a high concentration (100 μM) of GABA, the increase in peak response was not observed. The presence of the γ2 subunit in receptor-complexes was verified by zinc (please see Figure 2B). (**C**) Summary of the data. α1β2γ2(S195C) receptors exposed to 1 mM MTSES demonstrate an enhanced functional response to low (EC_7) concentrations of GABA suggesting that MTSES covalently modifies the receptor at the γ2(S195C) site. Coapplication of GABA or muscimol, but not the competitive antagonist of the GABA-site gabazine, with MTSES prevents productive labeling at the γ2(S195C) site. Data show mean ± S.D. from 3 to 6 cells. The statistical analyses apply to comparison with no effect and to comparison with the effect of labeling on resting receptors (see Methods for more details). *, $p < 0.05$; ***, $p < 0.001$; †, not significant. (**D**) Structure of MTSES.

Figure 4. Modification of the γ2(S195C) site by MTSES in the presence of allosteric ligands. (**A**) Cells expressing α1β2γ2(S195C) receptors were exposed to 5 s pulses of 0.3 μM GABA (~EC_7), separated by 115 s washout periods. The cell was then exposed to 1 mM MTSES + 5 μM etomidate (ETO) for 1 min, followed by a 3 min washout in bath, and further applications of 0.3 μM GABA. The experiment demonstrates an increase in the peak response following exposure to MTSES + ETO. (**B**) Cells expressing α1β2γ2(S195C) receptors were exposed to 5 s pulses of 0.3 μM GABA (~EC_7), separated by 115 s washout periods. The cell was then exposed to 1 mM MTSES + 100 μM pentobarbital (PB) for 1 min, followed by a 3 min washout in bath, and further applications of 0.3 μM GABA. The experiment

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demonstrates an increase in the peak response following exposure to MTSES + PB. (**C**) Summary of the data. Data show mean ± S.D. The statistical analyses apply to comparison with no effect and to comparison with the effect of labeling on resting receptors (see Methods for more details). *, $p < 0.05$; ***, $p < 0.001$; †, not significant. The data for the resting receptor are reproduced from Figure 3C.

Figure 5. Estimates of rates of modification of the γ2(S195C) site by MTSES. Normalized GABA current responses are plotted vs. cumulative time of MTSES application. MTSES was applied alone (**A**; resting receptors) or in the presence of 50 µM gabazine (**B**), 100 µM pentobarbital (**C**) or 5 µM etomidate (**D**). The concentration of MTSES was 20 µM for etomidate, and 100 µM under all other conditions. The plots show fits to data from individual cells (thin lines) and the data points and fits to averaged data (thick lines). The results of the fits are given in the text and in Table 1.

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Table 1.

Conditions	k_2 ($M^{-1}s^{-1}$)	n
Resting (Control)	$115 \pm 25^{\dagger,\dagger,***}$	5
Gabazine	$247 \pm 119^{\dagger,\dagger,***}$	6
Pentobarbital	$125 \pm 57^{\dagger,\dagger,***}$	4
Etomidate	$2484 \pm 1314^{***,***,***}$	3

Table 1. Second-order rate constants for MTSES derivatization of α1β2γ2(S195C) mutant receptors in the presence of gabazine, pentobarbital, or etomidate. Data represent mean ± S.D. from 3-6 cells. MTSES was applied alone or coapplied with 50 μM gabazine, 100 μM pentobarbital or 5 μM etomidate. The significance levels apply to comparison with all remaining three conditions using ANOVA with Bonferroni correction. ***, $p < 0.001$; †, not significant.

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FIGURE 1

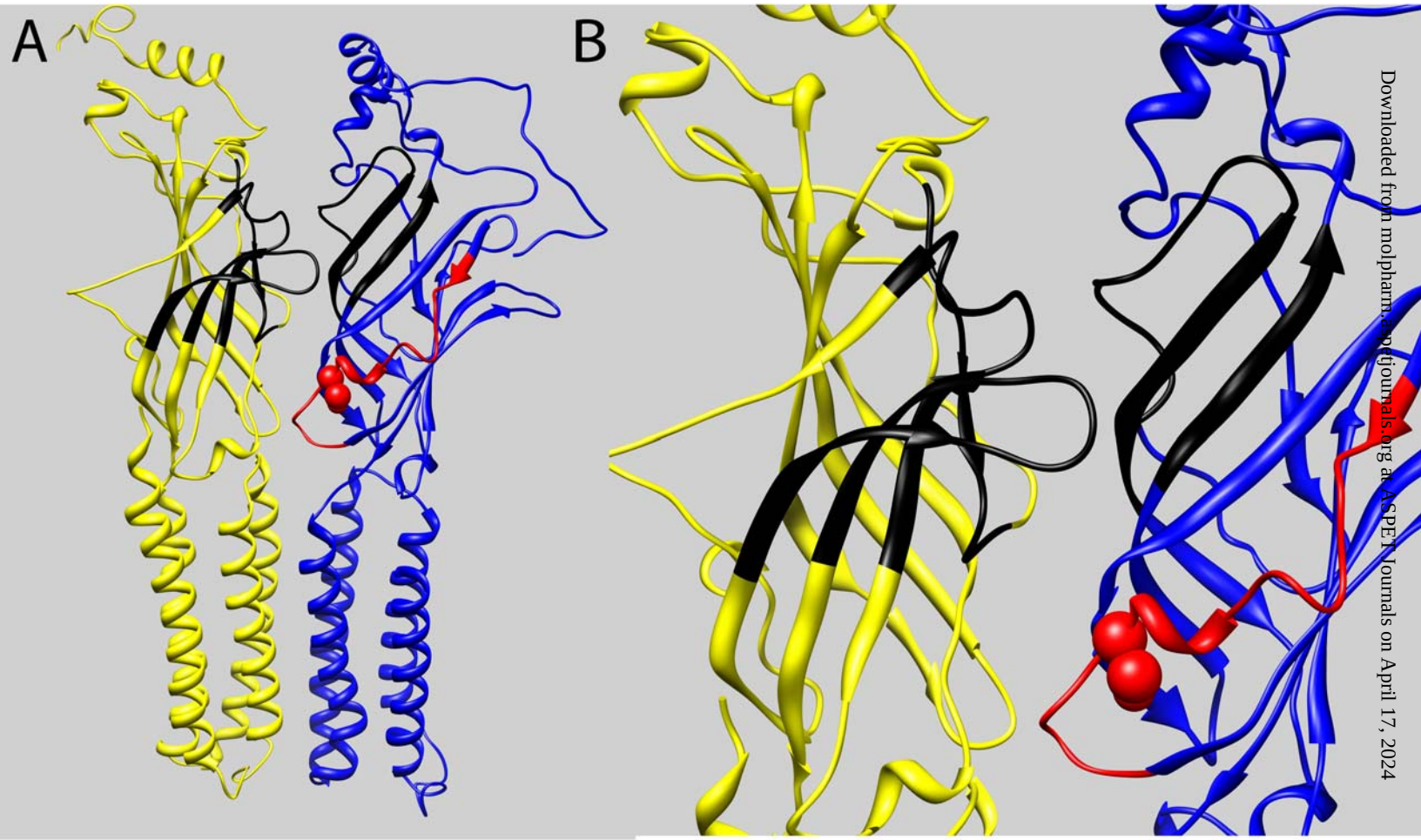

FIGURE 2

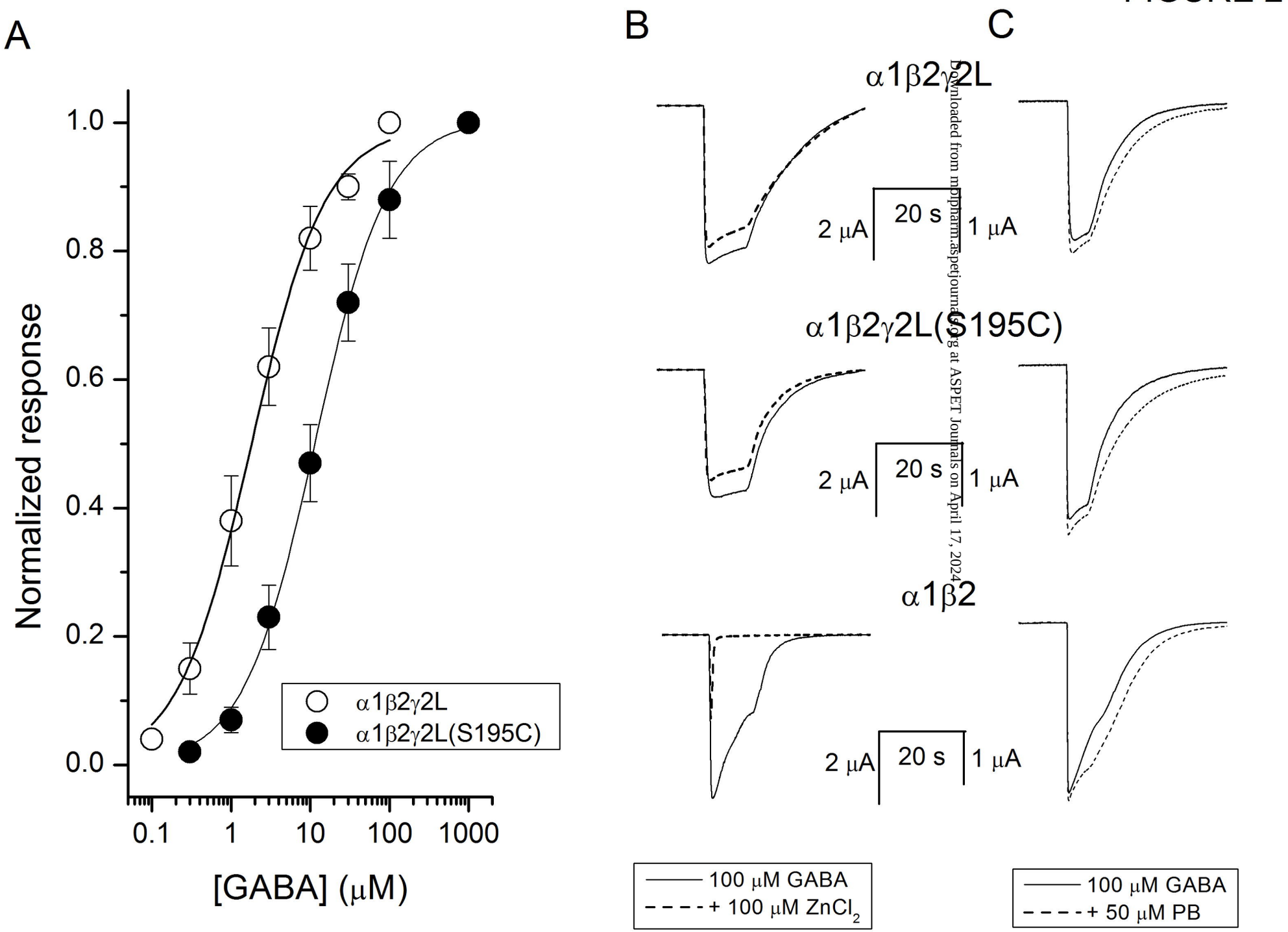

FIGURE 3

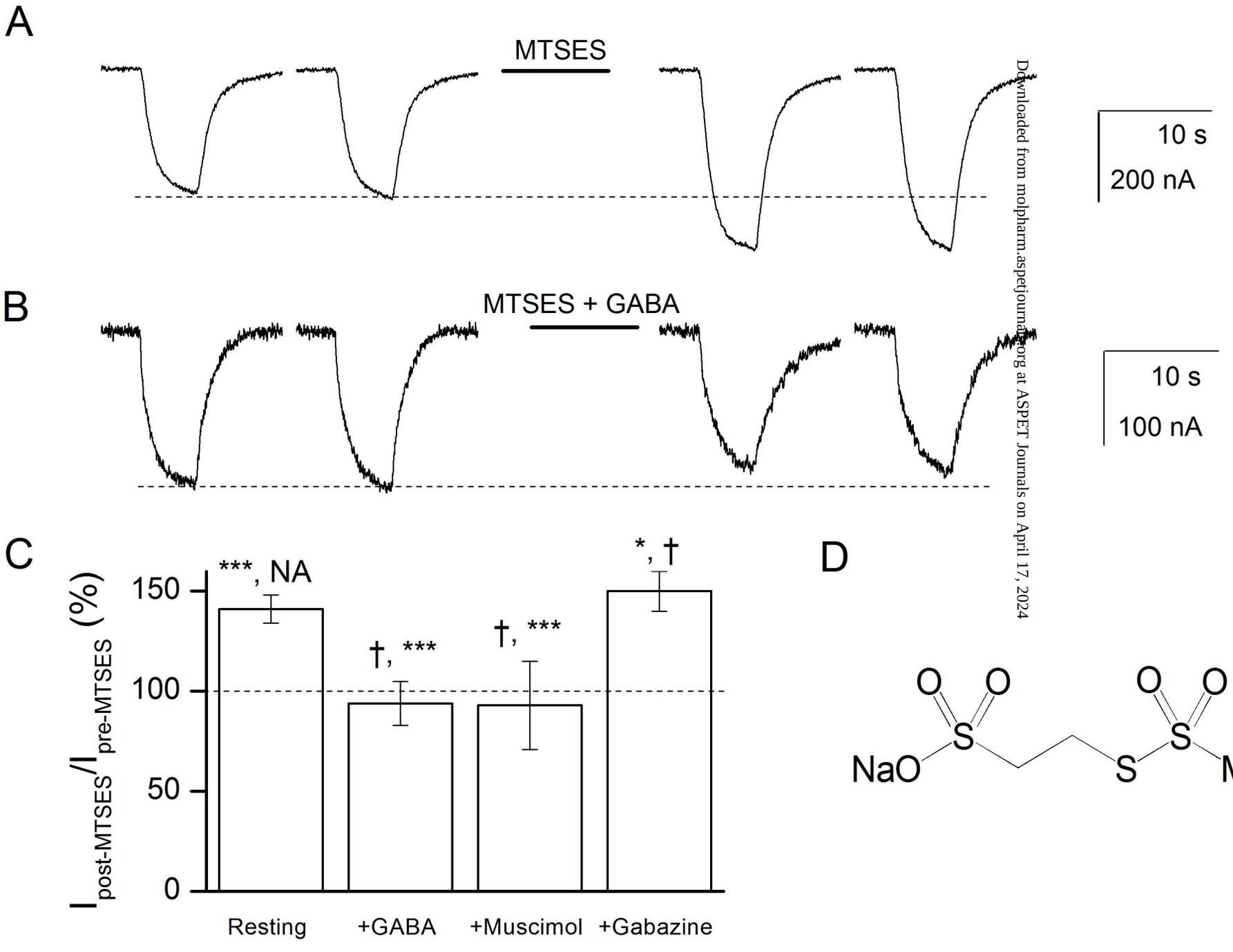

FIGURE 4

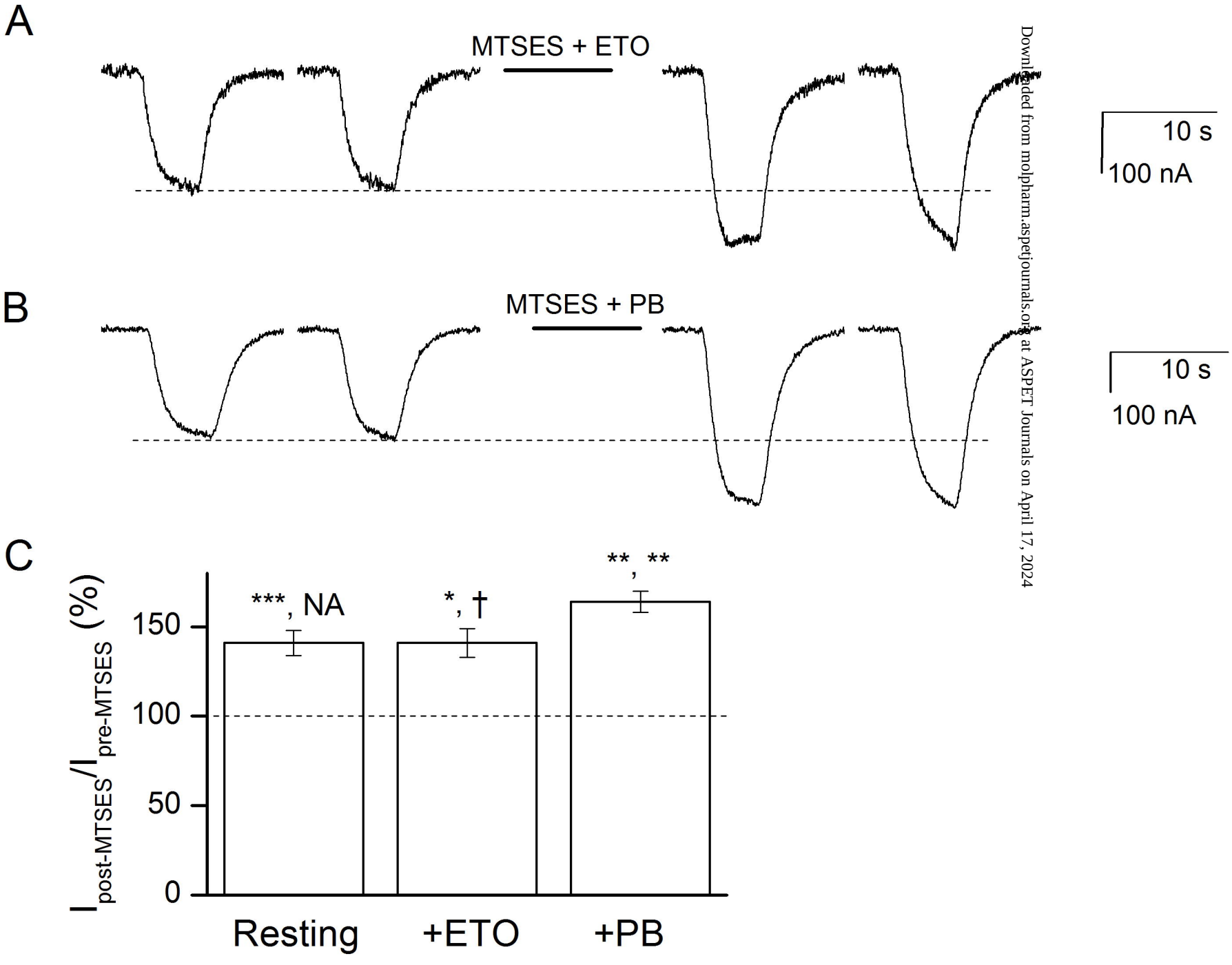

FIGURE 5