

Title page

A SINGLE AMINO ACID RESIDUE AT TRANSMEMBRANE DOMAIN 4 OF THE ALPHA
SUBUNIT INFLUENCES CARISOPRODOL DIRECT GATING EFFICACY AT GABA_A RECEPTORS

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Abbreviations: CSP, Carisoprodol; GABA, γ -aminobutyric acid; GABAA, type A GABA receptor; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol-bis (β -aminoethyl ether); HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; N, N, N', N'-tetra acetic acid; MEP, Meprobamate.

Abstract

The muscle relaxant carisoprodol (CSP, trade name Soma) has recently been controlled at the federal level as a Schedule IV drug due to its high abuse potential and consequences of misuse, such as withdrawal syndrome, delusions, seizures and even death. Recent work has shown that carisoprodol can directly gate and allosterically modulate the GABA_A receptor. These actions are subunit-dependent; compared to other GABA_A receptors, carisoprodol has nominal direct gating effects in $\alpha 3\beta 2\gamma 2$ receptors. Here, using site-directed-mutagenesis and whole cell patch clamp electrophysiology in transiently transfected HEK293 cells, we examined the role of GABA_A receptor α subunit transmembrane domain 4 (TM4) amino acids in direct gating and allosteric modulatory actions of carisoprodol. Mutation of $\alpha 3$ valine at position 440 to leucine (present in the equivalent position in the $\alpha 1$ subunit) significantly increased the direct gating effects of carisoprodol, without affecting allosteric modulatory effects. The corresponding reverse mutation, $\alpha 1$ (L415V), decreased carisoprodol direct gating potency and efficacy. Analysis of a series of amino acid mutations at the 415 position demonstrated amino acid volume correlated positively with CSP efficacy, while polarity inversely correlated with CSP efficacy. We conclude $\alpha 1$ (415) of TM4 is involved in the direct gating, but not allosteric modulatory, actions of carisoprodol. Also, orientation of alkyl or hydroxyl groups at this position influence direct gating effects. These findings support the likelihood that direct gating and allosteric modulatory effects of carisoprodol are mediated via distinct binding sites.

Introduction

The centrally acting muscle relaxant Carisoprodol (*N*-isopropyl meprobamate, CSP) is frequently prescribed for skeleton muscle pain (Luo et al., 2004; Toth and Urtis, 2004). In recent years, misuse and abuse of CSP has become a significant problem. Carisoprodol abuse causes psychomotor impairment and severe withdrawal that may predispose to seizures and death (Bramness et al., 2004; Fass, 2010; Reeves et al., 2012; Zacny and Gutierrez, 2011; Zacny et al., 2011). Tolerance to carisoprodol develops relatively quickly, facilitating the problems associated with withdrawal (Gatch et al., 2012; Reeves and Burke, 2010). As per the 2011 National Survey on Drug Use and Health, conducted by the Substance Abuse and Mental Health Services Administration, an estimated 2.9 million people in the United States admitted they had consumed carisoprodol for non-medical purpose in 2009 alone. Indeed, considering its alarming abuse rate, effective January of 2012, carisoprodol was controlled as a schedule IV substance at the federal level (Reeves et al., 2012).

Until recently, it was widely accepted that the sedative and muscle relaxing effects of carisoprodol were predominantly due to its primary metabolite, meprobamate (Bramness et al., 2004). More recent work has shown that carisoprodol itself allosterically modulates, directly activates and blocks γ -Aminobutyric acid, type A (GABA_A) receptors in a concentration-dependent manner (Gonzalez et al., 2009a; Gonzalez et al., 2009b). In vivo studies also support the fact that carisoprodol itself has significant CNS effects due to interaction with GABA_A receptors.

GABA_A receptors are member of the cys-loop family of ligand-gated ion channels; they are hetero-pentameric Cl⁻ channels and play a critical role in mediating fast inhibition in the brain (Corringer et al., 2012; Sigel and Steinmann, 2012). Multiple GABA_A receptor subunits and corresponding isoforms have been identified, including α (1-6), β (1-3), γ (1-3), ρ , δ , ε and θ (Olsen and Sieghart, 2008). Each subunit is composed of a large extracellular N terminus, four transmembrane helices (TM1–TM4), an extracellular TM2–TM3 loop, a large TM3–TM4 intracellular loop, and an extracellular C terminus (Cockcroft et al., 1995). The TM2 domains form the pore of the channel (Miyazawa et al., 2003; Xu and Akabas, 1996) (Fig. 1). In addition to the GABA binding site, GABA_A receptors have binding sites for several clinically

important drugs, including anxiolytics, sedative-hypnotics, muscle relaxants, and anesthetics. In $\alpha 1\beta 2\gamma 2$ receptors, the GABA binding site is located at the interface of the $\alpha 1$ and $\beta 2$ subunits, and benzodiazepines bind at the interface of the $\alpha 1$ and $\gamma 2$ subunits in the extracellular region (Newell and Czajkowski, 2003; Sigel and Steinmann, 2012) (Fig 1A). Barbiturate and general anesthetic (propofol, etomidate) binding sites are believed to be positioned in the water accessible region located between the TM helices of the receptor (Bali and Akabas, 2004; Siegwart et al., 2002; Zeller et al., 2007a; Zeller et al., 2007b). Carisoprodol actions are not mediated via reported sites of action for benzodiazepines or barbiturates (Gonzalez et al., 2009b). While the general anesthetics propofol and etomidate allosterically modulate and directly gate GABA_A receptors through a single site of action (Siegwart et al., 2002; Stewart et al., 2013), distinct GABA_A receptors sites confer these properties to neurosteroids (Hosie et al., 2006). Work to date suggests carisoprodol may mediate its allosteric modulatory and direct gating effects via distinct sites of action (Gonzalez et al., 2009b).

Our recent studies with carisoprodol on GABA_A receptors have shown the allosteric modulatory and direct gating properties of carisoprodol are subunit-dependent (Kumar et al., 2015). Allosteric modulatory actions of carisoprodol are most efficacious at receptors incorporating the $\alpha 1$ subunit, whereas $\alpha 3$ -expressing receptors show minimal direct gating effects. Characteristics of carisoprodol effects are consistent with it interacting at the transmembrane domains (Hosie et al., 2006). Aligned amino acid sequences of human α subunit isoforms ($\alpha 1$ -6) revealed that TM1, TM2 and TM3 are fully conserved in all α subunit isoforms. The TM4 region of α subunit isoforms is also largely conserved; however, I419, I423 and V440 residues of $\alpha 3$ differ compared to all other α subunit isoforms (Fig. 1C) (Barnard et al., 1998; Bergmann et al., 2013). We thus explored the extent to which these residues may contribute to the ability of carisoprodol to directly gate and allosterically modulate GABA_A receptors. We have identified L415 at TM4 of the $\alpha 1$ subunit (equivalent to V440 in the $\alpha 3$ subunit) as being critically involved in direct gating actions of carisoprodol, without affecting its allosteric modulatory effects.

Material and Methods

Plasmids and site-directed-mutagenesis. Human cDNA plasmids encoding $\alpha 1$, $\alpha 3$, $\beta 2$ and $\gamma 2$ GABA_A receptor subunits were used in the present study. Individual and combined mutations in $\alpha 1$ and $\alpha 3$ cDNA plasmids were created using Stratagene's Quik Change II ® site-directed-mutagenesis kit (Agilent Technologies; La Jolla, CA) and were sequenced to confirm mutations at West Virginia University's Genomics Core Facility.

Chemicals and solutions. Carisoprodol, meprobamate, pentobarbital, salts and buffers were purchased from Sigma Aldrich (St. Louis, MO), and GABA was obtained from Acros Organics (New Jersey, US). Pentobarbital and GABA stock solutions (500 mM) were prepared in deionized water. Carisoprodol stock solution (1 M) was made in DMSO. All stock solutions were stored at -20° C. On the day of experiment, fresh working drug concentrations were prepared from stock solution by dissolving in physiological buffer solution (below).

Cell Culture and Transfection. Human embryonic kidney 293 (HEK293) cells were transfected with human cDNA encoding desired GABA_A receptor subunits. To obtain $\alpha\beta\gamma 2$ GABA_A receptors, HEK293 cells were transfected with human GABA_A $\alpha 1/3$ mutant or wild type ; human $\beta 2$; and human $\gamma 2s$ (short isoform) subunit cDNA in a 1:1:5 (0.3 μ g : 0.3 μ g : 1.5 μ g) ratio using poly jet DNA in vitro transfection reagent (SigmaGen Laboratories, MD) and used for recording 24-48 h later. The $\gamma 2s$ subunit will be referred to as $\gamma 2$ from this point forward. Human GABA_A $\alpha 1$ subunit cDNA was generously provided by Neil Harrison (Columbia University Medical Center, New York). Cells were plated on glass coverslips coated with poly-L-lysine in 35-mm culture dishes and were incubated and maintained at 37°C in a humidified incubator with an atmosphere of 5% CO₂.

Whole-cell patch clamp electrophysiology. All experiments were conducted at room temperature (22-25°C) with the membrane potential clamped at -60 mV. Patch pipettes of borosilicate glass (1B150F; World Precision Instruments, Inc., Sarasota, FL) were pulled (Flaming/Brown, P-87/PC; Sutter Instrument Company, Novato, CA) to a tip resistance of 4–6 M Ω . Patch pipettes were filled with a solution consisting

of 140 mM CsCl, 10 mM EGTA- Na^+ , 10 mM HEPES- Na^+ , and 4 mM Mg^{2+} -ATP, pH 7.2. Coverslips containing cultured cells were placed in the recording chamber on the stage of an inverted light microscope and superfused continuously with an external solution consisting of 125 mM NaCl, 20 mM HEPES, 3 mM CaCl_2 , 5.5 mM KCl, 0.8 mM MgCl_2 , and 10 mM glucose, pH 7.4. Agonist-induced Cl^- currents were obtained with an Axopatch 200B amplifier with a rate of 50 samples per second (Molecular Devices, Sunnyvale, CA) equipped with a CV-203BU head stage. Currents were low-pass filtered at 5 kHz, monitored simultaneously on an oscilloscope and a chart recorder (Gould TA240; Gould Instrument Systems Inc., Cleveland, OH), and stored on a computer using an on-line data acquisition system (pCLAMP 6.0; Axon Instruments) for subsequent off-line analysis.

Experimental Protocol. GABA (with or without carisoprodol) or carisoprodol was prepared in external saline solution from stock solutions and applied to each cell by gravity flow using a Y-shaped tube positioned adjacent to the cell. Recordings were obtained from transfected cells only after establishing that two consecutive GABA EC_{20} -activated currents varied in amplitude by no more than $\pm 10\%$. For studies investigating direct activation, carisoprodol-mediated currents were normalized to currents elicited by saturating GABA concentrations. Modulatory effects of carisoprodol on GABA-gated currents were assessed using an EC_{20} gating concentration of GABA as the control (individually determined for each mutant and wild type receptor studied). This gating concentration was selected to ensure there was a sufficient range to observe the full allosteric potential of carisoprodol. At the initiation of each recorded cell, it was confirmed that gating concentration was approximately the EC_{20} (range of EC_{15} to EC_{25} accepted for an individual cell). In recordings displaying inhibition followed by a rebound current after termination of carisoprodol or carisoprodol plus GABA application (Gonzalez et al., 2009b), the maximal current amplitude achieved during active ligand application was taken as the peak current (Kumar et al., 2015).

Data Analysis. Concentration-response profiles for the positive modulatory actions of carisoprodol were generated (Origin 9.1; OriginLab Corp., Northampton, MA) using the equation $I/I_{\text{max}} = [\text{carisoprodol}]^n / ([\text{carisoprodol}]^n + \text{EC}_{50}^n)$, where I is the normalized current amplitude at a given concentration of carisoprodol, I_{max} is the maximum current induced by carisoprodol, EC_{50} is the half-

maximal effective concentration of carisoprodol, and n is the Hill coefficient. For concentration-response curves illustrating allosteric actions, a correction was applied to subtract direct gating effects. In some cases, the blocking actions of carisoprodol became notable at high concentrations; in these instances, curves were fitted to the data point corresponding to peak effect, and the curve was extrapolated. All data are presented as mean values \pm S.E. Statistical significance between control and test conditions was determined using Student's t -test (paired or unpaired) and one-way analysis of variance. Tukey-Kramer *post hoc* test for multiple comparisons was performed as needed. Correlation assessments were performed using linear fit in origin 9.1.

Results

Functional characterization of $\alpha 3$ and $\alpha 1$ TM4 mutant GABA_A receptors. For this study, an extensive series of mutations (single point or 2-3 residues) in $\alpha 1$ and $\alpha 3$ subunits were evaluated. In all cases, the mutant subunit was expressed with wild type $\beta 2$ and $\gamma 2$ subunits, and GABA concentration-response profiles were generated to assess overall receptor function and to establish gating concentrations for allosteric studies. GABA EC₅₀ for wild type $\alpha 1$ and $\alpha 3$ receptors were both approximately 35 μ M (Tables 1 and 2). In general, shifts in GABA EC₅₀ were modest. Mutations in $\alpha 3$ subunits caused a leftward shift in the GABA concentration-response curve of 1.9- to 4.6-fold (Table 1). Similarly, mutations in the $\alpha 1$ subunit had either insignificant or modest effects on GABA EC₅₀, with the maximal effect being a 2.5-fold increase in GABA EC₅₀ relative to wild type $\alpha 1$ receptors (Table 2). Thus the mutations had minimal effects on fundamental receptor gating.

Mutation of $\alpha 3$ TM4 amino acids to corresponding $\alpha 1$ amino acids increased direct gating effect of carisoprodol but not allosteric modulatory actions. Consistent with our previous report (Kumar et al., 2015), the ability of CSP to directly gate $\alpha 3\beta 2\gamma 2$ receptors was significantly less compared to GABA_A receptors expressing the $\alpha 1$ subunit (Fig. 2B, Tables 1, 2). To evaluate the direct gating efficacy of CSP, we normalized the CSP-gated currents to saturated GABA-gated current amplitudes. Maximal current amplitudes generated by 3 mM CSP were 41.8 ± 2.4 % in $\alpha 1\beta 2\gamma 2$ receptors and 8.5 ± 1.1 % in $\alpha 3\beta 2\gamma 2$ receptors, confirming low efficacy at $\alpha 3$ -expressing receptors. We thus assessed the potential involvement of three unique amino acids we identified in TM4 (I419, I423 and V440) of the $\alpha 3$ subunit in this attenuated direct gating effect of CSP. These amino acid residues were mutated to the amino acid found at the equivalent position in the $\alpha 1$ subunit, either individually or in combination. Mutation of a single amino acid $\alpha 3$ (V440L) significantly increased the direct gating effect of CSP compared to WT $\alpha 3$ -expressing receptors (Fig. 2A). Similarly, all $\alpha 3$ mutants resulted in a gain-of-function effect, significantly increasing direct gating currents such that current amplitudes in response to 3 mM CSP were not significantly different from that obtained in wild type $\alpha 1\beta 2\gamma 2$ receptors (Fig. 2B, summary values in Table 1).

As carisoprodol also has a diminished allosteric modulatory effect in $\alpha 3\beta 2\gamma 2$ receptors compared to $\alpha 1\beta 2\gamma 2$ receptors (Kumar et al., 2015), we also assessed the extent to which these mutations might affect sensitivity to the allosteric actions of CSP. Interestingly, allosteric modulatory effects of CSP were not affected by the $\alpha 3$ TM4 mutations (Fig. 2C). Carisoprodol potentiated GABA EC₂₀ currents in all mutated receptors, however the magnitude of the potentiation for each mutant variant was not significantly different from that observed in $\alpha 3\beta 2\gamma 2$ receptors, and it fell far short of that produced in $\alpha 1\beta 2\gamma 2$ receptors (Fig. 2D and Table 1). Thus the TM4 residues assessed here influence direct gating but not allosteric modulatory effects of CSP.

Carisoprodol's less potent metabolite, meprobamate, also displays reduced direct gating effects in $\alpha 3$ - expressing receptors (Kumar and Dillon, 2016) compared to all other α subunits. We thus evaluated the ability of these mutations to impact direct gating by meprobamate. These TM4 mutations also conferred gain-of-function effects for meprobamate direct gating, although the magnitude of effect was less than that observed with carisoprodol (Fig. 3).

A single mutation of $\alpha 1$ TM4 L415 amino acid to corresponding $\alpha 3$ V440 amino acid decreased direct gating effect of carisoprodol. To further assess the involvement of the identified α subunit TM4 residues in direct gating effects of carisoprodol, we conducted the converse set of studies; i.e., we mutated $\alpha 1$ TM4 amino acids to the corresponding $\alpha 3$ TM4 amino acids (L394I, A398I, and L415V) in all combinations of single, double or triple mutations, and assessed direct gating effects of CSP. GABA_A receptors expressing the $\alpha 1$ (L415V) subunit showed significantly decreased direct gating by CSP compared to WT receptors (Fig. 4A), whereas receptors expressing $\alpha 1$ (L394I) and $\alpha 1$ (A398I) subunits showed no significant alternation in direct gating actions of CSP. Out of seven mutations we generated, each of those containing the L415V mutation caused a significant loss of carisoprodol (3 mM) direct gating effect compared to wild type $\alpha 1$ receptors. Conversely, in receptors which did not incorporate the L415V mutation, carisoprodol's effects were not significantly different from wild type (Fig. 4B and Table 2).

These data indicate the leucine residue at position 415 in the $\alpha 1$ subunit has a key role in the direct gating action of carisoprodol.

Amino acid residue at $\alpha 1(415)$ subunit influences carisoprodol direct gating efficacy. In an attempt to gain additional insight into physicochemical determinants that influence CSP direct gating capability at the $\alpha 1(415)$ position, we generated and assessed the following series of mutations: $\alpha 1(L415S)$, $\alpha 1(L415G)$, $\alpha 1(L415T)$ $\alpha 1(L415Y)$ $\alpha 1(L415W)$, $\alpha 1(L415I)$, $\alpha 1(L415C)$ and $\alpha 1(L415R)$. These residues provide a range of amino acid side chain properties, including volume, polarity and hydrophathy. We assessed direct gating by carisoprodol in each mutant receptor. As with the (L415V) mutation, L415S, L415G and L415C all decreased maximal gating efficacy of CSP (to 5.5 ± 1.2 , 9.1 ± 1.6 , and 18.0 ± 1.6 % of saturating GABA current, respectively). Potency to carisoprodol was generally unaffected, with the exception that the L415W mutant induced a three-fold rightward shift in EC_{50} (Fig. 5 and Table 2). The significant rightward shift in potency in receptors expressing the L415W mutation precluded accurate determination of efficacy with this mutation. The presence of T, Y, I or R had no effect on either CSP efficacy or potency. Correlation analysis showed positive and negative correlations of amino acid volume and polarity (Grantham, 1974), respectively, at the 415 position with carisoprodol direct gating efficacy (Fig. 6), while hydrophobicity tended to positively correlate with gating efficacy ($r = 0.59$, critical region of -0.632 to 0.632). These data demonstrate the nature of the amino acid side chain at the $\alpha 1(415)$ position is critical for the direct gating effect of carisoprodol. We also observed that an increase in GABA EC_{50} correlated negatively with CSP efficacy (Fig. 6F).

The $\alpha 1$ (L415S) mutation does not affect allosteric modulation by carisoprodol or direct activation by pentobarbital. To further assess the extent to which the $\alpha 1(L415)$ residue may be differentially involved in direct gating compared to allosteric modulatory effects of carisoprodol, we tested if the L415S mutation had an effect on allosteric potentiation. In $\alpha 1(L415S)\beta\gamma 2$ receptors, CSP potentiation of GABA EC_{20} currents differed in neither maximum potentiation nor potency when compared to wild type receptors ($439.45 \pm 49.4\%$ potentiation and EC_{50} of $89.5 \pm 15 \mu M$, $n = 7$, in $\alpha 1(L415S)\beta\gamma 2$ receptors compared to $474.75 \pm 53.4\%$ and $102.2 \pm 16 \mu M$, $n = 5$ in wild type receptors, Fig. 7A, B). To assess

specificity of the L415S mutation, we evaluated if it had any effect on pentobarbital-activated currents. Direct gating by 1 mM pentobarbital was not significantly different in $\alpha 1$ (L415S) $\beta\gamma 2$ receptors compared to wild type receptors (current amplitude in comparison to saturating GABA was $70.2 \pm 4.2\%$, $n = 5$ and $84.1 \pm 6.4\%$, $n = 7$ in mutant and wild type receptors, respectively (Fig 7C, D). These results are consistent with distinct sites for direct and allosteric effects of CSP, and demonstrate effects of the L415S mutation are not due to non-specific effects on the ability of direct-gating ligands to activate the channel.

An $\alpha 1$ subunit mutation involved in direct gating by neurosteroids does not affect direct gating by carisoprodol. A number of neurosteroids also have the ability to directly gate and allosterically modulate GABA_A receptors. It has been demonstrated that mutation to isoleucine of the native threonine in position 236 of the $\alpha 1$ subunit (T236I) effectively abolishes direct gating by the neurosteroids tetrahydro-deoxy-corticosterone and allopregnanolone, without affecting their allosteric potentiating effects (Hosie et al., 2006). We thus tested whether the $\alpha 1$ (T236I) mutation affected carisoprodol direct gating actions. This mutation did not produce any change in the ability of carisoprodol to directly activate wild type receptors (Fig 8).

Discussion

In a recent report (Kumar et al., 2015), we found that direct gating effects of the skeletal muscle relaxant carisoprodol were reduced in $\alpha 3\beta\gamma 2$ GABA_A receptors, compared to those expressing any other α subunit variant ($\alpha 1$ -2, $\alpha 4$ -6). Here, we identified α subunit TM4 residues, in particular $\alpha 1415$ (equivalent to $\alpha 3440$), that are critical for direct gating, but not allosteric modulatory, effects of carisoprodol. Mutation of the native $\alpha 3$ 440V residue to the L residue found in the $\alpha 1$ subunit (V440L) resulted in a significant enhancement of carisoprodol-gated current; the converse mutation ($\alpha 1$ (L415V)) had the opposite effect. Direct gating efficacy of carisoprodol's primary metabolite, meprobamate, was also influenced by the L415 mutations. Subsequent evaluation of a series of mutations resulted in the following rank order effect on carisoprodol gating efficacy (L = I = T = R > Y > W = C = V > G > S), and correlation analysis demonstrated that both amino acid volume and polarity are important determinants of this position's effect on carisoprodol direct gating. The presence of a hydrophobic residue tended to correlate with enhanced CSP gating, although this effect did not reach statistical significance. Interestingly, except for tryptophan (which caused a 3-fold increase in CSP direct gating EC₅₀), none of the introduced mutants affected potency of carisoprodol's direct gating effect; the action was nearly exclusively an effect on efficacy.

Considering we have shown previously that carisoprodol inhibits the channel at high concentration (Gonzalez et al, 2009; Kumar et al., 2015; note also the rebound current in Fig. 2B following removal of carisoprodol), one might also consider the possibility that the effects of the mutations studied could be due to shifting carisoprodol's ability to inhibit the channel. For example, possibly the $\alpha 3$ V440L mutation attenuates CSP-mediated inhibition instead of enhancing CSP-mediated direct gating. Whereas we cannot definitively rule out this possibility, we consider it unlikely. We have reported in abstract form (Kumar and Dillon, 2014) that the ability of carisoprodol to block $\alpha 1\beta 2\gamma 2$ or homomeric $\beta 3$ GABA_A receptors is greatly attenuated or eliminated, respectively, when the 6' tyrosine residue in the second transmembrane domain is mutated to phenylalanine. The TM2 domain thus seems to be involved in CSP-mediated channel inhibition, while the TM4 residue targeted here is important for direct gating by carisoprodol.

Transmembrane 4 residues of the $\alpha 1$ subunit have been shown to be involved in allosteric modulatory effect of other GABA_A receptor ligands, such as neurosteroids and anesthetics (Hosie et al., 2006; Jenkins et al., 2002). Homology modeling has shown that N407 and Y410 donate a hydrogen bond to the ketone group of THDOC, and contribute to the binding pocket of neurosteroids. Substitution of polar residues to hydrophobic amino acids at N407A and Y410F reduced THDOC potency significantly (Hosie et al., 2006). Indeed, L415 itself has been implicated in effects mediated by anesthetic agents. In a tryptophan scanning study of TM4, it was found that introduction of tryptophan at position 415 (L415W) of the $\alpha 1$ subunit produced a significant decrease in the ability of the anesthetics halothane and chloroform to potentiate GABA-gated currents (Jenkins et al., 2002). It is possible TM domains form an important allosteric modulatory site on GABA_A receptors. However, our results would seem to rule out the potential involvement of the TM4 domain 415 position for allosteric effects of carisoprodol, as the (L415S) mutation had no effect on the ability of carisoprodol to allosterically enhance GABA-gated current. These results are most consistent with the conclusion that distinct sites exist for the allosteric modulatory and direct gating effects of carisoprodol.

Previous molecular and behavior studies of carisoprodol have demonstrated characteristics of barbiturate-like effects. Both ligands directly gate, allosterically modulate, and inhibit the receptor (at high concentrations). More notably, in drug discrimination studies, the barbiturate pentobarbital substituted for the discriminative stimulus effects of carisoprodol in carisoprodol-trained rats. In addition, the barbiturate antagonist bemegride blocked the locomotor depression effect of carisoprodol in mice, and also antagonized carisoprodol-gated currents in HEK293 cells expressing GABA_A receptors (Gonzalez et al., 2009b). These findings suggested that behavioral and molecular action of carisoprodol may be mediated by a barbiturate-like mechanism of action on GABA_A receptors. However, in the present study the $\alpha 1$ (L415S) mutation did not affect the ability of pentobarbital to directly gate GABA_A receptors. In addition, a rho receptor mutation that confers sensitivity to barbiturate (wild type is insensitive to barbiturates) did not confer sensitivity to carisoprodol (Gonzalez et al., 2009b). Thus, although previous studies have shown barbiturate-like action of carisoprodol, collectively the data support distinct binding sites and/or functional domains for

carisoprodol versus barbiturate direct gating effects in GABA_A receptors. We also found that the $\alpha 1$ T236I mutation, shown previously to abolish direct gating by neurosteroids (Hosie et al., 2006), did not affect carisoprodol-mediated activation. Thus, whereas the $\alpha 1$ (T236) position is critical for direct gating by neurosteroids, it does not have a significant role in direct gating in response to carisoprodol.

The primary metabolite of carisoprodol, meprobamate, also directly gates GABA_A receptors, with a potency several-fold lower than that of carisoprodol. The sole structural difference between the two ligands is the presence of an isopropyl group present on one of the two carbamyl nitrogens in carisoprodol; this functional group thus dictates the differences in potency between the two ligands. Whereas valine and leucine are similarly hydrophobic and both are of low polarity, one may speculate that the larger volume of leucine may make it more accessible for hydrophobic interaction with the isopropyl group present on the carbamyl nitrogen in carisoprodol. Although the $\alpha 3$ (V440L) mutation did result in enhanced gating of meprobamate (which lacks the isopropyl substituent), the fact that the magnitude of the effect was considerably smaller than that observed with carisoprodol would be consistent with this possibility. We should also note it is possible that carisoprodol is binding at a region distant from that studied here. In this scenario, the role of the $\alpha 1$ 415 leucine residue would be critical for transduction of the effects of carisoprodol subsequent to its binding at a distinct site. Additional studies, including molecular modeling, will be required to address these possibilities.

Carisoprodol is a relatively low affinity ligand. It is typically prescribed in 250 or 350 mg tablets, taken three times per day. With therapeutic administration, blood concentrations range from approximately 15-30 micromolar (Littrell et al, 1993; Olsen et al., 1994). Those abusing carisoprodol may be taking up to 50 tablets per day, and toxic concentrations of up to nearly 400 micromolar have been reported (Maes et al., 1969). Thus whereas therapeutic dosing should result in little to no direct gating by carisoprodol, the concentrations achieved in individuals abusing it are sufficient to result in direct activation. Indeed, this direct gating effect may be a critical factor in fatalities associated with carisoprodol abuse.

It is known that addictive drugs hijack the reward system by increasing dopamine levels in the mesolimbic system (Luscher and Ungless, 2006). For example, benzodiazepines mediate their addictive

actions by increasing dopamine release in the nucleus accumbens through activation of $\alpha 1$ -containing GABA_A receptors on GABAergic interneurons in the ventral tegmental area (Heikkinen et al., 2009; Rudolph and Knoflach, 2011; Tan et al., 2010). Moreover, behavior studies in transgenic mice expressing mutant α subunit isoforms have been instrumental in demonstrating distinct physiological effects of benzodiazepines associate with particular subunits. For instance, $\alpha 1$ -expressing receptors are involved in sedative effects and abuse potential, $\alpha 2$ -expressing receptors contribute to their anxiolytic effects, whereas $\alpha 2$ -, $\alpha 3$ - and $\alpha 5$ - expressing receptors are involved in the myorelaxant actions of benzodiazepines (Rudolph et al., 1999, Low et al., 2000, Crestani et al., 2001, van Rijnsoever et al., 2004; Licata and Rowlett, 2008) Drawing parallels to what is understood with regard to benzodiazepines, the robust effects of carisoprodol on $\alpha 1$ -expressing receptors (present report, also Kumar and Dillon, 2015) likely underlie its well-documented potential for abuse.

In summary, we have identified a transmembrane domain 4 residue of the GABA_A receptor that is critically involved in the direct gating actions of the skeletal muscle relaxant carisoprodol, and identified physicochemical traits that are important for this effect. Mutation of this residue did not impact allosteric modulatory effects of carisoprodol, and it also had no effect on the ability of the barbiturate pentobarbital to directly gate the receptor. These results are consistent with our contention that carisoprodol mediates these two actions through distinct sites on the GABA_A receptor. In addition, as noted recently (Kumar and Dillon, 2016), an array of meprobamate-related dicarbamate molecules was generated years ago, when both meprobamate and carisoprodol were being widely prescribed (Ludwig et al., 1969). Many of these molecules showed promise as muscle relaxants in pre-clinical studies, but to our knowledge none advanced to market. The potential reasons are many, including the fact that meprobamate was scheduled as a controlled substance soon thereafter. Given our current understanding of GABA_A receptor molecular pharmacology associated with therapeutic and adverse effects, it is feasible that reassessment of these molecules and potential derivatives would yield an efficacious muscle relaxant with considerably reduced abuse potential.

Authorship Contributions

Participated in research design: Kumar, Kumar and Dillon

Conducted mutagenesis: Manoj Kumar and Freund

Conducted experiments: Kumar and Kumar

Performed data analysis: Kumar, Kumar, Freund and Dillon

Wrote or contributed to writing of the manuscript: Kumar, Kumar and Dillon

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Footnotes

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Figure Legends

Figure 1. GABA_A receptor structure and alignment of transmembrane 4 amino acid residue of α (1-6) GABA_A receptor subunits. *Top left*, view from the membrane of a GABA_A receptor expressing α 1, β 2 and γ 2 subunits, and denoting GABA and benzodiazepine (BDZ) binding sites. *Top right*, lateral view of GABA_AR subunit, illustrating 4 transmembrane domains, the extracellular N-terminus, the C-terminus and the intracellular loop. *Bottom*, aligned amino acid sequence of TM4 region of human α subunit (α 1-6) isoforms showing conserved (*) and non-identical (shaded in gray) amino acids.

Figure 2. Influence of α 3 subunit TM4 mutations on direct activation and allosteric modulation by carisoprodol. **A**, representative traces demonstrating carisoprodol (CSP) activation of human α 3 β 2 γ 2 WT and α 3(V440L) β 2 γ 2 GABA_ARs. In this and all subsequent figures, WT or mutant α subunits are co-expressed with WT β 2 and γ 2 subunits (represented by “-”). Single mutation of V440 at TM4 of α 3 to L, present in α 1-expressing GABA_ARs, significantly increased CSP direct gating potency. **B**, bar graphs summarizing carisoprodol direct gating currents in human α 3-, α 3(V440L)-, α 3(I419L/I423A)-, α 3(I419L/I423A/V440L)- and α 1 β 2 γ 2 GABA_ARs. Single and combined mutation of TM4 domains of α 3 to those present in α 1 subunit significantly increased the direct gating potency of carisoprodol as compared to WT α 3 receptors. In this and all subsequent figures, carisoprodol-gated currents are normalized to currents elicited by saturating GABA (1 mM). **C**, representative traces demonstrating the potentiation of GABA-gated (EC₂₀) currents from human α 3 β 2 γ 2 WT and α 3(V440L) β 2 γ 2 GABA_ARs by carisoprodol. **D**, concentration-response curves for the allosteric modulation of GABA-gated currents in α 3-, α 3(V440L)-, α 3(I419L/I423A)-, α 3(I419L/I423A/V440L) and α 1 β 2 γ 2 GABA_ARs. Mutation of TM4 domains of α 3 to those present in α 1 subunit did not increase allosteric modulatory efficacy of carisoprodol. Carisoprodol-potentiated currents are normalized to currents elicited by GABA EC₂₀ concentrations. Each data point represents the mean \pm S.E.M. of a minimum of three cells. #, $p < 0.01$, *, $p < 0.05$.

Figure 3. Influence of $\alpha 3$ subunit TM4 mutations on direct activation of the carisoprodol metabolite meprobamate. **A**, representative traces demonstrating meprobamate (MEP) activation of human $\alpha 3\beta 2\gamma 2$ WT and $\alpha 3(V440L)\beta 2\gamma 2$ GABA_ARs. **B**, bar graph summarizing meprobamate direct gating currents in $\alpha 3$ -, $\alpha 3(V440L)$ -, $\alpha 3(I419L/I423A)$ -, $\alpha 3(I419L/I423A/V440L)$ and $\alpha 1\beta 2\gamma 2$ GABA_ARs. Single and combined mutation of TM4 residues of the $\alpha 3$ subunit to those present in the $\alpha 1$ subunit significantly increased the direct gating potency of meprobamate as compared to WT $\alpha 3$ receptors. Meprobamate-gated currents are normalized to currents elicited by saturating GABA (1 mM). Each data point represents the mean \pm S.E.M. of a minimum of three cells. #, $p < 0.01$, *, $p < 0.05$.

Figure 4. Influence of $\alpha 1$ subunit TM4 mutations on direct activation by carisoprodol. **A**, representative traces demonstrating CSP activation of human $\alpha 1\beta 2\gamma 2$ WT and $\alpha 1(L415V)\beta 2\gamma 2$ GABA_ARs. The converse mutation to those illustrated in Fig. 2 above significantly decreased carisoprodol direct gating potency. **B**, bar graphs summarizing carisoprodol direct gating currents for human $\alpha 3$ -, $\alpha 1(L394I)$ -, $\alpha 1(A398I)$ -, $\alpha 1(L415V)$ -, $\alpha 1(L394I/A398I)$ -, $\alpha 1(L394I/L415V)$ -, $\alpha 1(A398I/L415V)$ -, $\alpha 1(L394I/A398I/L415V)$ and $\alpha 1\beta 2\gamma 2$ GABA_ARs. All $\alpha 1$ - GABA_ARs containing the L415V mutation showed decreased CSP direct gating effects as compared to WT $\alpha 1\beta 2\gamma 2$ GABA_ARs. Each data point represents the mean \pm S.E.M. of a minimum of three cells. #, $p < 0.01$.

Figure 5. Influence of the $\alpha 1$ subunit TM4 L415 mutations on carisoprodol direct gating. **A**, representative traces demonstrating carisoprodol activation of human $\alpha 1$ (L415S) $\beta 2\gamma 2$ GABA_ARs. Nominal direct gating by CSP was present in this mutation, at concentrations up to 5 mM. **B**, concentration-response curves for the direct gating effect of carisoprodol from human $\alpha 1$ (L415S)-, $\alpha 1$ (L415V)-, $\alpha 1$ (L415G)-, $\alpha 1$ (L415W)-, $\alpha 1$ (L415C)- and $\alpha 1\beta 2\gamma 2$ GABA_ARs. These 5 mutations all decreased carisoprodol efficacy significantly without affecting carisoprodol EC₅₀, except for $\alpha 1$ (L415W) mutant which increased EC₅₀ an estimated 3-fold relative to wild type receptors. **C**, Bar graphs summarizing carisoprodol (CSP) direct gating currents for human $\alpha 1$ WT-, $\alpha 1$ (L415I)-, $\alpha 1$ (L415T)-, $\alpha 1$ (L415R)-, $\alpha 1$ (L415Y)-, $\alpha 1$ (L415W)-, $\alpha 1$ (L415V)-, $\alpha 1$ (L415C)-, $\alpha 1$ (L415G)-, $\alpha 1$ (L415S) $\beta 2\gamma 2$ GABA_ARs. Carisoprodol-gated current reached saturation at 5 mM; receptors containing $\alpha 1$ (L415T/R/Y/I) mutations did not affect carisoprodol direct gating efficacy and thus carisoprodol EC₅₀ values were not calculated. Each data point represents the mean \pm S.E.M of a minimum of three cells. #, $p < 0.01$.

Figure 6. Assessment of physiochemical traits at the $\alpha 1$ 415 residue on carisoprodol efficacy and GABA sensitivity. Correlation analysis of carisoprodol efficacy with amino acid hydrophathy (**A**), volume (**B**), and polarity (**C**) at the $\alpha 1$ 415 position. Analysis of potential correlation of GABA EC₅₀ with amino acid volume (**D**) and hydrophathy (**E**) at the 415 position was also assessed. Panel F illustrates the presence of a significant inverse correlation between direct gating efficacy of position 415 residues and GABA EC₅₀. Each data point represents the mean \pm S.E. of a minimum of three cells.

Figure 7. Influence of the alpha1 subunit TM4 L415S mutation on allosteric modulation by carisoprodol and pentobarbital direct activation. **A**, representative traces demonstrating carisoprodol potentiation of GABA-gated (EC_{20}) currents in $\alpha 1(L415S)\beta 2\gamma 2$ GABA_ARs. **B**, concentration-response curves for the allosteric modulation of GABA-gated currents in wild type $\alpha 1\beta 2\gamma 2$ and $\alpha 1(L415S)\beta 2\gamma 2$ GABA_ARs. Mutation of leucine to serine at the $\alpha 1(415)$ position did not affect allosteric modulation by carisoprodol. Carisoprodol-potentiated currents are normalized to currents elicited by GABA EC_{20} concentration. **C**, representative traces demonstrating pentobarbital (1mM) activation of human $\alpha 1(L415S)\beta 2\gamma 2$ GABA_ARs. **D**, bar graphs summarizing pentobarbital direct gating currents in wild type $\alpha 1\beta 2\gamma 2$ and $\alpha 1(L415S)\beta 2\gamma 2$ GABA_ARs. In contrast to effects on carisoprodol direct gating, his mutation did not affect direct gating by pentobarbital. Pentobarbital-gated currents are normalized to the currents elicited by saturating concentration of GABA (1 mM). Each data point represents the mean \pm S.E. of a minimum of three cells.

Figure 8. Mutation of threonine at 236 position of $\alpha 1$ subunit to isoleucine does not affect direct gating potency of carisoprodol at $\alpha 1\beta 2\gamma 2$ receptors. **A**, representative traces demonstrating carisoprodol (CSP) activates human $\alpha 1\beta 2\gamma 2$ WT and $\alpha 1(T236I)\beta 2\gamma 2$ GABA_ARs. **B**, bar graphs summarizing 1 mM carisoprodol direct gating currents for human $\alpha 1\beta 2\gamma 2$ WT (28.8 ± 2.5 , $n=6$) and $\alpha 1(T236I)\beta 2\gamma 2$ GABA_ARs (21.7 ± 4.0 , $n=3$). Mutation of $\alpha 1(T236I)$ did not show significant alteration in direct gating potency of carisoprodol as compared to WT $\alpha 1\beta 2\gamma 2$ GABA_ARs. Carisoprodol-gated currents are normalized to currents elicited by saturated GABA concentration. Human $\alpha 1\beta 2\gamma 2$ WT data reproduced from figure 3.

Table 1. Summary effects of GABA_A receptor $\alpha 3$ to $\alpha 1$ TM4 amino acid mutations on GABA EC₅₀, and carisoprodol direct and allosteric actions. Carisoprodol direct gating activation at 3 mM is normalized to peak GABA current, and carisoprodol allosteric modulatory effects are normalized to GABA EC₂₀ currents. Each data point represents the mean \pm S.E. of n cells. *, p < 0.05; **, p < 0.01 relative to wild type $\alpha 3\beta 2\gamma 2$ GABA_A receptors.

GABA _A R Configuration	GABA EC ₅₀		CSP gating (% of GABA max)		CSP modulation (% of GABA EC ₂₀)	
	(μ M)	n	3 mM	n	300 μ M	n
$\alpha 3$ WT	34.8 \pm 2.1	6	08.5 \pm 1.1	11	235 \pm 35	6
$\alpha 3$ (V440L)	7.5 \pm 0.9 **	7	37.6 \pm 3.5**	9	301 \pm 14	5
$\alpha 3$ (I419L/I423A)	18.1 \pm 2.2*	9	40.8 \pm 2.4**	8	252 \pm 14	4
$\alpha 3$ (I419L/I423A/V440L)	15.8 \pm 5.0 *	6	35.9 \pm 3.9**	10	156 \pm 22	6

Table 2. Summary table illustrating influence of GABA_A receptor $\alpha 3$ to $\alpha 1$ single and combination TM4 mutations and $\alpha 1$ (L415) amino acid substitutions on GABA EC₅₀ values and carisoprodol direct gating. Carisoprodol direct gating activation is normalized to saturated GABA current whereas carisoprodol modulation effect to potentiate GABA-gated current is normalized to GABA EC₂₀ current. Each data point represents the mean \pm S.E. of n cells. *, $p < 0.05$; **, $p < 0.01$ relative to wild type $\alpha 1\beta 2\gamma 2$ GABA_A receptors.

GABA _A R Configuration	GABA EC ₅₀		CSP Direct gating (% of GABA max)		
	(μ M)	n	Maximum Efficacy	EC ₅₀ (μ M)	n
$\alpha 1$ WT	35.5 \pm 0.6	5	41.8 \pm 2.4	685 \pm 32	15
$\alpha 1$ (L394I)	50.8 \pm 4.4	4	40.6 \pm 4.6	559 \pm 50	6
$\alpha 1$ (A398I)	35.2 \pm 4.1	5	36.1 \pm 2.9	617 \pm 83	6
$\alpha 1$ (L415V)	89.0 \pm 2.2**	5	17.7 \pm 3.1**	826 \pm 24	9
$\alpha 1$ (L394I/A398I)	27.0 \pm 3.4	3	42.0 \pm 5.7	443 \pm 28	9
$\alpha 1$ (A398I/L415V)	68.4 \pm 4.2 **	3	22.6 \pm 1.8**	456 \pm 40	5
$\alpha 1$ (L394I/L415V)	27.5 \pm 3.1	3	20.5 \pm 3.2**	864 \pm 21	9
$\alpha 1$ (L394I/A398I/L415V)	44.1 \pm 2.4	7	18.2 \pm 4.5**	380 \pm 20	7
$\alpha 1$ (L415C)	39.4 \pm 4.0	4	18.0 \pm 1.6**	697 \pm 41	9
$\alpha 1$ (L415W)	47.7 \pm 2.3	3	17.8 \pm 4.1**	2056 \pm 122**	6
$\alpha 1$ (L415G)	68.0 \pm 4.3**	4	09.1 \pm 1.6**	545 \pm 10	5
$\alpha 1$ (L415S)	65.0 \pm 4.2**	7	05.5 \pm 1.2**	651 \pm 24	13
$\alpha 1$ (L415T)	41.0 \pm 2.1	4	37.1 \pm 4.4	533 \pm 41	9
$\alpha 1$ (L415Y)	45.0 \pm 3.2	4	25.4 \pm 3.8	807 \pm 55	6
$\alpha 1$ (L415I)	40.3 \pm 2.2	4	31.9 \pm 4.1	450 \pm 30	7
$\alpha 1$ (L415R)	43.4 \pm 5.2	4	32.6 \pm 4.6	492 \pm 24	4

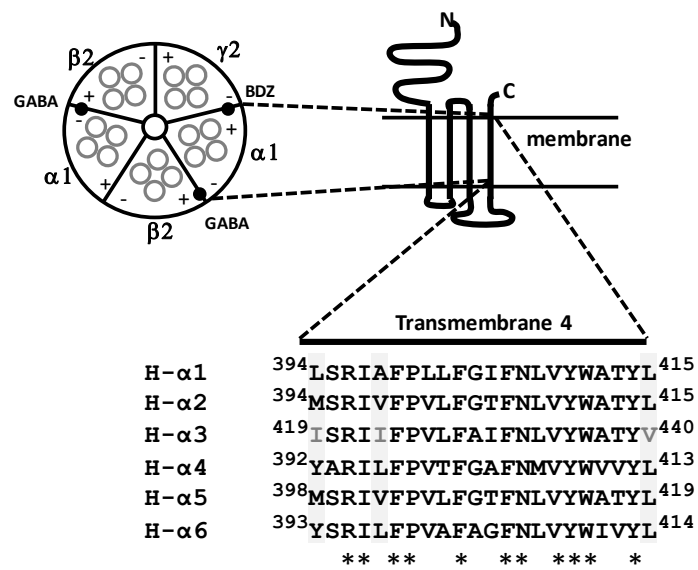


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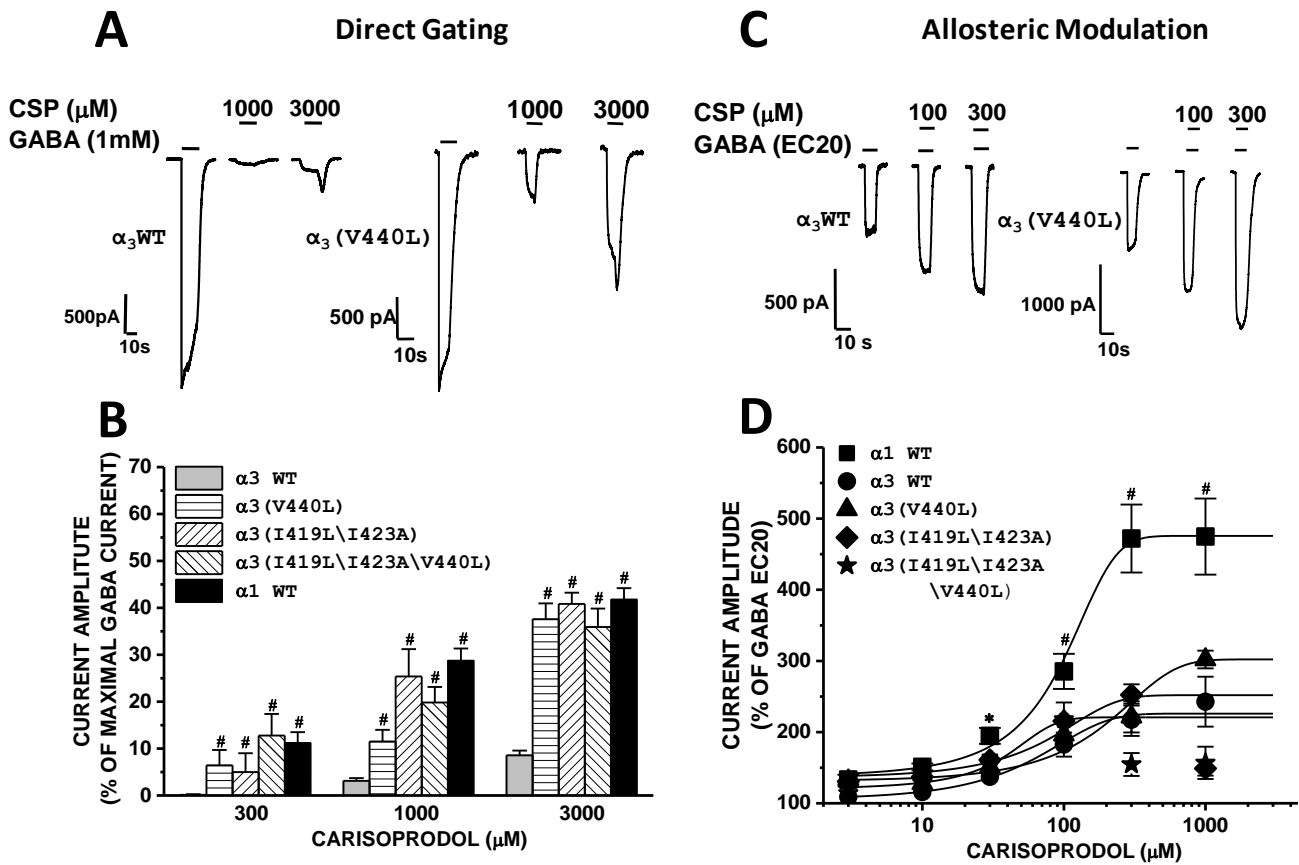


Figure 2.

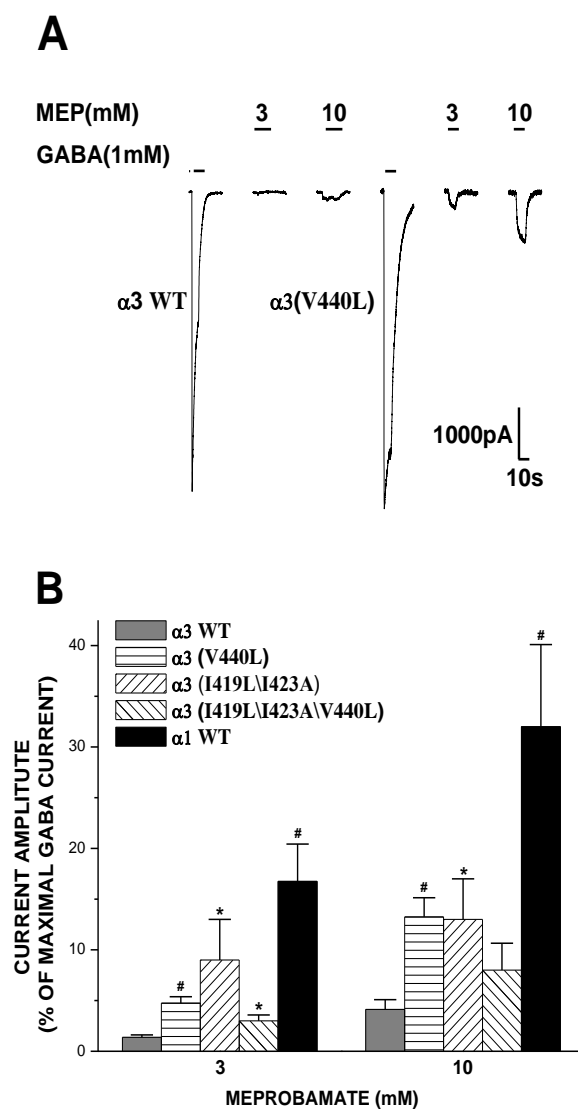


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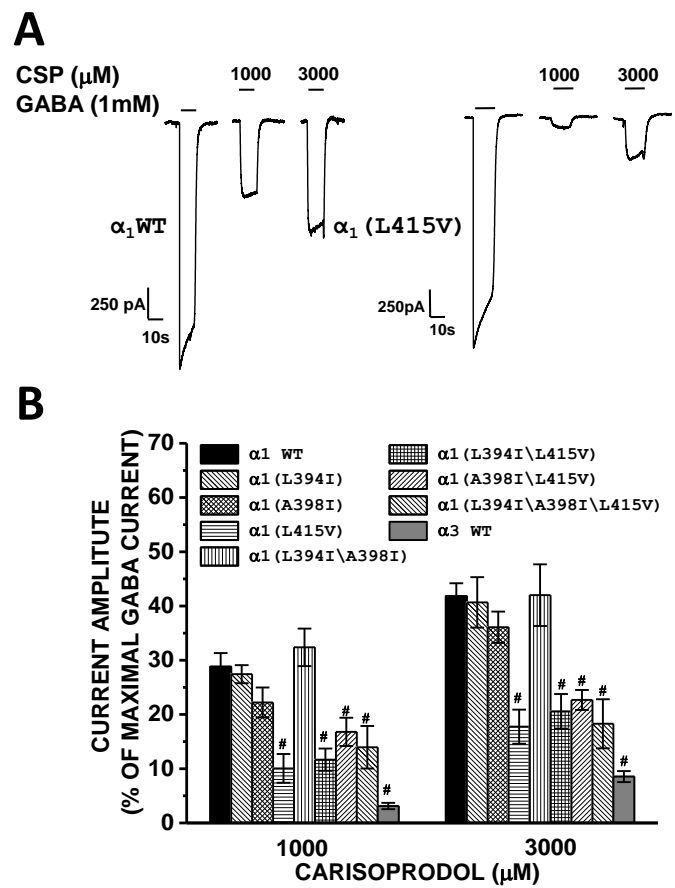


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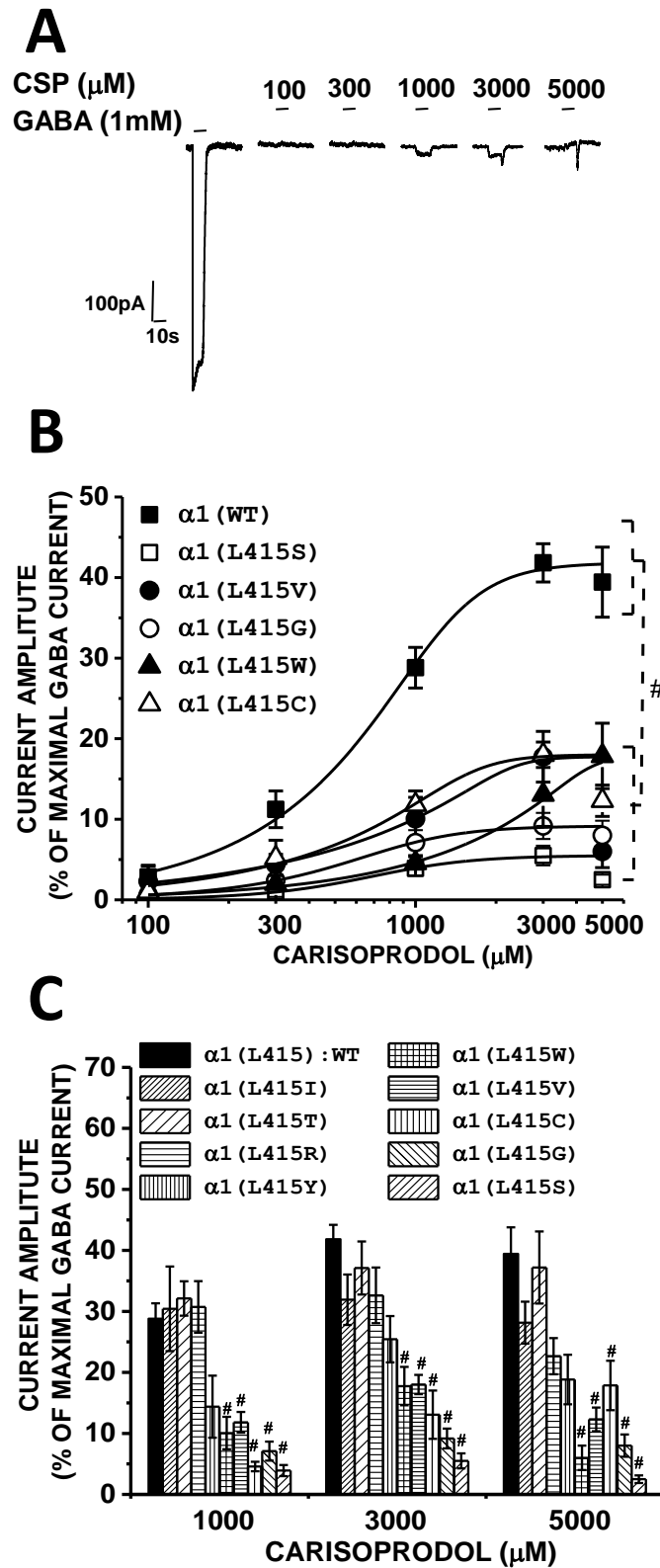
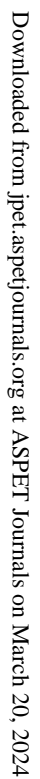


Figure 5.



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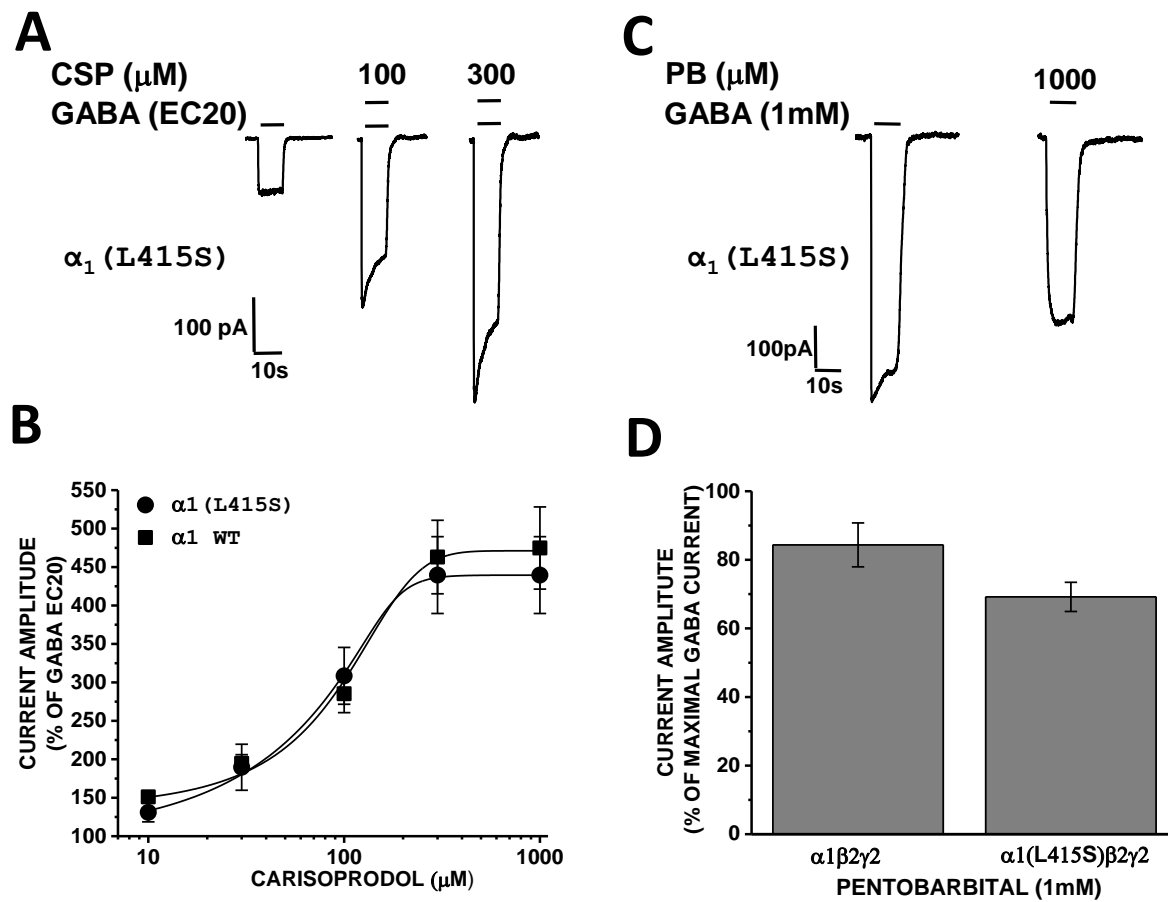


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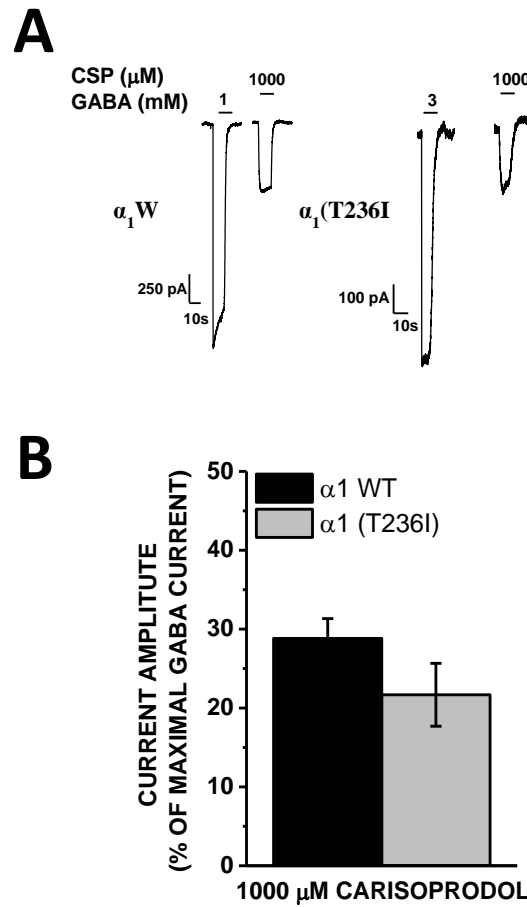


Figure 8.