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Title page

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

Manoj Kumar, Manish Kumar, John M. Freund and Glenn H. Dillon

Department of Physiology and Pharmacology and Center for Neuroscience, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, WV (MjK, MhK, JMF, GHD) and Center for Neuroscience Discovery of the Institute for Healthy Aging, University of North Texas Health Science Center, Fort Worth, TX (MhK, GHD)

Running title: $GABA_A$ receptor domains and direct gating by carisoprodol

Corresponding author: Glenn H. Dillon, Ph.D.
Center for Neuroscience Discovery, Institute for Healthy Aging
University of North Texas Health Science Center
Fort Worth, TX 76107
Phone: (817)-735-2427
E-mail: glenn.dillon@unthsc.edu

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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-etanesulfonicacid N, N, N', N'-tetra acetic acid; MEP, Meprobamate.

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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 $\alpha3\beta2\gamma2$ 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 α3 valine at position 440 to leucine (present in the equivalent position in the α1 subunit) significantly increased the direct gating effects of carisoprodol, without affecting allosteric modulatory effects. The corresponding reverse mutation, α1(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 α1(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.

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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

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important drugs, including anxiolytics, sedative-hypnotics, muscle relaxants, and anesthetics. In α1β2γ2 receptors, the GABA binding site is located at the interface of the α1 and β2 subunits, and benzodiazepines bind at the interface of the α1and γ2 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 α1 subunit, whereas α3-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 (α1-6) 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 α3 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 α1 subunit (equivalent to V440 in the α3 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 α1, α3, β2 and γ2 $GABA_A$ receptor subunits were used in the present study. Individual and combined mutations in α1 and α3 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 αxβ2γ2 $GABA_A$ receptors, HEK293 cells were transfected with human $GABA_A$ α1/3 mutant or wild type ; human β2; and human γ2s (short isoform) subunit cDNA in a 1:1:5 (0.3μg : 0.3μg : 1.5μg) ratio using poly jet DNA in vitro transfection reagent (SigmaGen Laboratories, MD) and used for recording 24-48 h later. The γ2s subunit will be referred to as γ2 from this point forward. Human $GABA_A$ α1 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% CO_2.

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 MΩ. Patch pipettes were filled with a solution consisting

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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 ± 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_{max} = [carisoprodol]^n/([carisoprodol]^n + $EC_{50}{}^n$), 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 ± 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.

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Results

Functional characterization of α3 and α1 TM4 mutant $GABA_A$ receptors. For this study, an extensive series of mutations (single point or 2-3 residues) in α1 and α3 subunits were evaluated. In all cases, the mutant subunit was expressed with wild type β2 and γ2 subunits, and GABA concentration-response profiles were generated to assess overall receptor function and to establish gating concentrations for allosteric studies. GABA EC_{50} for wild type α1 and α3 receptors were both approximately 35 μM (Tables 1 and 2). In general, shifts in GABA EC_{50} were modest. Mutations in α3 subunits caused a leftward shift in the GABA concentration-response curve of 1.9- to 4.6-fold (Table 1). Similarly, mutations in the α1 subunit had either insignificant or modest effects on GABA EC_{50}, with the maximal effect being a 2.5-fold increase in GABA EC_{50} relative to wild type α1 receptors (Table 2). Thus the mutations had minimal effects on fundamental receptor gating.

Mutation of α3 TM4 amino acids to corresponding α1 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 α3β2γ2 receptors was significantly less compared to $GABA_A$ receptors expressing the α1 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 α1β2γ2 receptors and 8.5 ± 1.1 % in α3β2γ2 receptors, confirming low efficacy at α3-expressing receptors. We thus assessed the potential involvement of three unique amino acids we identified in TM4 (I419, I423 and V440) of the α3 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 α1 subunit, either individually or in combination. Mutation of a single amino acid α3(V440L) significantly increased the direct gating effect of CSP compared to WT α3- expressing receptors (Fig. 2A). Similarly, all α3 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 α1β2γ2 receptors (Fig. 2B, summary values in Table 1).

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As carisoprodol also has a diminished allosteric modulatory effect in α3β2γ2 receptors compared to α1β2γ2 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 α3 TM4 mutations (Fig. 2C). Carisoprodol potentiated GABA EC_{20} currents in all mutated receptors, however the magnitude of the potentiation for each mutant variant was not significantly different from that observed in α3β2γ2 receptors, and it fell far short of that produced in α1β2γ2 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 α3- 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 α1 TM4 L415 amino acid to corresponding α3 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 α1 TM4 amino acids to the corresponding α3 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 α1(L415V) subunit showed significantly decreased direct gating by CSP compared to WT receptors (Fig. 4A), whereas receptors expressing α1(L394I) and α1(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 α1 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).

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These data indicate the leucine residue at position 415 in the α1 subunit has a key role in the direct gating action of carisoprodol.

Amino acid residue at α1(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 α1(415) position, we generated and assessed the following series of mutations: α1(L415S), α1(L415G), α1(L415T) α1(L415Y) α1(L415W), α1(L415I), α1(L415C) and α1(L415R). These residues provide a range of amino acid side chain properties, including volume, polarity and hydropathy. 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 α1(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 α1 (L415S) mutation does not affect allosteric modulation by carisoprodol or direct activation by pentobarbital. To further assess the extent to which the α1(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 α1(L415S)βγ2 receptors, CSP potentiation of GABA EC_{20} currents differed in neither maximum potentiation nor potency when compared to wild type receptors (439.45 ± 49.4% potentiation and EC_{50} of 89.5 ± 15 μM, n =7, in α1(L415S)βγ2 receptors compared to 474.75 ± 53.4% and 102.2 ± 16 μM, n =5 in wild type receptors, Fig. 7A, B). To assess

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specificity of the L415S mutation, we evaluated if it had any effect on pentorbarbital-activated currents. Direct gating by 1 mM pentobarbital was not significantly different in α1(L415S)βγ2 receptors compared to wild type receptors (current amplitude in comparison to saturating GABA was 70.2 ± 4.2%, n = 5 and 84.1 ± 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 α1 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 α1 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 α1(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 α3βγ2 $GABA_A$ receptors, compared to those expressing any other α subunit variant (α1-2, α4-6). Here, we identified α subunit TM4 residues, in particular α1415 (equivalent to α3440), that are critical for direct gating, but not allosteric modulatory, effects of carisoprodol. Mutation of the native α3 440V residue to the L residue found in the α1 subunit (V440L) resulted in a significant enhancement of carisoprodol-gated current; the converse mutation (α1(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 EC_{50}), 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 α3 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 α1β2γ2 or homomeric β3 $GABA_A$ 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 α1 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 α1 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 α1(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 α1T236I mutation, shown previously to abolish direct gating by neurosteroids (Hosie et al., 2006), did not affect carisoprodol-mediated activation. Thus, whereas the α1(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 α3(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 α1 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 meditate their addictive

actions by increasing dopamine release in the nucleus accumbens through activation of α1-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, α1-expressing receptors are involved in sedative effects and abuse potential, α2-expressing receptors contribute to their anxiolytic effects, whereas α2-, α3- and α5- 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 α1-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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Name and address of person to receive reprint requests:

Glenn H. Dillon, Ph.D.

Center for Neuroscience Discovery, Institute for Healthy Aging

University of North Texas Health Science Center

Fort Worth, TX 76107

Phone: (817)-735-2427

E-mail: glenn.dillon@unthsc.edu

Current Address of Manoj Kumar is Department of Otolaryngology, University of Pittsburgh, Pittsburgh, PA 15260.

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 α1, β2 and γ2 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 (α1-6) isoforms showing conserved (*) and non-identical (shaded in gray) amino acids.

Figure 2. Influence of α3 subunit TM4 mutations on direct activation and allosteric modulation by carisoprodol. A, representative traces demonstrating carisoprodol (CSP) activation of human α3β2γ2 WT and α3(V440L)β2γ2 $GABA_ARs$. In this and all subsequent figures, WT or mutant α subunits are co-expressed with WT β2 and γ2 subunits (represented by "-"). Single mutation of V440 at TM4 of α3 to L, present in α1-expressing $GABA_ARs$, significantly increased CSP direct gating potency. **B,** bar graphs summarizing carisoprodol direct gating currents in human α3-, α3(V440L)-, α3(I419L/I423A)-, α3(I419L/I423A/V440L)- and α1β2γ2 $GABA_ARs$. Single and combined mutation of TM4 domains of α3 to those present in α1 subunit significantly increased the direct gating potency of carisoprodol as compared to WT α3 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 (EC_{20}) currents from human α3β2γ2 WT and α3(V440L)β2γ2 $GABA_ARs$ by carisoprodol. **D,** concentration-response curves for the allosteric modulation of GABA-gated currents in α3-, α3(V440L)-, α3(I419L/I423A)-, α3(I419L/I423A/V440L) and α1β2γ2 $GABA_ARs$. Mutation of TM4 domains of α3 to those present in α1 subunit did not increase allosteric modulatory efficacy of carisoprodol. Carisoprodol-potentiated currents are normalized to currents elicited by GABA EC_{20} concentrations. Each data point represents the mean ± S.E.M. of a minimum of three cells. **#,** $p< 0.01$, ***,** $p< 0.05$.

Figure 3. Influence of α3 subunit TM4 mutations on direct activation of the carisoprodol metabolite meprobamate. A, representative traces demonstrating meprobamate (MEP) activation of human α3β2γ2 WT and α3(V440L)β2γ2 $GABA_A$Rs. **B,** bar graph summarizing meprobamate direct gating currents in α3-, α3(V440L)-, α3(I419L/I423A)-, α3(I419L/I423A/V440L) and α1β2γ2 $GABA_A$Rs. Single and combined mutation of TM4 residues of the α3 subunit to those present in the α1 subunit significantly increased the direct gating potency of meprobamate as compared to WT α3 receptors. Meprobamate-gated currents are normalized to currents elicited by saturating GABA (1 mM). Each data point represents the mean ± S.E.M. of a minimum of three cells. **#,** $p< 0.01$, ***,** $p< 0.05$.

Figure 4. Influence of α1 subunit TM4 mutations on direct activation by carisoprodol. A, representative traces demonstrating CSP activation of human α1β2γ2 WT and α1(L415V)β2γ2 $GABA_A$Rs. 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 α3-, α1(L394I)-, α1(A398I)-, α1(L415V)-, α1(L394I/A398I)-, α1(L394I/L415V)-, α1(A398I/L415V)-, α1(L394I/A398I/L415V) and α1β2γ2 $GABA_A$Rs. All α1- $GABA_A$Rs containing the L415V mutation showed decreased CSP direct gating effects as compared to WT α1β2γ2 $GABA_A$Rs. Each data point represents the mean ± S.E.M. of a minimum of three cells. **#**, $p< 0.01$.

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Figure 5. Influence of the α1 subunit TM4 L415 mutations on carisoprodol direct gating. A, representative traces demonstrating carisoprodol activation of human α1(L415S)β2γ2 $GABA_A$Rs. 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 α1(L415S)-, α1(L415V)-, α1(L415G)-, α1(L415W)-, α1(L415C)- and α1β2γ2 $GABA_A$Rs. These 5 mutations all decreased carisoprodol efficacy significantly without affecting carisoprodol EC_{50}, expect for α1(L415W) mutant which increased EC_{50} an estimated 3-fold relative to wild type receptors. **C,** Bar graphs summarizing carisoprodol (CSP) direct gating currents for human α1 WT-, α1(L415I)-, α1(L415T)-, α1(L415R)-, α1(L415Y)-, α1(L415W)-, α1(L415V)-, α1(L415C)-, α1(L415G)-, α1(L415S)β2γ2 $GABA_A$Rs. Carisoprodol-gated current reached saturation at 5 mM; receptors containing α1 (L415T/R/Y/I) mutations did not affect carisoprodol direct gating efficacy and thus carisoprodol EC_{50} values were not calculated. Each data point represents the mean ± S.E.M of a minimum of three cells. #, $p< 0.01$.

Figure 6. Assessment of physiochemical traits at the α1 415 residue on carisoprodol efficacy and GABA sensitivity. Correlation analysis of carisoprodol efficacy with amino acid hydropathy **(A)**, volume **(B)**, and polarity **(C)** at the α1 415 position. Analysis of potential correlation of GABA EC_{50} with amino acid volume **(D)** and hydropathy **(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 EC_{50}. Each data point represents the mean ± 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 α1(L415S)β2γ2 $GABA_A$Rs. **B,** concentration-response curves for the allosteric modulation of GABA-gated currents in wild type α1β2γ2 and α1(L415S)β2γ2 $GABA_A$Rs. Mutation of leucine to serine at the α1(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 α1(L415S)β2γ2 $GABA_A$Rs. **D,** bar graphs summarizing pentobarbital direct gating currents in wild type α1β2γ2 and α1(L415S)β2γ2 $GABA_A$Rs. 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 ± S.E. of a minimum of three cells.

Figure 8. Mutation of threonine at 236 position of α1 subunit to isoleucine does not affect direct gating potency of carisoprodol at α1β2γ2 receptors. A, representative traces demonstrating carisoprodol (CSP) activates human α1β2γ2 WT and α1(T236I)β2γ2 $GABA_A$Rs. B, bar graphs summarizing 1 mM carisoprodol direct gating currents for human α1β2γ2 WT (28.8 ± 2.5, n= 6) and α1(T236I)β2γ2 $GABA_A$Rs (21.7 ± 4.0, n= 3). Mutation of α1(T236I) did not show significant alteration in direct gating potency of carisoprodol as compared to WT α1β2γ2 $GABA_A$Rs. Carisoprodol-gated currents are normalized to currents elicited by saturated GABA concentration. Human α1β2γ2 WT data reproduced from figure 3.

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Table 1. Summary effects of $GABA_A$ receptor α3 to α1 TM4 amino acid mutations on GABA EC_{50}, 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 EC_{20} currents. Each data point represents the mean ± S.E. of n cells. *, $p < 0.05$; **, $p < 0.01$ relative to wild type α3β2γ2 $GABA_A$ receptors.

$GABA_AR$ Configuration	GABA EC_{50}		CSP gating (% of GABA max)		CSP modulation (% of GABA EC_{20})	
	(μM)	n	3 mM	n	300 μM	n
α3 WT	34.8 ± 2.1	6	08.5 ± 1.1	11	235 ± 35	6
α3(V440L)	7.5 ± 0.9 **	7	37.6 ± 3.5**	9	301 ± 14	5
α3(I419L/I423A)	18.1 ± 2.2*	9	40.8 ± 2.4**	8	252 ± 14	4
α3(I419L/I423A/V440L)	15.8 ± 5.0 *	6	35.9 ± 3.9**	10	156 ± 22	6

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Table 2. Summary table illustrating influence of $GABA_A$ receptor α3 to α1 single and combination TM4 mutations and α1 (L415) amino acid substitutions on GABA EC_{50} 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 EC_{20} current. Each data point represents the mean ± S.E. of n cells. *, $p < 0.05$; **, $p< 0.01$ relative to wild type α1β2γ2 $GABA_A$ receptors.

$GABA_AR$ Configuration	GABA EC50		CSP Direct gating (% of GABA max)		
	(μM)	n	Maximum Efficacy	EC50 (μM)	n
α1 WT	35.5 ± 0.6	5	41.8 ± 2.4	685 ± 32	15
α1(L394I)	50.8 ± 4.4	4	40.6 ± 4.6	559 ± 50	6
α1(A398I)	35.2 ± 4.1	5	36.1 ± 2.9	617 ± 83	6
α1(L415V)	89.0 ± 2.2**	5	17.7 ± 3.1**	826 ± 24	9
α1(L394I/A398I)	27.0 ± 3.4	3	42.0 ± 5.7	443 ± 28	9
α1(A398I/L415V)	68.4 ± 4.2 **	3	22.6 ± 1.8**	456 ± 40	5
α1(L394I/L415V)	27.5 ± 3.1	3	20.5 ± 3.2**	864 ± 21	9
α1(L394I/A398I/L415V)	44.1 ± 2.4	7	18.2 ± 4.5**	380 ± 20	7
α1(L415C)	39.4 ± 4.0	4	18.0 ± 1.6**	697 ± 41	9
α1(L415W)	47.7 ± 2.3	3	17.8 ± 4.1**	2056 ± 122**	6
α1(L415G)	68.0 ± 4.3**	4	09.1 ± 1.6**	545 ± 10	5
α1(L415S)	65.0 ± 4.2**	7	05.5 ± 1.2**	651 ± 24	13
α1(L415T)	41.0 ± 2.1	4	37.1 ± 4.4	533 ± 41	9
α1(L415Y)	45.0 ± 3.2	4	25.4 ± 3.8	807 ± 55	6
α1(L415I)	40.3 ± 2.2	4	31.9 ± 4.1	450 ± 30	7
α1(L415R)	43.4 ± 5.2	4	32.6 ± 4.6	492 ± 24	4

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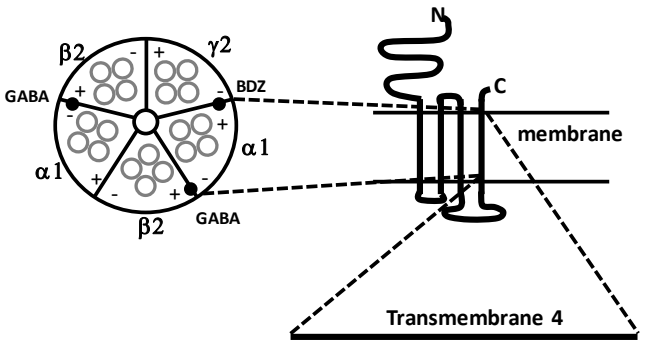


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H-α1   394LSRIAFPLLFGIFNLVYWATYL415
H-α2   394MSRIVFPVLFGTFNLVYWATYL415
H-α3   419ISRIIFPVLFAIFNLVYWATYV440
H-α4   392YARILFPVTFGAFNMVYWVVYL413
H-α5   398MSRIVFPVLFGTFNLVYWATYL419
H-α6   393YSRILFPVAFAGFNLVYWIVYL414
          ** **   *  **  ***  *
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Figure 1.

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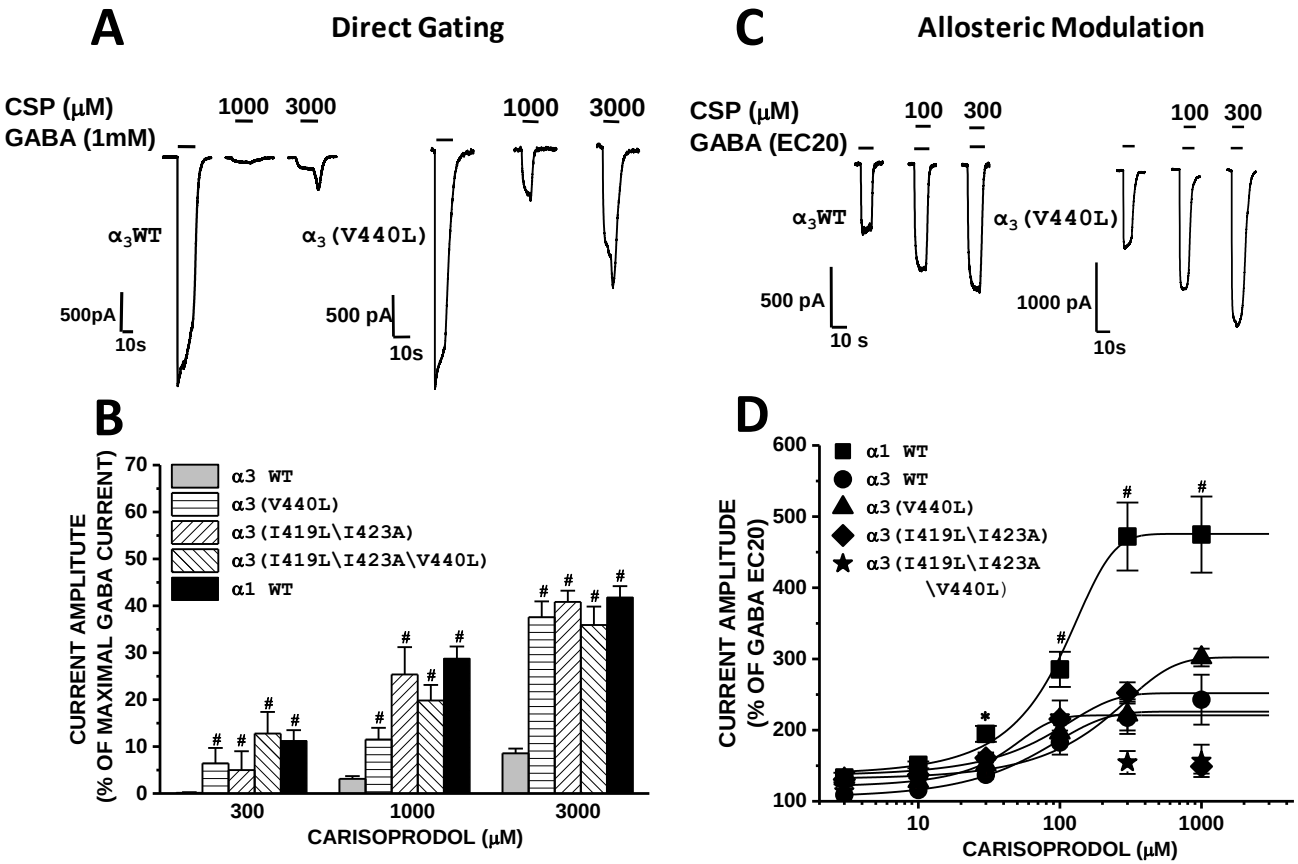

Figure 2.

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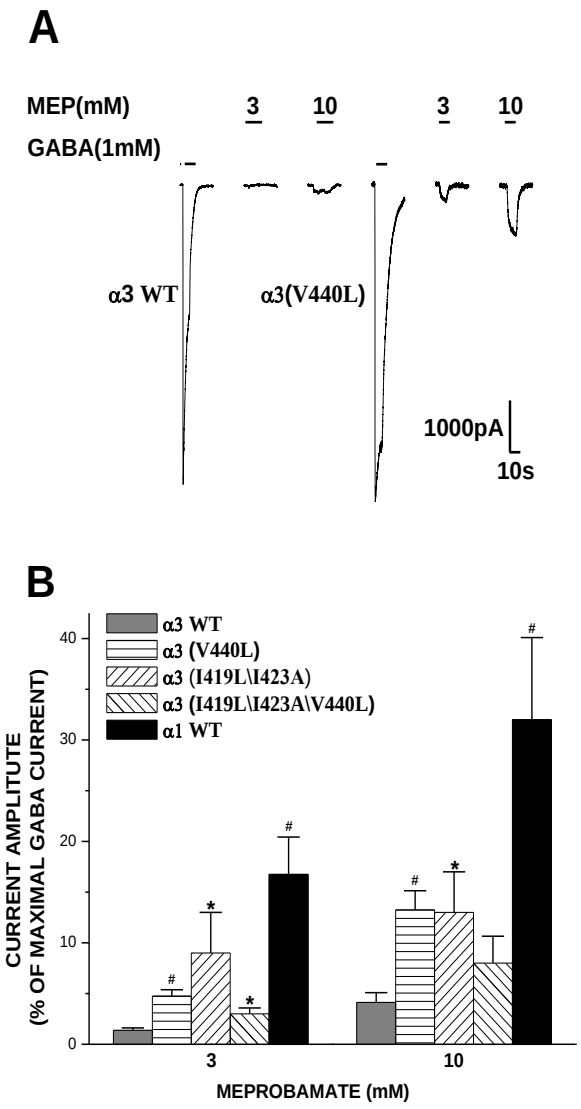

Figure 3.

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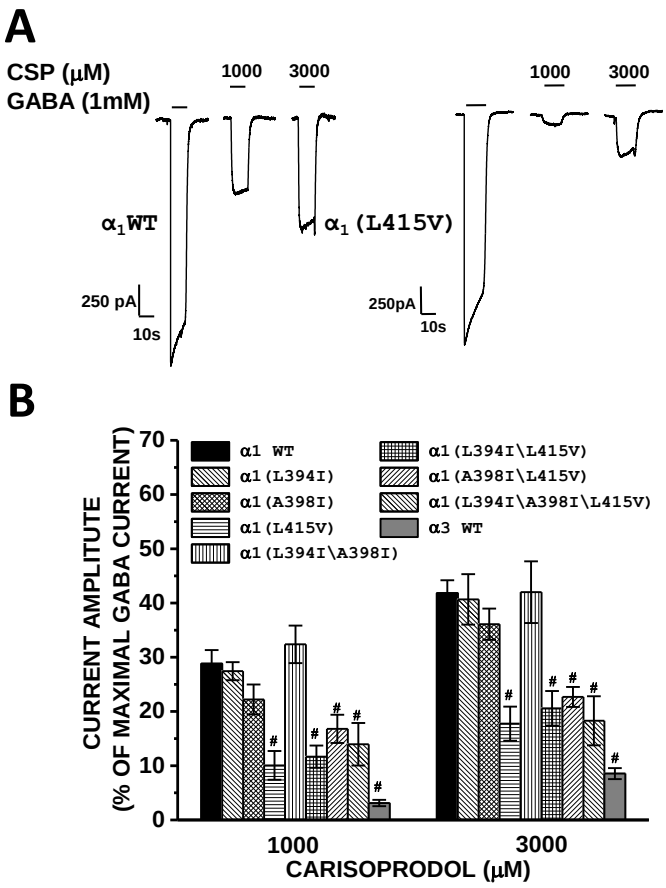

Figure 4.

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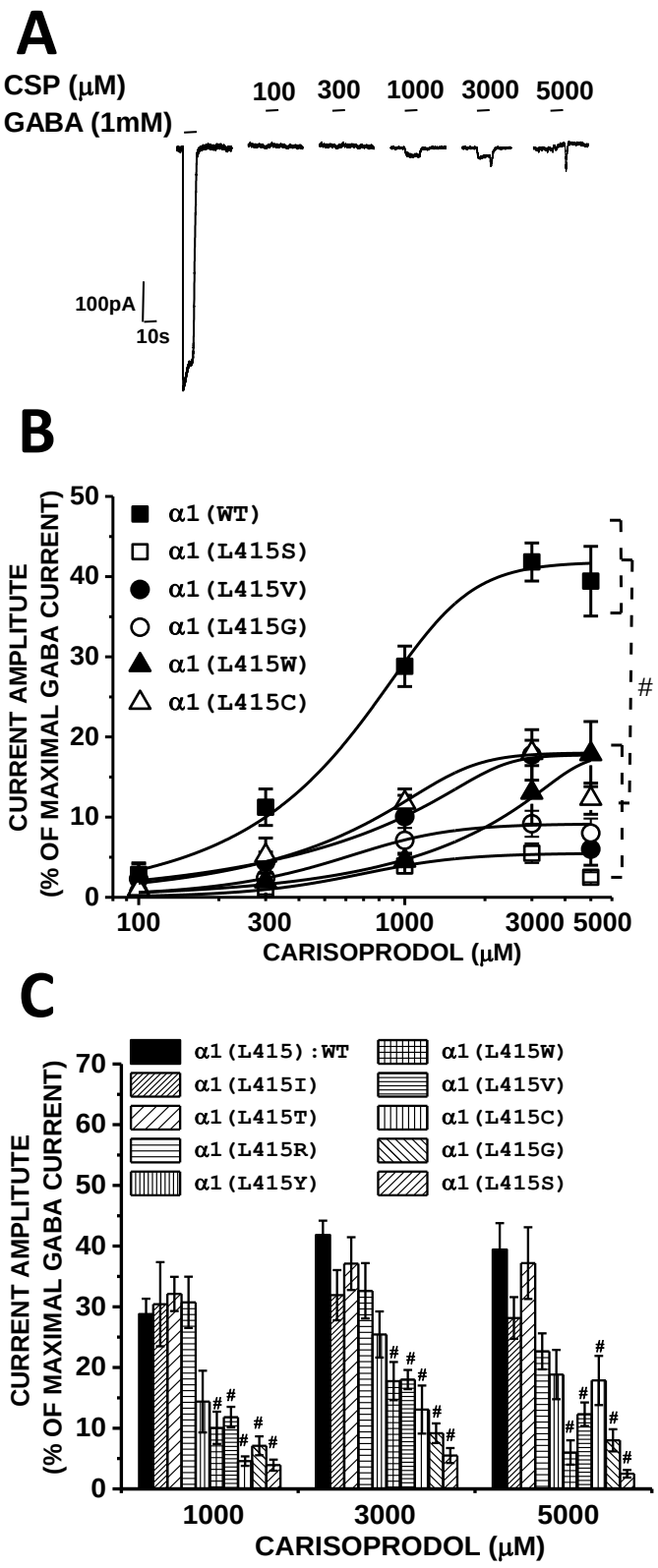

Figure 5.

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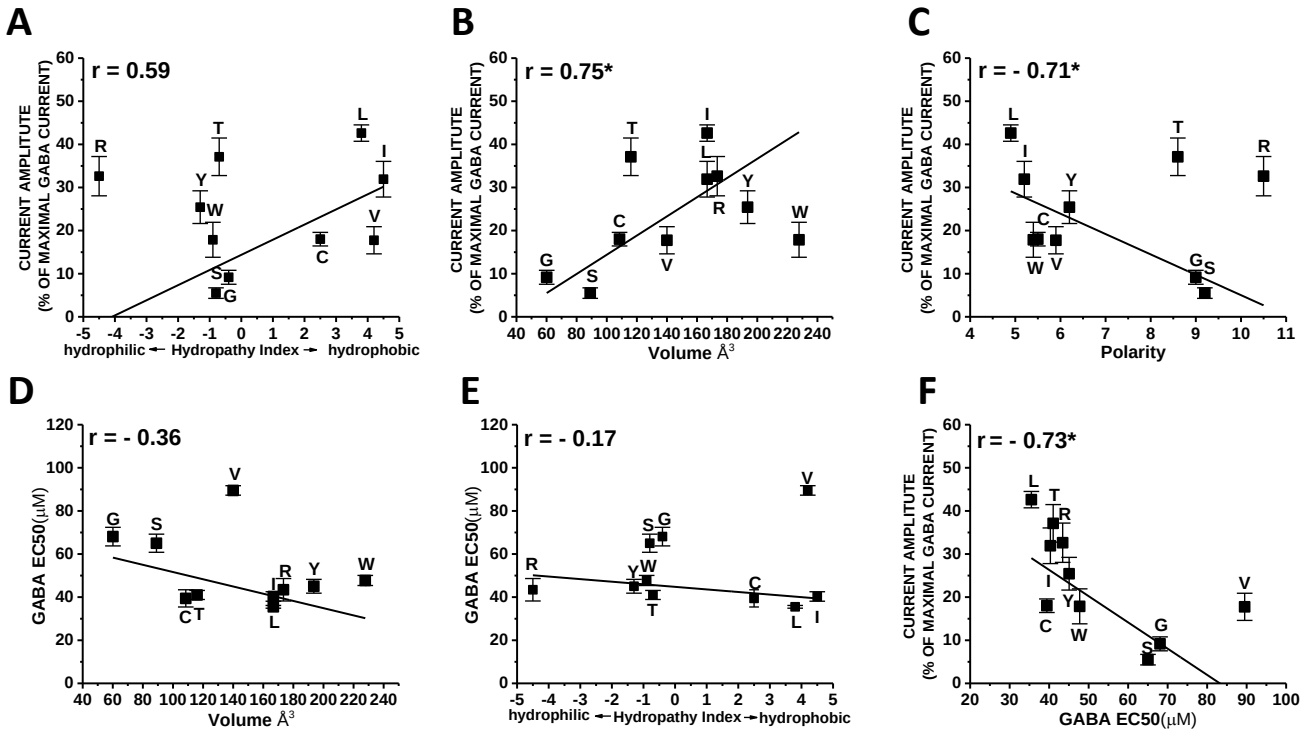

Figure 6.

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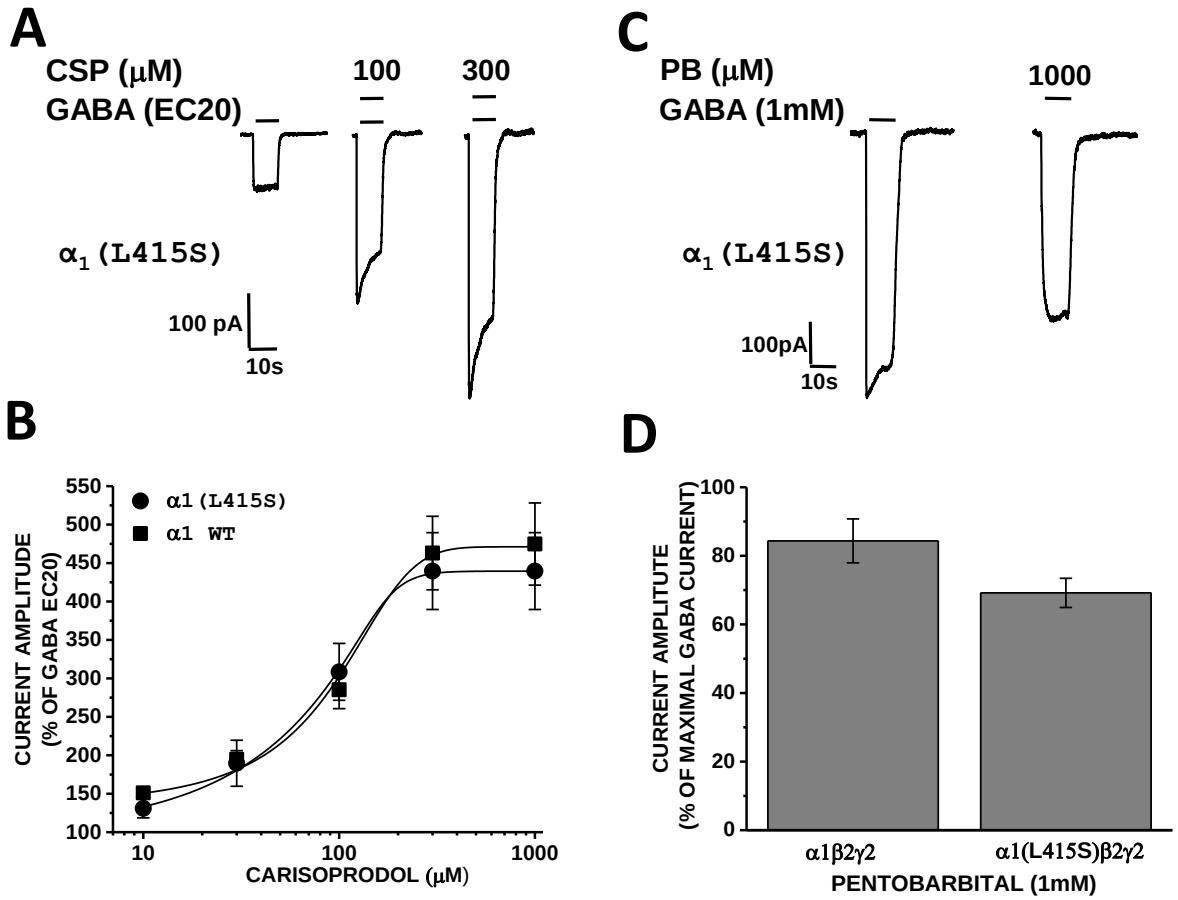

Figure 7.

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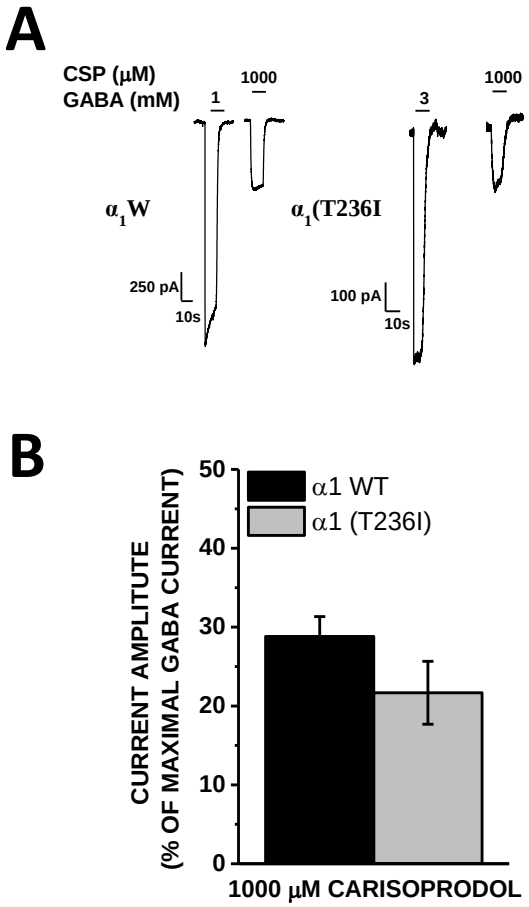

Figure 8.