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Carisoprodol is an increasingly abused, centrally-acting muscle relaxant. Its sedative effects, which contribute to its therapeutic and recreational use, are attributed to its metabolite, meprobamate, a controlled substance with barbiturate-like activity at $GABA_A$ receptors ($GABA_ARs$). $GABA_ARs$ are ion channel-coupled protein complexes underlying the majority of fast synaptic inhibition in the central nervous system. Recent evidence suggests carisoprodol may act independently of meprobamate. Thus, we used behavioral and pharmacological approaches to investigate carisoprodol's effects on $GABA_AR$ function with the ultimate goal of elucidating its mechanism of action at these receptors. In mice, the time course of locomotor depression was comparable for carisoprodol (intraperitoneal or oral) versus meprobamate (intraperitoneal). GABAergic ligands substituted for carisoprodol in drug discrimination studies using carisoprodol-trained rats. As observed in vitro, carisoprodol's effects were antagonized by bemegride, a barbiturate antagonist, but not by the benzodiazepine site antagonist flumazenil, suggesting carisoprodol produces barbiturate-like effects in vivo. Moreover, whole-cell patch clamp recordings were obtained from HEK293 cells expressing human $\alpha1\beta2$ and $\alpha x\beta z\gamma2$ (where x = 1-4 and z = 1-2) $GABA_ARs$. Each receptor configuration was directly activated and allosterically modulated by carisoprodol in a barbiturate-like manner. Carisoprodol efficacy, but not potency, was subunit-dependent with α and β isoforms

contributing to carisoprodol site(s) of action. Notably, carisoprodol was more efficacious at α1-containing receptors, consistent with its sedative effects and abuse potential. Homomeric glycine α1 and GABA ρ1 receptors were carisoprodol-insensitive. Despite similarities between carisoprodol and barbiturates, their sites of action are likely not equivalent as barbiturate-sensitive ρ1W328M subunits were carisoprodol-insensitive. However, chimeric ρ1/α1 receptors gained sensitivity to modulation, but not direct activation by carisoprodol. Our findings indicate carisoprodol modulates $GABA_A$Rs in a subunit- and receptor-dependent manner, contributing to its pharmacological profile and possibly its abuse potential. Furthermore, partial restoration of modulation, but not direct gating by carisoprodol suggests this drug may mediate its effects via multiple sites on $GABA_A$Rs.

ELUCIDATION OF THE MECHANISM OF ACTION OF CARISOPRODOL AT $GABA_A$ RECEPTORS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

CHAPTER I

INTRODUCTION

Carisoprodol was approved for clinical use as a muscle relaxant by the Food and Drug Administration (FDA) in 1959 and was marketed under the trade name Soma® (Wallace Laboratories, Cranbury, NJ). It is currently available from other pharmaceutical companies as well. In 2000, carisoprodol was the second most frequently prescribed muscle relaxant, accounting for 21% of all skeletal muscle relaxant prescriptions in the United States (Luo et al., 2004). According to IMS Health™, there were approximately 10 million prescriptions of carisoprodol issued in 2006 (United States Department of Justice Drug Enforcement Administration Office of Diversion Control, 2008), supporting its continued use in clinical settings. However, much of the attention this drug has received has little to do with its therapeutic use.

Carisoprodol has gained notoriety as a drug of abuse. The incidence of carisoprodol abuse is rising at such an alarming rate that it has prompted the states of Alabama, Arizona, Arkansas, Connecticut, Florida, Georgia, Hawaii, Kentucky, Massachusetts, Minnesota, Nevada, New Mexico, Oklahoma, Oregon, Virginia, and West Virginia to classify it as a schedule IV controlled substance (United States Department of Justice Drug Enforcement Administration Office of Diversion Control, 2008; Reeves and Burke, 2008). It is believed its low cost and accessibility relative to illegal drugs make it an ideal target for substance abusers.

While the number of reports regarding the potential dangers of carisoprodol continues to increase, there are few reports concerning its mechanism of action. Given the present and potential dangers posed by carisoprodol abuse, it is of crucial importance to determine how this drug mediates its effects as there is currently no standard regimen for treating tolerance, dependence, and withdrawal from carisoprodol. Thus, the following studies were aimed at contributing to the current knowledge regarding the mechanism of action of carisoprodol as a therapeutic agent and as a drug of abuse.

Chemistry and Physiological Effects of Carisoprodol

In 1946, Berger and Bradley introduced mephenesin—a centrally-acting muscle relaxant characterized by its tranquilizing effects. The therapeutic potential of mephenesin was never fully realized since oxidation of its primary hydroxyl group resulted in a short time course (Riley and Berger, 1949). To overcome the rapid pharmacokinetics and low potency of mephenesin, various derivatives of mephenesin were synthesized. One such derivative was 2-methyl-2-propyl-1,3-propanediol dicarbamate, or meprobamate (Miltown®, Equanil®). The taming effects and muscle relaxant properties associated with meprobamate catapulted this drug to prominence as a sedative, muscle relaxant, and anxiolytic in the 1950s and 1960s (Berger, 1952; Ludwig and Potterfield, 1971).

In the hopes of isolating the sedative effects of meprobamate from its muscle relaxant properties, derivatives of meprobamate were synthesized. It was observed that substituting a short-chain alkyl group for a hydrogen on one of the carbamyl nitrogen atoms resulted in exceptional muscle-paralyzing capabilities in mice (Ludwig et al.,

1969). Although several of the derivatives exhibited promise as therapeutic agents, *N*-isopropyl-2-methyl-2-propyl-1,3-propanediol dicarbamate, or carisoprodol, was unique in several respects.

Despite the structural similarities between meprobamate and carisoprodol (Figure I-1), there were striking differences in the physiological effects of these drugs on the central nervous system (CNS) (Berger et al., 1960). For meprobamate, lower doses (10-30 mg/kg) did not have significant effects on brainwave activity (Hendley et al., 1954; Gangloff, 1959), yet higher doses (100-200 mg/kg) decreased electrical activity in the cortex and subcortex and had a profound sedative effect. In addition, meprobamate did not produce any behavioral or arousal deficits at the lower doses (Gangloff, 1959; Berger et al., 1960). Moreover, it was demonstrated the effects of meprobamate on the limbic system were likely to underlie its tranquilizing properties (Kletzkin and Berger, 1959).

In contrast, initial studies demonstrated low doses of carisoprodol (5-10 mg/kg) significantly decreased the frequency and increased the amplitude of cortical and subcortical recordings in cats and rabbits without producing any behavioral or neurological deficits (Berger et al., 1960). The absence of any behavioral deficits was not surprising as similar doses had no significant effects on the limbic system. At doses of 40-60 mg/kg, the decrease in frequency was more pronounced and was accompanied by periods of electrical silence. Furthermore, the lower doses of carisoprodol depressed cortical and hippocampal arousal, relaxed muscles, and produced paralysis. Like other centrally-acting muscle relaxants, carisoprodol produced paralysis via depression of multineuronal reflexes; however, its actions were predominantly at the level of the

reticular formation as opposed to the myoneural junction (Del Castillo and Nelson, 1960). Lower doses of carisoprodol (relative to LD_{50} values) were sufficient to produce paralysis, providing for a greater therapeutic window than that of meprobamate (Berger et al., 1960). Although carisoprodol possessed remarkable characteristics, perhaps the most promising property of carisoprodol was its potency and efficacy in abolishing decerebrate rigidity in cats (Berger et al., 1960). Effects on decerebrate rigidity are often used as a means to evaluate the usefulness of centrally-acting muscle relaxants. In these studies, the potency of carisoprodol was approximately eight-fold greater than that of meprobamate.

In many respects, the effects of carisoprodol were similar to those of other compounds. At low doses, its effects on the central nervous system were likened to those of atropine; however, carisoprodol produced sedation at high doses whereas atropine had an excitatory effect (Wescoe et al., 1948; Longo, 1956; Bradley and Elkes, 1957; Berger et al., 1960). Carisoprodol differed from barbiturates in that brainwave activity with the latter demonstrates spindling—a brainwave pattern associated with stage II sleep. In addition, depression of the CNS by barbiturates is accompanied by drowsiness. Again, this was not the case with carisoprodol (Berger et al., 1960). While depression of electrical activity is often observed with other depressants, carisoprodol was unique in its ability to elicit its effects on the CNS without significantly affecting reactivity to sensory stimuli. The CNS effects of carisoprodol—specifically, its effects on pain perception—were also implicated in its analgesic properties since it was unlike antipyretic or narcotic analgesics (Berger et al., 1959; Berger et al., 1960). Given the unique actions of

carisoprodol as a muscle relaxant and an analgesic, this drug held great promise for use in therapeutic applications.

Clinical Use of Carisoprodol

The analgesic properties and muscle relaxant effects of carisoprodol are the bases for its use in the alleviation of lower back pain and in the short-term treatment of painful, acute musculoskeletal conditions. Like other muscle relaxants, carisoprodol is often prescribed as an adjunct to rest or physical therapy and is also available in preparations with other analgesics such as aspirin or codeine (Soma® Compound or Soma® Compound with codeine).

When indicated in the treatment of acute muscular pain, the recommended dose of carisoprodol is four 350 mg tablets daily. According to Soma® prescribing information, the most common adverse effects associated with this drug are drowsiness, dizziness, and headaches (MedPointe Pharmaceuticals, Somerset, NJ). Other side effects include nausea, vomiting, tachycardia, hypotension, tremor, ataxia, vertigo, nystagmus, and seizures. These are experienced most often when carisoprodol is ingested in excess or after abrupt cessation. Interestingly, agitation and myclonus have also been reported with toxic doses of carisoprodol (Goldberg, 1969; Roth et al., 1998).

Although the most common side effects of carisoprodol are consistent with the actions of other centrally-acting drugs, they are not always well-tolerated by patients and may lead to noncompliance with prescribed treatment. In light of these findings, the FDA approved a 250 mg tablet preparation of the drug available through MedPointe Pharmaceuticals in September 2007. Using the same dosing regimen, the new preparation

has a more favorable tolerability profile while providing a comparably efficacious result (Medical News Today, 2007).

Pharmacology of Carisoprodol

Because of its lipophilic nature, carisoprodol is rapidly absorbed from the gastrointestinal tract and rapidly distributed throughout the CNS. With therapeutic doses, the effects of carisoprodol begin within 30 minutes of ingestion, and peak plasma concentrations reach 4-7 μg/mL in 2 to 4 hours (Littrell et al., 1993a). The half-life of carisoprodol is approximately 100 minutes in humans, but may increase significantly for individuals who are poor metabolizers of mephenytoin (Olsen et al., 1994; Dalen et al., 1996).

Carisoprodol undergoes hepatic biotransformation by the cytochrome P450 enzyme 2C19 (CYP2C19). Hydroxylation and dealkylation produce three metabolic products—hydroxycarisoprodol, hydroxymeprobamate, and meprobamate—all of which are excreted by the kidneys (Douglas et al., 1962; Olsen et al., 1994; Dalen et al., 1996). Interestingly, initial studies involving carisoprodol were conducted in dogs; they metabolize the drug primarily to hydroxycarisoprodol. However, in mice and humans, the primary metabolite of carisoprodol is meprobamate (Douglas et al., 1962; van der Kleijn, 1969).

As previously noted, meprobamate experienced success as an anxiolytic, sedative, and muscle relaxant. Although carisoprodol, itself, has been shown to have analgesic and muscle relaxant properties, it is widely accepted that the therapeutic effects of carisoprodol occur via the sedative effects of its metabolite. Within 2.5 hours of a single

700 mg dose of carisoprodol, serum levels of meprobamate surpass those of carisoprodol with approximately 92 ± 5% of the parent drug metabolized to meprobamate within 6 hours (Olsen et al., 1994). Peak serum concentrations of meprobamate are 18.4 ± 2.7 μmol/L. These concentrations are within the range achieved with a single 400 mg dose of meprobamate. Thus, it is possible that conversion to meprobamate underlies the therapeutic effects of carisoprodol. Ironically, its metabolism to meprobamate is also believed to be the primary reason for its abuse liability as meprobamate is currently a schedule IV controlled substance at the federal level.

Carisoprodol as a Drug of Abuse

Initial pharmacokinetic studies for carisoprodol were conducted in dogs. In these studies, serum concentrations of unchanged carisoprodol were highest, and only 2-3% of the administered dose of carisoprodol was converted to meprobamate—a drug known to have barbiturate-like properties. Thus, it was concluded the conversion of carisoprodol to meprobamate was pharmacologically insignificant (Douglas et al., 1962). In a separate study, however, carisoprodol administration prevented abstinence symptoms in barbiturate-dependent dogs, indicating potential abuse liability for carisoprodol (Deneau and Weiss, 1968). In humans, acute carisoprodol administration did not substitute for morphine, and chronic administration of the drug did not produce morphine- or barbiturate-like intoxication or withdrawal patterns (Fraser et al., 1961). In light of these findings, it was determined carisoprodol did not have abuse- or dependence-producing potential in man (Eddy et al., 1969).

Contrary to this assertion, the first case of carisoprodol dependence was reported less than 10 years later, and cases of carisoprodol abuse have been widely reported in the literature since (Morse and Chua, 1978; Luehr et al., 1990; Elder, 1991; Littrell et al., 1993a; Rust et al., 1993; Sikdar et al., 1993; Reeves et al., 1997; Forrester, 2006). The illicit use of this drug to combat opiate withdrawal or to enhance the sedative or euphoric effects of other CNS depressants is well-documented (Chop, 1993; Reeves et al., 1999; Reeves and Liberto, 2001; Drug Abuse Warning Network, 2004b; Drug Abuse Warning Network, 2004a). Ironically, carisoprodol is frequently prescribed as adjunct therapy with analgesics, benzodiazepines, and opiates.

Although it is not currently a federally controlled substance, carisoprodol is considered a drug/chemical of concern and is listed as such on the U.S. Department of Justice Drug Enforcement Agency Office of Diversion Control website (http://www.deadiversion.usdoj.gov/index.html), suggesting its prominence as a drug of abuse is recognized on a national level. Its widespread abuse is evident (Schwilke et al., 2006; Reeves et al., 2007a). According to the Dallas DEA Field Division, carisoprodol is one of the six most commonly diverted drugs in its region (Maxwell, 2008). Along with benzodiazepines, Vicodin®, and OxyContin®, carisoprodol is one of the most commonly abused prescription drugs in Northern California (United States Drug Enforcement Administration, 2008). In Florida, the number of carisoprodol-/meprobamate-related deaths in 2005 exceeded those attributed to opioids, including heroin and fentanyl. In May 2008, illegal sales of carisoprodol were featured on “Keeping Them Honest”—a segment on Cable News Network’s (CNN) “Anderson Cooper 360 Degrees” (Cable

News Network, 2008). More importantly, carisoprodol has also been directly and indirectly implicated in fatalities and suicide attempts (Adams et al., 1975; Bailey and Briggs, 2002; Robertson and Marinetti, 2003; Akins et al., 2009).

Carisoprodol abuse is not only an issue in the United States of America. Reports of its abuse have been reported in India, Korea, Norway, and Sweden (Sikdar et al., 1993; Chung et al., 2004; Jonsson et al., 2004; Bramness et al., 2007). Recently, the Committee for Medicinal Products for Human Use (CHMP) concluded the abuse potential associated with carisoprodol outweighs its benefits as a therapeutic drug (World Health Organization, 2007). Based on these findings, the European Medicines Agency recommended the suspension of the marketing authorization for all carisoprodol-containing products (World Health Organization, 2007).

Recent studies have demonstrated the risk for tolerance, dependence, and withdrawal (Reeves and Parker, 2003; Heacock and Bauer, 2004; Reeves et al., 2004; Reeves, 2005; Rohatgi et al., 2005; Ni et al., 2007; Reeves et al., 2007b). Given its metabolic products, carisoprodol toxicity and abuse were previously dismissed as being caused by meprobamate (Littrell et al., 1993b). However, as previously noted, there is a distinction between carisoprodol toxicity and meprobamate toxicity, with the former being characterized by agitation and bizarre movement disorders and the latter involving mainly CNS depression (Goldberg, 1969; Ellenhorn and Barceloux, 1988; Roth et al., 1998). Roth et al. (1998) reported carisoprodol-induced myoclonic encephalopathy. In that case study, serum levels of carisoprodol were elevated while those of meprobamate

were within therapeutic range. These findings suggest the actions of carisoprodol, itself, are dangerous and can be distinguished from those of meprobamate.

The true mechanism of action of carisoprodol is unknown; however, previous studies have suggested carisoprodol may act via the γ-aminobutyric acid (GABA)-ergic system. Roberge et al. (2000) reported the use of flumazenil to reverse carisoprodol intoxication. Flumazenil is a benzodiazepine antagonist known to block the actions of benzodiazepines at $GABA_A$ receptors ($GABA_ARs$). An interaction with $GABA_ARs$ would explain the benzodiazepine-like effects of carisoprodol. Moreover, recent studies have reported the barbiturate-like actions of meprobamate at $GABA_ARs$ (Rho et al., 1997).

$GABA_A$ Receptors

$GABA_ARs$ are members of the cys-loop ligand-gated ion channel (LGIC) superfamily, which includes serotonin type-3, glycine, $GABA_{A0r}$, and nicotinic acetylcholine receptors. The LGIC superfamily is composed of cationic and anionic receptors. $GABA_ARs$ belong to the latter group and mediate the majority of fast synaptic inhibition in the adult CNS. Depending upon the brain region, GABA-mediated transmission is used by approximately 20-50% of all neuronal synapses (Bloom and Iversen, 1971; Chu et al., 1990). Because of the vital contributions of $GABA_AR$ function to CNS regulation, deviations from normal $GABA_AR$ function—either through mutations or dysregulation—can disrupt inhibitory tone in the CNS, resulting in pathological consequences. For instance, abnormal receptor function has been implicated in Angelman syndrome, anxiety, epilepsy, insomnia, and schizophrenia (DeLorey et al., 1998; Minassian et al., 1998; Mohler, 2006; Benarroch, 2007).

$GABA_A$ Receptor Subunit Classes

Functional $GABA_A$Rs are protein complexes, assembled through the combination of individual subunits. Subunit composition determines channel conductance, kinetics, and gating properties of the receptor (Verdoorn et al., 1990; Mathers, 1991; Picton and Fisher, 2007) in addition to its pharmacological profile (Sigel et al., 1990).

Based on cloning and amino acid sequence analyses, the various subunits and their isoforms have been divided into the following classes in mammals: α(1-6), β(1-3), γ(1-3), ρ(1-3), δ, ε, π, and θ (Macdonald and Olsen, 1994; McKernan and Whiting, 1996; Davies et al., 1997; Korpi et al., 2002). Subunits are categorized into classes based upon the extent of their amino acid identity. Between classes, the level of amino acid identity is approximately 30-40% (Hevers and Luddens, 1998); within subunit classes, homology ranges between 70-80%. Each subunit is encoded by a separate gene with some genes producing splice variants of the subunits (i.e. γ2L and γ2s for the long and short isoforms, respectively) (Whiting et al., 1990; Kofuji et al., 1991).

The variance between subunit isoforms is not limited to amino acid identity; mRNA localization, immunohistochemical staining, and autoradiography have demonstrated the subcellular and regional distribution of $GABA_A$R subunits differ as well (Hevers and Luddens, 1998; Mehta and Ticku, 1999). For example, α1, α6, β2, and δ subunits are highly expressed in cerebellum with α6 subunit distribution limited to the cerebellar granule cells of this brain region. The highest levels of α2, α3, α4, α5, β1, and β3 are found in the hippocampus with the localization of α5 subunits predominantly in the soma, dendrites, and axons of neurons and α2 subunits at the axon initial segment

(Nusser et al., 1996; Fritschy et al., 1998). Intermediate levels of these subunits are also present in the cerebral cortex with the exception of α5 which is present in low levels. High levels of α4 are expressed in the thalamus where δ subunits are present in intermediate levels. β1 and β3 subunits are present in the cortex and cerebellum. Unlike the other subunits, γ1 subunits are found predominantly in the amygdala and septum. Expression of the γ3 subunit is highest in the cortex and basal nuclei. Like the α1 and β2 subunits, γ2 subunits are expressed throughout the brain. The localization of receptors containing γ2 subunits is highest at synapses, suggesting their involvement in phasic inhibition (Farrant and Nusser, 2005). The ε subunit is expressed in the thalamus, subthalamic nucleus, and amygdala (Hevers and Luddens, 1998) whereas the π subunit is expressed in peripheral tissues such as lung, prostate, uterus, and thymus (Hedblom and Kirkness, 1997). Although the ρ subunit isoforms were once believed to be expressed solely in the retina, more recent evidence suggests these isoforms are expressed in other regions of the rat brain (Boue-Grabot et al., 1998).

Many of these subunits colocalize (i.e. α4/δ, α6/δ, α2/β3, and α5/β3), suggesting they may preferentially assemble to form channels. In fact, it has been demonstrated receptors containing α4, α5, α6, and δ subunits are likely to colocalize extrasynaptically where they form receptors that mediate tonic inhibition (Caraiscos et al., 2004; Michels and Moss, 2007). Because of their generalized distribution, the α1, β2, and γ2 subunits are likely to colocalize as well. Thus, it is highly likely the most physiologically abundant

$GABA_AR$ configuration is $\alpha1\beta2\gamma2$. It is estimated this configuration represents over 60% of the $GABA_AR$ configurations present in the brain (McKernan and Whiting, 1996).

Structure of $GABA_A$ Receptors

Despite subunit diversity, all subunits share a common architecture. Each subunit is characterized by a large amino-terminal domain, four hydrophobic transmembrane domains (TM1-TM4), a large intracellular loop between TM3 and TM4, and an extracellular carboxyl-terminal domain (Figure I-2) (Schofield et al., 1987; Unwin, 1993). The TM3-TM4 intracellular loop contains several sites that allow for protein-protein interactions, thereby facilitating trafficking and phosphorylation of the receptor (Moss and Smart, 2001; Brandon et al., 2002). Located within the amino-terminal domain is the "cys-loop"—a conserved feature, comprised of two cysteine residues separated by thirteen highly variable amino acids.

A functional channel is formed when five subunits associate in a pentameric fashion surrounding a central, ion-permeable pore that is lined by the TM2 domains of the individual subunits (Unwin, 1993; Xu and Akabas, 1996). Typically, the five subunits form heteromeric receptors; however, $\rho1$ subunits can form homomeric receptors (Enz and Cutting, 1999). There is also evidence for homomeric receptors composed of $\beta1$, $\beta3$, or $\gamma2L$ subunits (Sanna et al., 1995; Cestari et al., 1996; Connolly et al., 1996; Martinez-Torres and Miledi, 2004); however, the GABA sensitivity of these receptors is highly variable. Although the subunit composition of native receptors is unknown, the current consensus is that the majority of $GABA_A$ receptors in the brain are pentamers of

2α:2β:1γ stoichiometry (Im et al., 1995; Chang et al., 1996; Tretter et al., 1997; Farrar et al., 1999).

$GABA_A$ Receptor Activation

In response to an action potential, synaptic vesicles fuse with the presynaptic membrane, releasing GABA in millimolar concentrations into the synaptic cleft (Mody et al., 1994). Activation of $GABA_A$Rs occurs when GABA binds extracellularly at the interface of the α and β subunits, triggering a conformational change and permitting chloride ions (Cl^-) to flow through the channel. Early in development, Cl^- concentrations inside cells are higher than extracellular concentrations due, in part, to the Na^+-K^+-Cl^- cotransporter (Payne et al., 2003; Rivera et al., 2005). Thus, receptor activation at this stage of neuronal development results in depolarization as Cl^- follows its electrochemical gradient. In mature neurons, however, the K^+-Cl^- cotransporter type 2 (KCC2) maintains low intracellular Cl^- concentrations (Lu et al., 1999; Payne et al., 2003). Upon activation, Cl^- influx results in membrane hyperpolarization and reduces membrane excitability.

Inhibitory neurotransmission can be phasic or tonic (Semyanov et al., 2004; Farrant and Nusser, 2005). The former is the result of transient exposure to GABA at high concentrations and results in neuronal inhibition for approximately 10-100 milliseconds. This type of inhibition is associated with synaptic neurotransmission and is likely to facilitate synchronization and integration of networks. Tonic inhibition is mediated by extrasynaptic, high-affinity, non-desensitizing receptors responding to ambient levels of GABA. Spillover and non-synaptic release of GABA result in ambient GABA concentrations in the nanomolar to low micromolar range (Farrant and Nusser,

2005). Tonic inhibition serves a modulatory role in the CNS. Although phasic and tonic forms of inhibition differ greatly, one of their similarities is that each is subject to pharmacological modulation.

Pharmacology of $GABA_A$ Receptors

Aside from the GABA-binding site, these receptors have binding sites for several clinically important drugs (Kittler and Moss, 2003). These compounds bind to their respective sites on the receptor and allosterically modulate channel activity. To date, distinct sites have been described for several clinically important drugs including, but not limited to, anticonvulsants, general anesthetics, neurosteroids, benzodiazepines, barbiturates, and meprobamate. Further attention will be given to several of these drug classes.

Interactions of Benzodiazepines with $GABA_A$ Receptors

Members of the benzodiazepine class of drugs include diazepam (Valium®), chlordiazepoxide (Librium®), alprazolam (Xanax®), midazolam (Versed®), and triazolam (Halcion®). These drugs are used as muscle relaxants, anxiolytics, sedatives, hypnotics, and anticonvulsants. They bind to $GABA_A$Rs at sites distinct from the GABA binding site and increase the frequency of channel opening (Study and Barker, 1981; Macdonald and Olsen, 1994). As true allosteric modulators, benzodiazepines mediate their effects only in the presence of GABA and can potentiate GABA-gated currents only to the extent produced by saturating concentrations of GABA. Because of this property, benzodiazepines are considered safer than barbiturates and are not likely to be fatal in overdose when ingested alone.

High-affinity binding of benzodiazepines in a recombinant system was first demonstrated in cells expressing the human α1β1γ2 $GABA_A$Rs (Pritchett et al., 1989). Since then, it has been accepted that incorporation of the γ subunit is essential for benzodiazepine-sensitivity. Not all γ subunit isoforms contribute equally, however. Affinity and efficacy are consistently greater at γ2-containing receptors whereas these properties for γ1- and γ3-containing receptors depend on the benzodiazepine (Ymer et al., 1990; Wafford et al., 1993; Hadingham et al., 1995; Benke et al., 1996). Thus, γ subunit isoforms play a significant role in determining benzodiazepine pharmacology; the same is true for α subunit isoforms.

The α subunit isoform determines the selectivity with which benzodiazepines bind to $GABA_A$Rs. Benzodiazepine-sensitive receptors contain either α1, α2, α3, or α5 subunits. Notably absent are the α4 and α6 isoforms; incorporation of these subunits produces benzodiazepine-insensitive receptors even in the presence of a γ subunit (Luddens et al., 1990; Wisden et al., 1991). A conserved histidine residue located in the amino-termini of α1 (H101), α2 (H101), α3 (H126), and α5 (H105) subunits renders these isoforms sensitive to benzodiazepines (Wieland et al., 1992; Wieland and Luddens, 1994). This residue is replaced by arginine in the remaining α subunit isoforms. The benzodiazepine binding site is located extracellularly at the interface of the γ and α subunits (Sigel, 2002).

Interactions of Barbiturates with $GABA_A$ Receptors

Barbiturates comprise a unique class of drugs that interact with $GABA_A$Rs in a manner similar to the benzodiazepines. Before the advent of benzodiazepines, these drugs were commonly used as sedative-hypnotics. However, their toxicity and potential for abuse have limited their current therapeutic uses to anesthesia and treatment of seizure disorders. Like benzodiazepines, barbiturates potentiate GABA-gated currents; however, they do so by increasing the duration, not the frequency, of channel opening (Study and Barker, 1981; MacDonald et al., 1989). At therapeutic concentrations, barbiturates can also directly gate the channel in the absence of GABA (Akaike et al., 1985; Akaike et al., 1987). Moreover, at suprathreshold concentrations, barbiturates inhibit GABA-gated currents and produce a characteristic rebound current upon termination of drug application (Rho et al., 1996).

Although the effects of barbiturates mediated by $GABA_A$Rs have been fairly well-characterized, the barbiturate binding site has yet to be elucidated. Whereas the allosteric effects of benzodiazepines require the γ subunit (Pritchett et al., 1988; McKernan et al., 1995; Wingrove et al., 1997), barbiturates can allosterically and directly modulate $GABA_A$Rs in the absence of the γ subunit (Levitan et al., 1988; Pritchett et al., 1989). Previous studies have implicated the α subunit in determining the efficacy of barbiturates, but not their potency (Thompson et al., 1996; Krasowski et al., 1997). Other reports have suggested that the β subunit forms the binding site for barbiturates. Homomeric receptors consisting of β1 subunits formed GABA-sensitive chloride channels that were directly activated by pentobarbital (Sanna et al., 1995). Furthermore,

mutation of a glycine residue at the entrance of the TM1 domain of the β2 subunit diminishes the allosteric effects of pentobarbital, providing further evidence for the role of the β subunit in barbiturate-mediated activity (Carlson et al., 2000; Chang et al., 2003). The involvement of α and β subunits in barbiturate sensitivity is supported by the fact that receptors of αβ, αβγ, and αβδ combinations are sensitive to barbiturates.

However, not all combinations of $GABA_AR$ subunits produce barbiturate-sensitive receptors. Those consisting of ρ subunits or αβε combinations are barbiturate-insensitive (Shimada et al., 1992; Davies et al., 1997). Recent studies have used chimeric receptors to identify amino acids critical for conferring barbiturate sensitivity (Koltchine et al., 1996; Amin, 1999). Amin (1999) demonstrated that replacing the TM3 domain of the barbiturate-insensitive ρ subunit with that of the β2 subunit was sufficient to produce a barbiturate-sensitive homomeric ρ receptor. Likewise, Koltchine et al. (1996) used a similar approach in which barbiturate-insensitive glycine receptor α1 subunits were used to generate chimeric $GABA_A$/glycine receptors. This unique study demonstrated that neither the amino- nor the carboxyl-terminus of the α2 or β1 subunits was involved in barbiturate-mediated actions. Several laboratories continue to focus their research towards elucidating the barbiturate binding site.

Interactions of Meprobamate with $GABA_A$ Receptors

Early studies regarding the interactions of meprobamate with the GABAergic system have produced various findings. In one study, meprobamate had no effect on inhibiting ^{3}[H]-diazepam (Squires and Brastrup, 1977). Other reports, however, demonstrate meprobamate is a weak inhibitor of benzodiazepine binding in the absence

of GABA, and it is more potent at inhibiting GABA-enhanced benzodiazepine binding (Olsen, 1981; Paul et al., 1981). While these findings suggest meprobamate may interact with the benzodiazepine site, there is also evidence meprobamate is similar to the barbiturate class of drugs.

With respect to binding, meprobamate is barbiturate-like in its enhancement of benzodiazepine binding and inhibition of [^{35}S]*t*-butylbicyclophosphorothionate binding at $GABA_A$Rs (Squires et al., 1983; Koe et al., 1986). Functionally, the actions of meprobamate in vivo have been likened to those of barbiturates (Roache and Griffiths, 1987). Furthermore, Rho et al. (1997) demonstrated the barbiturate-like modulation of $GABA_A$R function by meprobamate in vitro. Meprobamate potentiated GABA-gated currents by prolonging burst duration of single-channel currents, and it directly activated $GABA_A$Rs at millimolar concentrations. These actions are characteristic of barbiturates and likely underlie the dangers associated with meprobamate toxicity and its potential for abuse.

Carisoprodol: Potential Actions at $GABA_A$ Receptors

Given the structural similarities between carisoprodol and meprobamate, it seems highly likely that carisoprodol, too, acts at $GABA_A$Rs. Its metabolism to meprobamate provides a reasonable explanation for the depressant effects attributed to carisoprodol. However, as previously noted, a distinction can be made between carisoprodol and meprobamate toxicity, each being equally dangerous. This indicates carisoprodol, itself, may mediate its effects via interaction with $GABA_A$Rs.

Objectives of the Dissertation

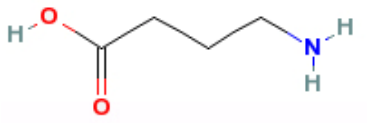
Based upon the hypothesis that carisoprodol acts at $GABA_A$Rs, the overall goal of the studies presented herein was to identify the mechanism of action for carisoprodol at these receptors. Preliminary studies conducted in the laboratories of Dr. Michael Forster and Dr. Michael Gatch suggested carisoprodol elicits behavioral effects in mice with a profile similar to that of other GABAergic compounds, but not entirely consistent with that of its primary metabolite, meprobamate. These findings raised several questions. Does carisoprodol have the potential to mediate these effects via the GABAergic system independently of its metabolite? If so, do its actions vary in a subunit- or receptor-dependent manner? Does carisoprodol share its sites of action with other clinically relevant GABAergic compounds?

Since initial studies were conducted at the whole-animal level, the answers to these questions were confounded by metabolism of the parent compound. In order to gain a better understanding of carisoprodol's mechanism and site of action, functional studies were conducted using an in vitro system, circumventing the issue of metabolism. The purpose of these electrophysiological studies was to characterize potential carisoprodol-mediated activity at $GABA_A$Rs with respect to subunit- and receptor-dependence. The aim of these experiments was two-fold: 1) to investigate regional differences in the actions of carisoprodol in the CNS and 2) to provide insight into critical domains involved in mediating carisoprodol-mediated activity at $GABA_A$Rs. To assess potential sites of action for carisoprodol, molecular and pharmacological approaches were used in conjunction with electrophysiology. As discussed previously, binding sites and sites of

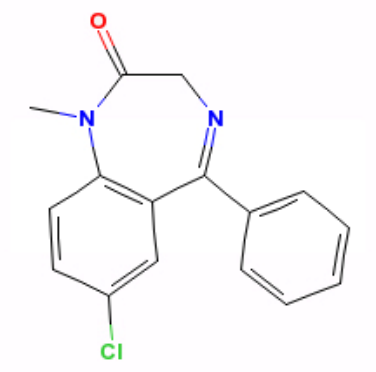
action have been reported for several GABAergic compounds. Although described for other compounds, these sites are not compound-exclusive and may also serve as sites of action for carisoprodol. While this possibility was addressed, a chimeric strategy was also utilized to identify subunit domains responsible for conferring sensitivity to carisoprodol.

Taken together, the studies included herein will substantially increase our understanding of the mechanism of action of carisoprodol as a therapeutic agent and as a drug of abuse. $GABA_AR$ subunit configuration varies regionally; thus, elucidating the subunit-dependence of carisoprodol may provide insight into its effects on certain areas of the brain that contribute to its therapeutic effects as well as its abuse potential. Moreover, while the number of reports regarding carisoprodol abuse continues to increase, there has been little progress in the treatment of carisoprodol dependence and withdrawal. At present, treatment consists of brief courses with benzodiazepines or phenobarbital to combat anxiety and insomnia. Furthermore, treatment of carisoprodol overdose is complicated as it is often characterized by agitation and seizures, and the administration of anticonvulsants and sedatives exacerbates CNS depression, leaving supportive therapy as a preferred course of action. Identification of the mechanism and site of action of carisoprodol may lead to a better understanding of the therapeutic effects of this drug and a more effective means of treating carisoprodol tolerance, dependence, and withdrawal.

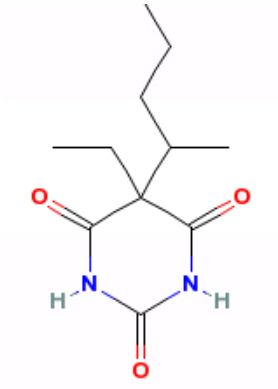
Figure I-1. Chemical structures of $GABA_A$ receptor agonists and allosteric modulators. GABA (γ-aminobutyric acid) is the endogenous agonist for $GABA_A$ receptors. Diazepam is a member of the benzodiazepine class of drugs. It allosterically modulates $GABA_A$ receptor function. Pentobarbital is a barbiturate capable of directly activating and allosterically modulating receptor function. Carisoprodol and meprobamate are propanediol dicarbamates. Meprobamate has barbiturate-like actions at $GABA_A$ receptors. Chemical structures were obtained from PubChem, an online resource made available through the United States National Library of Medicine (http://pubchem.ncbi.nlm.nih.gov/).



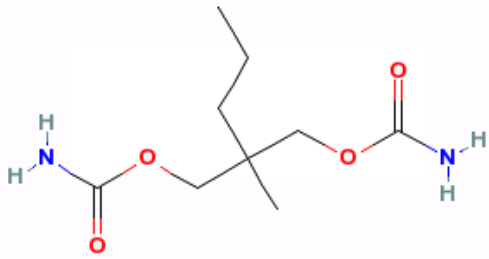
GABA



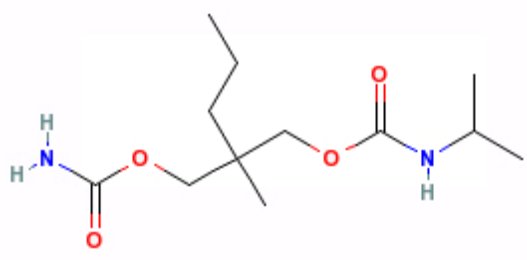
DIAZEPAM



PENTOBARBITAL

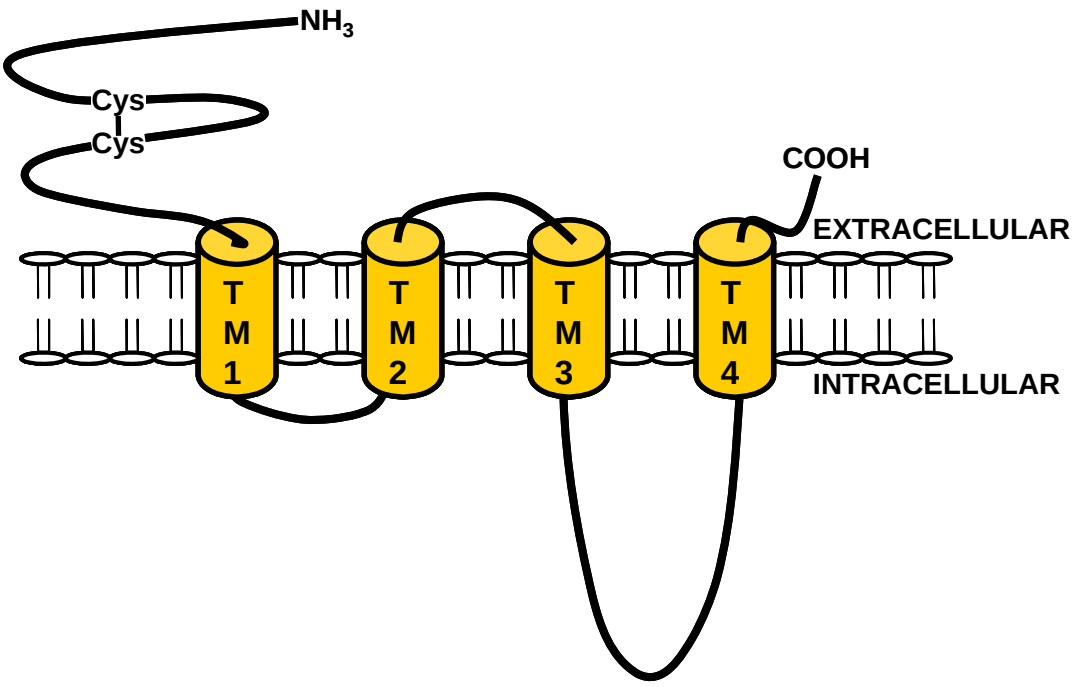


MEPROBAMATE



CARISOPRODOL

Figure I-2. Subunit structure of GABA receptors. Subunit structure is conserved throughout $GABA_A$ receptor subunits. They are composed of a large extracellular amino-terminal domain, four hydrophobic, α-helical transmembrane domains, a large intracellular loop, and an extracellular carboxyl-terminal domain.


NH3
Cys
Cys
COOH
EXTRACELLULAR
TM1
TM2
TM3
TM4
INTRACELLULAR

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CHAPTER II

CARISOPRODOL-MEDIATED MODULATION OF $GABA_A$ RECEPTORS: IN VITRO AND IN VIVO STUDIES

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ABSTRACT

Carisoprodol is a frequently prescribed muscle relaxant. In recent years, this drug has been increasingly abused. The effects of carisoprodol have been attributed to its metabolite, meprobamate, a controlled substance that produces sedation via $GABA_A$ receptors ($GABA_ARs$). Given the structural similarities between carisoprodol and meprobamate, we used electrophysiological and behavioral approaches to investigate whether carisoprodol directly affects $GABA_AR$ function. In whole-cell patch clamp studies, carisoprodol allosterically modulated and directly activated human $\alpha1\beta2\gamma2$ $GABA_AR$ function in a barbiturate-like manner. At millimolar concentrations, inhibitory effects were apparent. Similar allosteric effects were not observed for homomeric $\rho1$ GABA or glycine $\alpha1$ receptors. In the absence of GABA, carisoprodol produced picrotoxin-sensitive, inward currents that were significantly larger than those produced by meprobamate, suggesting carisoprodol may directly produce GABAergic effects *in*

vivo. When administered to mice via intraperitoneal or oral routes, carisoprodol elicited locomotor depression within 8 to 12 min. following injection. Intraperitoneal administration of meprobamate depressed locomotor activity in the same time frame. In drug discrimination studies with carisoprodol-trained rats, the GABAergic ligands pentobarbital, chlordiazepoxide, and meprobamate each substituted for carisoprodol in a dose-dependent manner. In accordance with findings in vitro, the discriminative stimulus effects of carisoprodol were antagonized by a barbiturate antagonist, bemegride, but not by the benzodiazepine site antagonist flumazenil. The results of our studies in vivo and in vitro collectively suggest the barbiturate-like effects of carisoprodol may not be due solely to its metabolite, meprobamate. Furthermore, the functional traits we have identified likely contribute to the abuse potential of carisoprodol.

INTRODUCTION

Carisoprodol (N-isopropylmeprobamate, Soma®) is a centrally-acting skeletal muscle relaxant frequently prescribed for the alleviation of lower back pain (Elenbaas, 1980). In 2000, carisoprodol was the second most frequently prescribed muscle relaxant, accounting for greater than 20% of all skeletal muscle relaxant prescriptions in the United States (Luo et al., 2004). Although evidence of carisoprodol abuse has been reported for several years (Morse and Chua, 1978; Elder, 1991; Rust et al., 1993; Reeves et al., 1997), its abuse is on the rise. A report by Elder (1991) ranked carisoprodol 54th among 234 drugs with abuse potential. Only 9 years later, the Drug Abuse Warning Network (2000) identified carisoprodol as the 20th most abused drug, ranking higher than oxycodone, methadone, and *d*-lysergic acid diethylamide.

Once ingested, carisoprodol is metabolized to hydroxycarisoprodol, hydroxymeprobamate, and meprobamate (Olsen et al., 1994; Dalen et al., 1996). Meprobamate (Miltown®, Equanil®) is a sedative-hypnotic that was commonly used in the treatment of anxiety and is currently classified as a schedule IV controlled substance at the federal level. Although the central actions of meprobamate have not been fully elucidated, one target of its effects appears to be $GABA_A$ receptors ($GABA_AR$s), the predominant inhibitory neurotransmitter receptor in the brain. Rho et al. (1997) demonstrated meprobamate potentiates GABA-gated currents by prolonging burst duration of single-channel currents, and it directly activates $GABA_AR$s at millimolar concentrations.

It is believed generally that both the sedative and adverse effects of carisoprodol are due to its metabolic conversion to meprobamate. The known ability of meprobamate to modulate $GABA_AR$ function does provide a reasonable explanation for the depressant effects attributed to carisoprodol. However, there is a distinction between carisoprodol toxicity and meprobamate toxicity, with the former being characterized by agitation and bizarre movement and the latter involving mainly CNS depression (Goldberg, 1969; Ellenhorn and Barceloux, 1988; Roth et al., 1998). Moreover, these signs of toxicity are observed early in overdose, before carisoprodol is significantly dealkylated to meprobamate (Roth et al., 1998). These findings suggest the actions of carisoprodol are dangerous in their own right and can be distinguished from those of meprobamate.

In light of these observations, we sought to determine whether carisoprodol, independently of its conversion to meprobamate, can modulate $GABA_ARs$. We assessed the actions of carisoprodol at both the molecular pharmacologic and behavioral pharmacologic level. Results from our in vitro studies demonstrate carisoprodol allosterically modulates and directly activates $GABA_ARs$, with an efficacy and potency greater than that of meprobamate. Moreover, in vivo behavioral experiments demonstrate that carisoprodol has GABAergic activity, with a pharmacologic profile consistent with that observed in our in vitro studies. Our results collectively provide strong evidence that carisoprodol can directly produce notable CNS depressant activity, and this activity may contribute to its abuse potential.

MATERIALS AND METHODS

In Vitro Studies

Cloned Receptors. Both stably and transiently transfected cells were used in the present study. Because the α1β2γ2 configuration of the $GABA_A$ receptor is the predominant configuration expressed in the brain (Huang et al., 2006) and because it is known to be associated with effects of GABAergic agents on locomotor activity (Rudolph et al., 1999; McKernan et al., 2000), it was the focus of the in vitro studies. Human embryonic kidney (HEK) 293 cells stably expressing human α1β2γ2 (short isoform of γ2) $GABA_A$Rs or homomeric V5-His-tagged α1 glycine receptors (below) were used in the current investigation. A complete description of the preparation and maintenance of the human α1β2γ2 (short isoform) cell line has been published previously (Hawkinson et al., 1996).

A cell line stably expressing human glycine α1 receptors was generated in our laboratory. In brief, the α1 subunit was subcloned into the vector pcDNA3.1/V5-His B (Invitrogen, Carlsbad, CA) using *BamHI* and *EcoRI* restriction sites. The glycine α1 cDNA was linearized with *PvuI*, and the linearized cDNA was transfected into HEK293 cells using the modified calcium phosphate transfection method. Forty-eight hours after transfection, cells were transferred to a medium containing minimum essential media, 10% fetal bovine serum, L-glutamine (200mM), penicillin and streptomycin (10,000U/ml), and the selection agent G-418 (500-1000mg/ml). Cells were maintained in the selection media for 2 weeks. Resistant cells were split at a high dilution and plated in

multi-well plates. Single-cell clones were selected and grown in selective media for another week. Each of these clones was tested for expression of functional glycine receptors using whole-cell patch clamp. Clones that responded robustly to the application of a saturating concentration of glycine were selected and maintained in media containing 500 mg/ml G-418. This cell line, established to stably express human glycine α1 receptors, was used to conduct subsequent experiments. To study both $GABA_A$ and glycine receptors, cells stably expressing the respective receptors were plated on glass coverslips coated with poly-L-lysine (Sigma, St. Louis, MO) in 35-mm culture dishes and used for electrophysiological analysis 24-48 h after plating.

The wild-type human GABA ρ1 subunit was generously provided by David Weiss (University of Texas Health Science Center, San Antonio, TX). To generate barbiturate-sensitive ρ1(W328M) subunits, tryptophan 328 of the wild-type ρ1 subunit was mutated to methionine using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Incorporation of the mutation was verified by DNA sequencing. For studies involving GABA ρ1 receptors, HEK293 cells were transiently transfected using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's specifications. In brief, HEK293 cells were plated onto coverslips and transfected using 0.5 μg of wild-type or mutant GABA ρ1 cDNA. Cells were washed and placed in fresh culture medium after incubation (6 h) at 37°C in a humidified incubator with an atmosphere of 5% CO_2. Cells were used in electrophysiological studies 24-48 h after transfection.

Electrophysiology. Whole-cell patch clamp electrophysiology was used to assess GABA-, meprobamate-, carisoprodol-, or glycine-activated Cl^- currents. All electrophysiology 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 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 (Olympus IX71; Olympus, Tokyo, Japan) 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 7.3. Agonist-induced Cl^- currents were obtained with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) equipped with a CV-203BU headstage. 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. The modulatory effects of carisoprodol on GABA-gated currents were assessed using an EC_{20} concentration of GABA (Huang et al., 2001). GABA (with or without carisoprodol or other $GABA_A$ receptor agonists) was prepared in external solution and applied to each cell by gravity flow using a Y-shaped tube positioned adjacent to the cell. For studies investigating direct activation by carisoprodol,

carisoprodol (with or without $GABA_A$ receptor antagonists) was dissolved in external solution and applied in the manner described above. In the bemegride studies, cells were incubated in external solution containing bemegride at the indicated concentration for 2 min. Control responses were established by observing two consecutive agonist-activated currents that varied in amplitude by no more than ±10%. After establishing the control response, effects of the test drug were determined.

Data Analysis. Concentration-response profiles for the positive modulatory actions of carisoprodol were generated (Origin 5.0; OriginLab Corp., Northampton, MA) using the equation $I/I_{max} = [\text{carisoprodol}]^n/([\text{carisoprodol}]^n + EC_{50}^n)$, where I is the normalized current amplitude at a given concentration of carisoprodol, I_{max} is the maximum GABA current induced by carisoprodol, EC_{50} is the half-maximal effective concentration of carisoprodol, and n is the Hill coefficient. All data are presented as mean values ± S.E. Statistical significance ($p<0.05$) between control and test conditions was determined using Student's t test (paired or unpaired) and one-way analysis of variance. Dunnett's post hoc test was performed as needed.

In Vivo Studies

Animals. Male Sprague-Dawley rats were obtained from Harlan-Sprague Dawley (Indianapolis, IN). All rats were housed individually and were maintained on a 12-/12-h light/dark cycle (lights on at 7:00 AM). Body weights were maintained at 320-350 g by limiting food to 20 g/day, which included the food received during operant sessions. Water was freely available. Male Swiss–Webster mice were obtained from Harlan at approximately 8 weeks of age and tested at approximately 10 weeks of age. Mice were

group-housed in cages on a 12-/12-h light/dark cycle and were allowed free access to food and water. All in vivo testing of rats and mice was done during the light portion of the cycle. All housing and procedures were in accordance with the guidelines of the *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) and were approved by the University of North Texas Health Science Center Animal Care and Use Committee.

Discrimination training. Standard operant chambers (Coulbourn Instruments, Allentown, PA) were connected to IBM-PC compatible computers via LVB interfaces (MED Associates, St. Albans, VT). The computers were programmed in MED-PC IV (MED Associates) for the operation of the chambers and collection of data. Rats were trained to discriminate carisoprodol (100 mg/kg p.o.) from vehicle (2% methylcellulose) using a two-lever choice methodology. Food (45-mg food pellets; Bio-Serv, Frenchtown, NJ) was available as a reinforcer under a fixed-ratio 10 schedule when responding occurred on the injection-appropriate lever. There was no consequence for responses on the incorrect lever. The rats received approximately 60 training sessions before they were used in substitution or antagonism experiments. Animals were selected for use in experiments when they had met the criteria of emitting 85% of responses on the injection-correct lever for both the first fixed ratio and for the remainder of the session during their last 10 training sessions. Training sessions occurred in a double alternating fashion (D-D-S-S-D, etc.), and tests were conducted between pairs of identical training sessions (i.e., between either two vehicle or two carisoprodol training sessions). Rats were tested only if they had achieved 85% drug-lever responding for both first fixed-ratio

and total session on the two prior training sessions. Before each session, the rats received an injection of either vehicle or carisoprodol. Ten minutes later, the rats were placed in an operant chamber. Each training session lasted a maximum of 10 min, and the rats could earn up to 20 food pellets.

Discrimination test procedures. In contrast with training sessions, both levers were active during the discrimination test sessions, such that 10 consecutive responses on either lever led to reinforcement. Data were collected until the first reinforcer was obtained or for a maximum of 20 min. At least 3 days elapsed between test sessions. Groups of nine or 10 rats were tested with each test compound. A repeated measures design was used, such that each rat was tested at all doses. During substitution experiments, oral administration of carisoprodol (by gavage, 1 ml/kg) or its vehicle (2% methylcellulose) occurred 20 min before the start of the test session, and a dose range of 10 to 100 mg/kg was examined. Meprobamate (10-175 mg/kg), pentobarbital (1-25 mg/kg), and chlordiazepoxide (1-10 mg/kg) were administered via intraperitoneal injections (1 ml/kg). Administration of meprobamate or its vehicle (2% methylcellulose) occurred 30 min before the start of the test session. Administration of pentobarbital, chlordiazepoxide, or their vehicle (0.9% saline) occurred 15 min before the start of the test session. During antagonism experiments, intraperitoneal injections (1 ml/kg) of bemegride (1-5 mg/kg), flumazenil (0.5 to 25 mg/kg), or their vehicles occurred 30 min before the start of the test session. Oral administration of carisoprodol (by gavage, 1 ml/kg) occurred 20 min before the start of the test session.

Locomotor Activity. Studies of locomotor activity were conducted using a Digiscan apparatus (model RXYZCM-16, Omnitech Electronics, Columbus, OH) and clear acrylic locomotor activity testing chambers (40.5 X 40.5 X 30.5 cm) housed in sets of two, within sound-attenuating chambers. A panel of infrared beams (16 beams) and corresponding photodetectors were located in the horizontal direction along the sides of each activity chamber. A 7.5-W incandescent light above each chamber provided dim illumination. Fans provided an 80-dB ambient noise level within the chamber.

In studies of the time-course of locomotor depression elicited by meprobamate, separate groups of eight mice received either vehicle (2% methylcellulose) or meprobamate (10, 30, 100, or 300 mg/kg) by intraperitoneal injection immediately before locomotor activity testing. Time-course studies of carisoprodol involved separate groups of 16 mice that received either vehicle (2% methylcellulose) or carisoprodol (10, 30, 100, 300, or 560 mg/kg p.o.) (by gavage) immediately before locomotor activity testing. In the time-course studies, photocell interruptions (ambulation counts) within the horizontal plane of the testing chambers were recorded within 10-min epochs for a period of 8 h.

Additional studies were performed using separate groups of eight mice to compare the times of onset of locomotor depression following 300 mg/kg of carisoprodol (administered orally versus intraperitoneally) or meprobamate (administered intraperitoneally). In these studies, locomotor activity was recorded in 4-min epochs for a period of 24 min after administration.

Data Analysis. Drug discrimination data were expressed as the mean percentage of responses made on the carisoprodol-appropriate lever. Percentage carisoprodol-

appropriate responding and response rate were plotted as a function of the dose of the test compound (log scale). Percentage carisoprodol-appropriate responding was calculated for a given dose only if at least three rats completed the fixed ratio. Full substitution was defined as ≥80% carisoprodol-appropriate responding, and full antagonism as ≤20% carisoprodol-appropriate responding. Rates of responding were expressed as a function of the number of responses made divided by the total session time. The potencies of carisoprodol, meprobamate, chlordiazepoxide, pentobarbital, and bemegride were calculated by fitting straight lines to the individual dose-response data for each compound by means of TableCurve 2D (SPSS, Inc., Chicago, IL). Straight lines were fitted to the linear portion of dose-effect curves, defined by doses producing 20 to 80% of the maximal effect, including not more than one dose producing <20% of the maximal effect and not more than one dose producing >80% of the maximal effect. Other doses were excluded from the analyses. The slopes of the dose-effect curves were compared using parallel line procedures (Kenakin, 1997). Comparison of the ED_{50} values was performed by one-way analysis of variance. Individual comparisons were made using Bonferonni-adjusted *F* tests. Criterion for significance was set a priori at $p<0.05$. Response rate data were analyzed by one-way repeated measures analyses of variance. In the context of a significant overall effect, individual doses were compared with the appropriate control value using individual *F* tests.

For assessment of time-course effects, ambulation counts within each time sampling interval were considered in a two-way analysis of variance with treatment and time (repeated) as the factors. Only the first 5 h of the test session was considered

because no treatment effects were evident over the last 3 h of testing. The 10- to 20- min time period was selected for analysis of dose-response data because this was the earliest period in which maximal locomotor depression first appeared as a function of dose for both compounds. An ED_{50} (dose producing one half-maximal depressant activity, where maximal depression = 0 counts/10 min), was calculated based on a linear fit to the descending portion of the dose-response curve. A one-way analysis of variance was conducted on ambulation counts for the 10- to 20-min time period for each compound, and individual comparisons of each dose with vehicle control were considered using *F* tests. For studies of the onset of depression after carisoprodol and meprobamate, data were considered in a two-way analysis of variance, with treatment and time (repeated) as the factors.

Drugs

Carisoprodol ($C_{12}H_{24}N_2O_4$), meprobamate ($C_9H_{18}N_2O_4$), diazepam, chlordiazepoxide, flumazenil, picrotoxin, and pentobarbital sodium were purchased from Sigma-Aldrich. Bemegride was obtained from Pfaltz & Bauer Ltd. (Waterbury, CT). For the electrophysiology studies, stock solutions of these compounds were made by dissolving the compounds in dimethyl sulfoxide. Drugs were diluted in normal saline, so that the final dimethyl sulfoxide concentration (v/v) of the test solutions was ≤0.3%. GABA and bemegride stock solutions were prepared using H_2O. For the in vivo studies, carisoprodol, meprobamate, and flumazenil were suspended in 2% methylcellulose. Chlordiazepoxide, bemegride, and pentobarbital were dissolved in 0.9% saline.

Carisoprodol was administered by oral gavage in a volume of 1 ml/kg. All remaining drugs were administered intraperitoneally in a volume of 1 ml/kg.

RESULTS

Carisoprodol Allosterically Modulates GABA-Activated Currents. Using whole-cell patch clamp electrophysiology, we investigated carisoprodol-mediated effects on HEK293 cells stably expressing human $\alpha1\beta2\gamma2$ $GABA_A$Rs. This approach circumvents the metabolism of carisoprodol to meprobamate, allowing us to focus solely on the effects of the parent drug. When co-applied with GABA, carisoprodol potentiated GABA-gated currents in a concentration-dependent manner (Fig. 1). The actions of carisoprodol were rapid and reversible, suggesting carisoprodol-mediated effects are due to direct interaction with the receptor. The EC_{50} value for carisoprodol was estimated at 142 ± 13 μM with a Hill coefficient of 2.46 ± 0.42, although the direct-gating effects of carisoprodol (below) may contribute to recorded current amplitude. Potentiation of GABA-gated currents is characteristic of central nervous system depressants such as benzodiazepines, barbiturates, neurosteroids, and anesthetics (see Huang et al., 2006). At millimolar concentrations, we observed "rebound currents" upon termination of drug application, and we also observed inhibitory actions of carisoprodol on GABA-gated currents. This phenomenon is observed with some other GABA modulators, including barbiturates (Rho et al., 1996) and lactones (Gonzales et al., 2004). In a previous study, meprobamate was shown to allosterically modulate GABA-activated currents (Rho et al, 1997). In the present study, we also found that meprobamate could allosterically enhance GABA-gated currents (Fig. 1, C and D), although both the potency and the efficacy were less than that observed with carisoprodol.

Carisoprodol Activates Inward Currents in the Absence of GABA. Several agents that potentiate GABA-gated currents can directly activate $GABA_A$Rs in the absence of GABA (see Huang et al., 2006); meprobamate is among these (Rho et al., 1997). Thus, we assessed whether carisoprodol can directly activate $GABA_A$Rs. As shown in Fig. 2, application of carisoprodol in the absence of GABA elicited a concentration-dependent inward current activation. As was seen with allosterically enhanced GABA currents, we observed rebound currents in response to millimolar carisoprodol (Fig. 2, A and B). In addition, we confirmed the results of Rho et al. (1997) demonstrating direct activation of GABA currents by meprobamate. Although both drugs directly activated $GABA_A$Rs, carisoprodol was more potent and efficacious than its metabolite (Fig. 2, A and B). Our studies collectively suggest that carisoprodol has the potential to produce sedative effects similar to those of meprobamate, and it can do so without being metabolized to meprobamate.

To confirm that carisoprodol-activated inward currents were, in fact, mediated by $GABA_A$Rs, we investigated carisoprodol's effects in the presence of picrotoxin (PTX), a widely utilized $GABA_A$R antagonist. Carisoprodol-activated current amplitude was antagonized by PTX in a concentration-dependent manner (Fig. 2, C and D), indicating that the inward currents are carried by $GABA_A$Rs.

Carisoprodol Does Not Mediate Its Effects via the Benzodiazepine Site of the $GABA_A$ Receptor. Aside from the GABA binding site, these receptors have distinct binding sites for several clinically important drugs, including barbiturates and benzodiazepines, among others (see Huang et al., 2006). To assess the potential

involvement of the benzodiazepine site in the actions of carisoprodol, we tested its ability to modulate GABA-gated currents in the presence of the benzodiazepine antagonist flumazenil. As shown in Fig. 3, flumazenil blocked the ability of diazepam to augment GABA-gated currents, yet it had no significant effect on the allosteric effects of carisoprodol. Likewise, the presence of flumazenil did not attenuate the amplitude of carisoprodol-mediated currents. These data demonstrate that neither the direct nor allosteric potentiating effects of carisoprodol are mediated via the benzodiazepine site of $GABA_ARs$.

Carisoprodol-Mediated Currents Are Blocked by Bemegride. The actions of meprobamate, the metabolite of carisoprodol, have been characterized as "barbiturate-like" (Rho et al., 1997). Moreover, as the effects of carisoprodol reported here are in several ways also reminiscent of barbiturates, we considered whether the barbiturate site is involved in mediating the effects of carisoprodol. Although not necessarily considered a pure barbiturate antagonist, bemegride has been shown to antagonize the stimulus effects of pentobarbital (Krimmer et al., 1978; Schechter, 1984). Thus, antagonism of carisoprodol's effects by bemegride might provide some insight into whether carisoprodol and barbiturates share a site or mechanism of action. Because high concentrations of bemegride also inhibit GABA-gated currents, we did not assess its effects on allosteric modulation by carisoprodol. Instead, we examined whether bemegride incubation affects carisoprodol-mediated currents. As shown in Fig. 4, carisoprodol-activated currents were significantly and reversibly attenuated in the

presence of bemegride. Although not definitive, these findings are consistent with the possibility that the barbiturate site may influence the direct-gating effects of carisoprodol.

Carisoprodol Does Not Modulate Homomeric ρ1 or Glycine α1 Receptors. To assess the extent to which carisoprodol might selectively potentiate $GABA_A$Rs, we evaluated its ability to modulate homomeric ρ1 GABA and homomeric α1 glycine receptors, two other anion-selective members of the Cys-loop family of LGICs with pharmacology distinct from that of the $GABA_A$R. Transient transfection of ρ1 cDNA into HEK293 cells resulted in GABA-gated currents that displayed GABA sensitivity (EC_{50}, 0.9 ± 0.08 μM) and activation and deactivation kinetics consistent with those previously reported (Amin and Weiss, 1994). In these homomeric ρ1 receptors, 300 μM carisoprodol had no effect on GABA-activated current (Fig. 5, A and B). In α1 glycine receptors, concentrations of carisoprodol up to 1 mM had no stimulatory effect on the current amplitude of glycine-gated currents; at 1 mM, modest attenuation of the glycine-gated current was observed (Fig. 5, C and D). Thus, both homomeric ρ1 GABA receptors and homomeric α1 glycine receptors show no potentiation in response to carisoprodol, in contrast to the severalfold potentiation observed in α1β2γ2 $GABA_A$Rs (Fig. 1 above).

W328M Mutation in ρ1 Receptors Confers Sensitivity to Pentobarbital but Not Carisoprodol. Amin (1999) has shown that introduction of a methionine residue at position 328 in homomeric ρ1 receptors confers sensitivity to both direct-gating and allosteric effects of pentobarbital. Thus, we generated this mutant and assessed whether it could similarly confer sensitivity to carisoprodol. HEK293 cells transfected with the ρ1

subunit cDNA yielded functional receptors with GABA sensitivity and channel kinetics consistent with those previously reported (Amin and Weiss, 1994). As reported by Amin (1999), the W328M mutation did confer sensitivity to both the allosteric modulating and direct gating effects of pentobarbital (Fig. 6, A and B). In contrast, this mutation did not confer to carisoprodol the ability to either allosterically potentiate or directly gate the ρ1 receptor (Fig. 6, A and B). Thus, although our data, in general, demonstrate the actions of carisoprodol are barbiturate-like, these experiments indicate the binding and/or functional domains for the two ligands are not equivalent.

Carisoprodol Produces Time-Dependent Depression of Locomotor Activity. Behavioral studies were carried out to assess the in vivo actions of carisoprodol. First, we investigated the ability of carisoprodol to depress the locomotor activity of non-habituated, male Swiss-Webster mice. As depicted in Fig. 7, left, treatment with carisoprodol resulted in maximal depression of locomotor activity (lasting 40 min to 2 h) after doses of 300 or 560 mg/kg, respectively. For both doses, maximal depressant effects were first evident within the time period from 10 to 20 min after treatment, and an ED_{50} of 240 mg/kg was estimated based on dose-response data for this period. A significant interaction of treatment and time period supported the overall observation of dose- and time-dependent effects [$F(145,2610) = 3.47$, $p < 0.001$], and individual comparisons with the vehicle group for the 10- to 20-min time period confirmed a significant depressant effect for 300 and 560 mg/kg (all $p < 0.001$).

Although the above results are consistent with previous reports that carisoprodol produces sedation, loss of balance, and increased reaction time (Robertson and Marinetti,

2003), they did not address whether the effects are due to carisoprodol or its metabolite, meprobamate. To address this question, experiments were also conducted with meprobamate to determine whether the extent of locomotor depression and its time course would be consistent with that observed after oral carisoprodol. Intraperitoneal treatment with meprobamate resulted in partial depression of locomotor activity (lasting approximately 40 min) after 100 mg/kg and maximal depression (lasting 2.5 h) after 300 mg/kg (Fig. 7, right). Maximal depressant effects of meprobamate were first evident 10 to 20 min after 300 mg/kg, and an ED_{50} of 135 mg/kg was estimated based on dose-response data for this time period. A significant interaction of treatment and time period [$F(116,1015) = 4.89$, $p < 0.001$] supported the overall observation of time- and dose-dependent effects, and individual comparisons with the vehicle group for the 10- to 20-min time period confirmed significant depression for the 100 and 300 mg/kg doses (all $p < 0.001$). It is noteworthy that after 300 mg/kg, maximum depression relative to vehicle control was evident for both compounds within 20 min after injection, yet the time-course of depression was dramatically longer for meprobamate. This observation would be consistent with reports suggesting a shorter plasma half-life of carisoprodol compared with meprobamate (e.g., Bramness et al., 2004).

To determine the influence of the oral route of administration on the rate of onset of locomotor depression after carisoprodol relative to the intraperitoneal administration of meprobamate, we compared the effects of those treatments when locomotor activity was monitored within 4-min epochs for 24 min. As suggested by the results shown in Fig. 8, administration of 300 mg/kg i.p. carisoprodol failed to significantly accelerate (or delay)

the onset of locomotor depression relative to the same dose administered orally. Moreover, carisoprodol by either route produced locomotor depression that was equivalent in magnitude and rate of onset to meprobamate injected intraperitoneally. However, carisoprodol administered intraperitoneally resulted in stimulation of locomotor activity during the first 4 min after injection, an effect not evident after the other treatments. Analysis of these data yielded a significant treatment × time period interaction [$F(20,175) = 9.7$, $p < 0.001$], in accordance with the decrease in locomotor activity after all drug treatments that began 8 min after administration of carisoprodol or meprobamate. Individual comparisons at each time period confirmed a significant difference from control for all treatments during periods 3 to 6 and a significant difference between intraperitoneal carisoprodol and the intraperitoneal vehicle control during the first 4 min of testing ($p < 0.01$).

Discriminative Stimulus Effects of Carisoprodol. Our functional studies performed in vitro strongly suggested carisoprodol has the potential to mediate GABAergic effects in vivo. Thus, we sought to determine whether the stimulus effects of carisoprodol generalized to those of other compounds known to modulate $GABA_A$Rs. The purpose of these experiments was 2-fold: 1) to train carisoprodol as a discriminative stimulus and 2) to test whether the mechanism for the discriminative stimulus effects of carisoprodol is GABA-like by testing for substitution with a number of GABAergic compounds.

After training of the discrimination for 100 mg/kg carisoprodol, different doses (10, 25, 50, 100 mg/kg p.o.) produced dose-dependent increases in carisoprodol-appropriate responding (Fig. 9). Intraperitoneal injections of pentobarbital (1, 5, 10, 25

mg/kg), meprobamate (10, 25, 50, 100, 175 mg/kg), and chlordiazepoxide (1, 2.5, 5, 10 mg/kg) each produced dose-dependent substitution for the discriminative stimulus effects of carisoprodol (Fig. 9). ED_{50} values are shown in Table 1. Vehicles for the test compounds produced predominately vehicle-appropriate responding. Pentobarbital and chlordiazepoxide were more potent than carisoprodol or meprobamate [$F(3,27) = 9.23$, $p < 0.001$]. Pentobarbital and chlordiazepoxide did not differ in potency, nor did carisoprodol and meprobamate.

Carisoprodol dose-dependently increased response rate [$F(4,64) = 9.13$, $p < 0.001$], and chlordiazepoxide increased response rate after 2.5 and 5 mg/kg [$F(4,36) = 5.46$, $p = 0.002$]. In contrast, pentobarbital increased response rate after 5 and 10 mg/kg and decreased response rate after 25 mg/kg [$F(4,36) = 12.35$, $p < 0.001$]. Meprobamate dose-dependently decreased response rate [$F(5,45) = 6.65$, $p < 0.001$].

Next, we examined whether the discriminative stimulus effects of carisoprodol could be antagonized in vivo. To determine whether carisoprodol may be acting at barbiturate- or benzodiazepine-sensitive sites, carisoprodol testing was performed in combination with antagonists for those sites on the receptor. Bemegride (1, 2.5, 5 mg/kg) dose-dependently attenuated the discriminative stimulus effects of the training dose of carisoprodol (Fig. 10). The ED_{50} value was 2.83 mg/kg (95% confidence interval = 2.27-3.36 mg/kg). In contrast, flumazenil failed to fully antagonize the discriminative stimulus effects of carisoprodol (defined as less than or equal to 20% drug-appropriate responding). An intermediate dose (2.5 mg/kg) reduced carisoprodol-appropriate responding to 36%, but a higher dose (25 mg/kg) resulted in 91% carisoprodol-

appropriate responding. Response rate was decreased to 79% carisoprodol control after 5 mg/kg bemegride [$F(3,27) = 4.45$, $p = 0.012$] whereas flumazenil did not affect response rate [$F(6,425) = 0.903$, $p = 0.504$]. These results are consistent with the in vitro data in which the benzodiazepine site antagonist flumazenil had no significant effect on either the allosteric or the direct activity of carisoprodol, whereas bemegride significantly reduced carisoprodol-mediated currents.

DISCUSSION

The mechanism of action of carisoprodol is unclear. The general consensus has been that the therapeutic and addictive properties associated with carisoprodol are due to its metabolism to meprobamate. This assertion is supported by the findings of Rho et al. (1996, 1997) which demonstrated the propanediol dicarbamates meprobamate and felbamate act in a barbiturate-like manner at $GABA_A$Rs. Given that carisoprodol is also a dicarbamate, the current study examined whether carisoprodol acts in a similar manner in vitro and in vivo.

Whole-cell patch clamp studies demonstrated potentiation of GABA-gated currents at micromolar concentrations. High concentrations of carisoprodol in the presence of GABA produced inhibition, followed by rebound currents upon termination of drug application. This phenomenon is consistent with the proposed channel block observed with some GABAergic compounds at high concentrations (Rho et al., 1996; Williams et al., 1997). In the absence of GABA, micromolar concentrations of carisoprodol produced rapid and reversible inward currents that were blocked by picrotoxin, indicating the currents were mediated by $GABA_A$Rs.

As reported previously (Rho et al., 1997), we found the metabolite meprobamate also had direct-gating and allosteric effects at $GABA_A$ receptors. For both actions, our studies demonstrate carisoprodol is more potent and efficacious (up to the 3 mM concentration assessed) than its metabolite. After therapeutic use in humans, the effects of carisoprodol begin within 30 minutes of ingestion, with peak plasma concentrations reaching 4 to 7

μg/mL (Littrell et al., 1993). This translates to a concentration of approximately 27 μM of carisoprodol, suggesting even the therapeutic use of this drug can result in allosteric and direct effects.

Our functional studies performed in vitro strongly suggested carisoprodol has the potential to mediate barbiturate-like effects in vivo. Carisoprodol produced maximal depression of locomotor activity in mice within 8 min when administered via oral or intraperitoneal routes of administration. Behavioral depression elicited by 300 mg/kg carisoprodol followed a relatively short time course, with full recovery after 40 min. This pattern matches that reported for the time-course of its plasma concentrations in mice reported in the literature (Bossoni et al., 1979; Chan, 2000). In a National Toxicology Program study, Chan (2000) reported that a single oral dose of 300 mg/kg carisoprodol yielded a peak plasma concentration of 15.7 μg/mL at 15 min after treatment and a decline to 4.5 μg/mL by 60 min, after which carisoprodol was not detectable. After a 600 mg/kg dose, the plasma concentration peaked and fell to 5 μg/mL within 2 h. Compared to the current studies of locomotor activity employing the same doses, it seems that falling plasma concentrations of carisoprodol closely parallel the offset of behavioral depression.

Hepatic conversion of carisoprodol to meprobamate in mice is relatively rapid (van der Kleijn, 1969), and in the current study, this metabolite administered by itself could indeed elicit behavioral depression with potency comparable to or greater than carisoprodol. However, it seems unlikely that hepatic conversion to meprobamate could fully account for the rapid time course of behavioral depression elicited by carisoprodol.

The accumulation of meprobamate in the brain is markedly slower than carisoprodol (van der Kleijn, 1969), attributable to a greater lipid solubility of carisoprodol. In addition, in accordance with its markedly longer duration of depressant action demonstrated in the current study, the plasma half-life of meprobamate is nearly 8-fold longer than that of carisoprodol (Olsen et al., 1994; Bramness et al., 2004). If depressant effects of carisoprodol are fully attributable to formation of meprobamate, it is not clear why recovery of depression should parallel the disappearance of carisoprodol from plasma, a period when concentrations of meprobamate should persist.

Given that carisoprodol, itself, also has a barbiturate-like action in vitro, it would seem reasonable to consider that carisoprodol, itself, or perhaps a product of carisoprodol and meprobamate, may be largely responsible for the initial behavioral depression after its oral administration. Additional results reported in the literature are consistent with this view. The induction of hepatic microsomal enzymes that increase metabolism of carisoprodol (and presumably accelerate the appearance of meprobamate) has been reported to result in a shortening of the duration of carisoprodol-induced behavioral depression (Kato and Takanaka, 1968). In human studies, high plasma concentrations of carisoprodol, but not meprobamate, have been linked to impaired driving ability (Bramness et al., 2004).

Drug discrimination studies are often used to identify similarities in the stimulus effects of drugs and are likely to indicate a drug's potential for abuse. Thus, we investigated whether the stimulus effects of GABAergic compounds substituted for those of carisoprodol. Meprobamate, pentobarbital, and chlordiazepoxide all substituted for the

discriminative stimulus effects of carisoprodol. These findings indicate carisoprodol produces at least part of its discriminative stimulus effects through actions at $GABA_AR$s and provide further support for the barbiturate-like actions of carisoprodol. It is interesting that we anticipated generalization would occur at a lower dose of pentobarbital (~5 mg/kg); however, we did not observe full substitution in this range. We hypothesize this disparity may be due to cross-tolerance between these two drugs. Although these studies are promising, they do not allow us to definitively conclude the generalization of pentobarbital is due to carisoprodol-mediated events rather than metabolism of carisoprodol to meprobamate. In addition, non-GABAergic compounds have not been tested in the carisoprodol-trained rats, so it is possible other receptors may also contribute to the mechanism of action of carisoprodol. For example, meprobamate has been shown to inhibit NMDA-activated currents (Rho et al., 1997), and carisoprodol toxicity has been described as having characteristics similar to those of serotonin syndrome (Bramness et al., 2005).

Although subjects trained to discriminate barbiturates do not generally distinguish between benzodiazepines and barbiturates in substitution studies (Ator and Griffiths, 1989; De Vry and Slangen, 1986; Woolverton and Nader, 1995), antagonists relatively selective for these sites will selectively block the discriminative stimulus effects of test compounds. That is, bemegride, a barbiturate antagonist, blocks the discriminative stimulus effects of pentobarbital but not benzodiazepines, whereas flumazenil, a benzodiazepine site antagonist, blocks the discriminative stimulus effects of benzodiazepines but not pentobarbital (De Vry and Slangen, 1986; Herling and Shannon,

1982; Schechter, 1984). Furthermore, the antagonists also block cross-substitution; for example, bemegride blocks both the discriminative stimulus effects of pentobarbital and the ability of pentobarbital to substitute for chlordiazepoxide (Schechter, 1984). This is important because it provides a method for distinguishing the effects of benzodiazepines and barbiturates in rats trained to discriminate pentobarbital.

In the present study, bemegride fully antagonized the discriminative stimulus effects of carisoprodol whereas flumazenil failed to produce a consistent, dose-dependent blockade. These findings are in agreement with the electrophysiological studies and suggest the behavioral effects of carisoprodol are barbiturate-like, but not benzodiazepine-like. It is important to note that Roberge et al. (2000) reported the use of flumazenil to reverse carisoprodol intoxication. Flumazenil was considered a benzodiazepine-specific antagonist known to block the actions of benzodiazepines at $GABA_A$Rs. This seems contradictory to our findings; however, it has also been demonstrated that this drug can reverse the actions of non-benzodiazepine drugs such as ethanol, tetrahydrocannabinol, and meprobamate in vivo (Roberge et al., 2000).

Although both our in vitro and in vivo studies are consistent with barbiturate-like effects of carisoprodol, we are not concluding that carisoprodol is acting at the barbiturate site of the receptor. As noted, other $GABA_A$R modulators, such as the lactones, have the ability to allosterically modulate, directly gate, and antagonize the receptor (Williams et al., 1997; Gonzales et al., 2004). In addition, we found that homomeric ρ1 GABA receptors and homomeric α1 glycine receptors, which are insensitive to barbiturates, are also insensitive to carisoprodol. However, although we confirmed the W328M mutation

in ρ1 GABA receptors confers sensitivity to barbiturates (Amin, 1999), this mutation did not confer sensitivity to carisoprodol. Thus, although the characteristics of carisoprodol can be described as barbiturate-like at the receptor and whole-animal levels, the distinct effects of the two ligands on the mutant receptor suggest the functional domain(s) for these ligands are distinct.

In recent years, there has been increasing concern regarding carisoprodol's potential as a drug of abuse. In light of our findings, it seems highly likely that the barbiturate-like activity of carisoprodol may underlie its capacity to enhance the sedative effects of CNS depressants, contributing to its potential for abuse. In our hands, carisoprodol was more potent and efficacious than its metabolite, suggesting carisoprodol is equally as dangerous as meprobamate. Thus, the question remains: Why is carisoprodol, the parent drug, a noncontrolled substance? Despite its emerging role as a drug of abuse, there is currently no standard treatment for carisoprodol dependence and withdrawal. Given the present and potential dangers posed by carisoprodol abuse, it is of crucial importance to determine the mechanism of action of this drug. This knowledge may provide insight into effectively treating carisoprodol tolerance, dependence, and withdrawal

Table II-1. ED_{50} values of substitution for carisoprodol. Intraperitoneal injections of pentobarbital (1, 5, 10, 25 mg/kg), meprobamate (10, 25, 50, 100, 175 mg/kg), and chlordiazepoxide (1, 2.5, 5, 10 mg/kg) each produced dose-dependent substitution for the discriminative stimulus effects of carisoprodol. ED_{50} values and confidence intervals for each compound are listed in Table 1.

Compound	ED_{50} (mg/kg)	95% Confidence Interval
Carisoprodol	46.71	34.08 - 59.34
Meprobamate	60.08	22.36 – 97.80
Pentobarbital	4.46	2.39 – 6.53
Chlordiazepoxide	3.32	2.41 – 4.23

Figure II-1. Potentiation of GABA-gated currents by carisoprodol and meprobamate. A, representative traces demonstrating potentiation of GABA-gated currents by carisoprodol in a concentration-dependent manner. At millimolar concentrations, offshoot currents were observed upon termination of drug application, and currents were inhibited at carisoprodol concentrations above 1 mM. B, concentration-response curve for the allosteric effects of carisoprodol on GABA-gated currents. Relatively large variance at high concentrations is due to onset of inhibition of some cells. C, representative traces demonstrating potentiation of GABA-gated currents by meprobamate. D, concentration-response curve for the allosteric effects of meprobamate on GABA-gated currents. For both data sets, each point represents the mean ± S.E. of data collected from three to 17 cells.

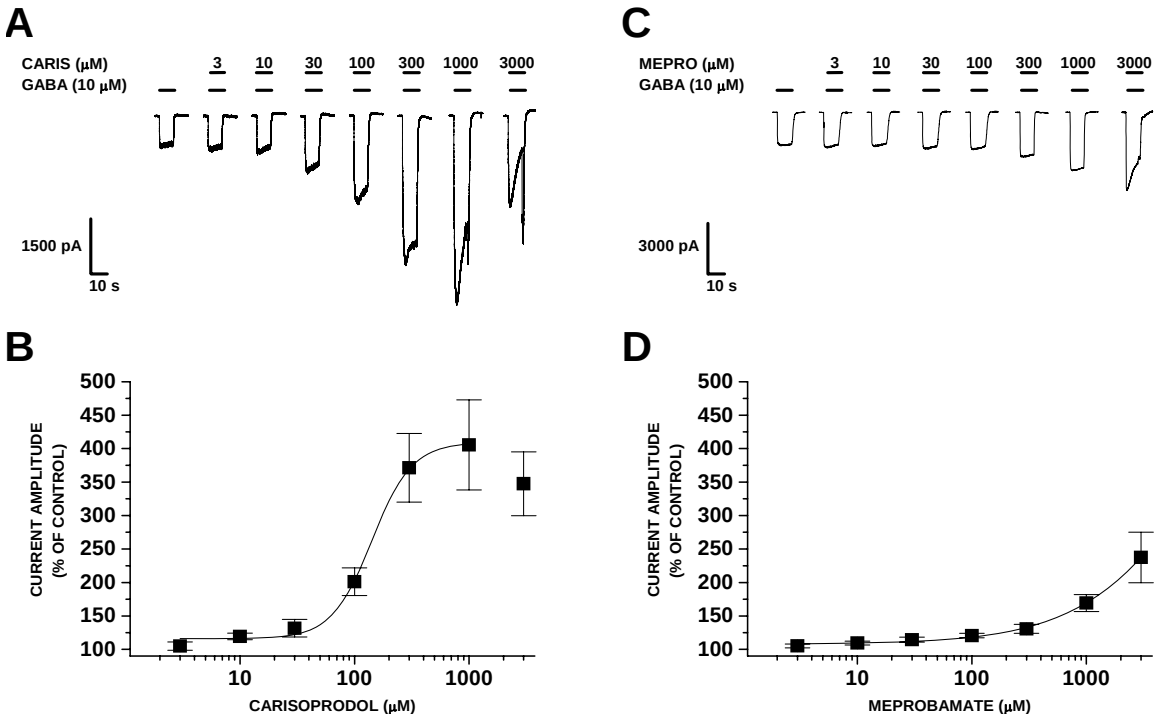

A
CARIS (μM)
3
10
30
100
300
1000
3000
GABA (10 μM)
1500 pA
10 s
C
MEPRO (μM)
3
10
30
100
300
1000
3000
GABA (10 μM)
3000 pA
10 s
B
CURRENT AMPLITUDE
(% OF CONTROL)
500
450
400
350
300
250
200
150
100
10
100
1000
CARISOPRODOL (μM)
D
CURRENT AMPLITUDE
(% OF CONTROL)
500
450
400
350
300
250
200
150
100
10
100
1000
MEPROBAMATE (μM)

Figure II-2. Direct activation of $GABA_A$ receptors by carisoprodol and meprobamate. A, representative traces demonstrating inward currents evoked by carisoprodol and meprobamate in the absence of GABA. B, current amplitude of carisoprodol- or meprobamate-evoked currents relative to currents evoked by 10 μM GABA. Each bar represents the mean ± S.E. of a minimum of three cells; ***, significant difference relative to an equal concentration of meprobamate ($p < 0.001$). C, representative traces of carisoprodol-mediated currents in the presence of various concentrations of PTX. Current amplitude was measured at the end of the 10 second co-application period. D, summary of results illustrated in C; carisoprodol-activated currents were reduced to 45.7 ± 2.5 % and 16.6 ± 4.3% of control in the presence of 30 and 100 μM PTX, respectively. Recovery from PTX was 78.7 ± 9.0% of control (not shown). Antagonism by PTX suggests the carisoprodol-activated current was conducted via $GABA_A$ receptors. Each bar represents the mean ± S.E. of four cells.

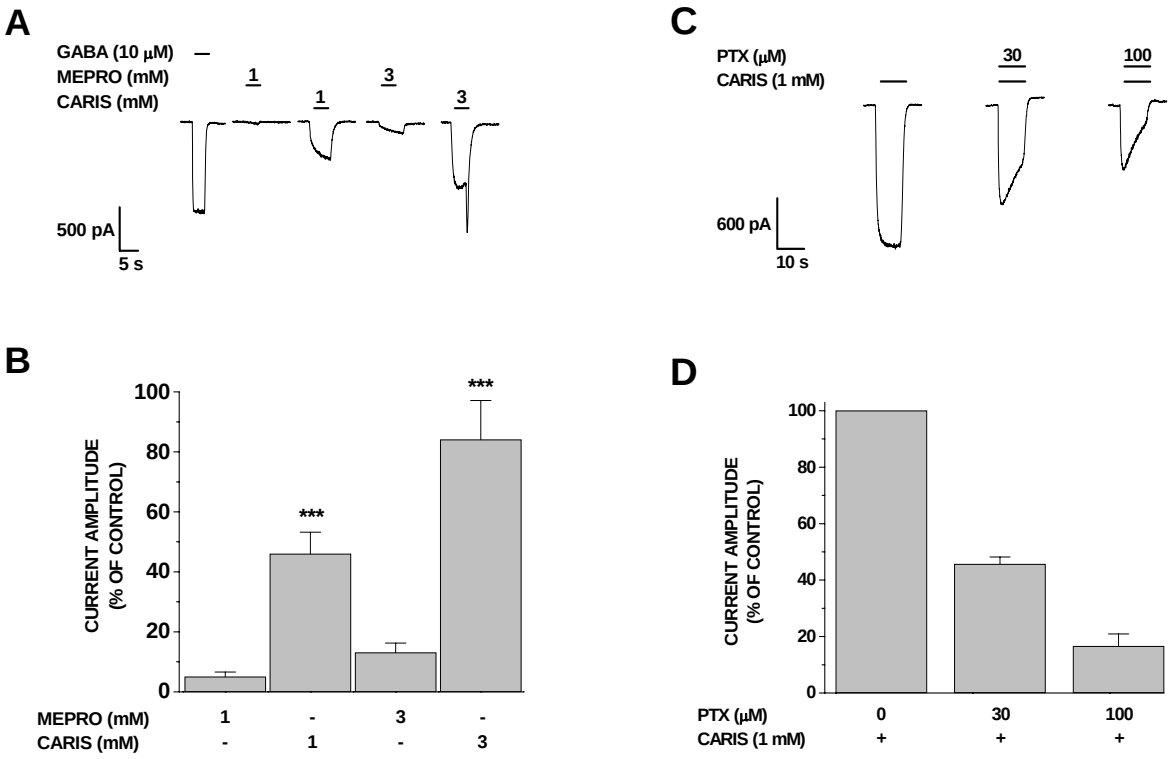

A
GABA (10 μM)
MEPRO (mM)
CARIS (mM)
1
1
3
3
500 pA
5 s
C
PTX (μM)
CARIS (1 mM)
30
100
600 pA
10 s
B
CURRENT AMPLITUDE
(% OF CONTROL)
100
80
60
40
20
0
***
***
MEPRO (mM)
CARIS (mM)
1 - 3 -
- 1 - 3
D
CURRENT AMPLITUDE
(% OF CONTROL)
100
80
60
40
20
0
PTX (μM)
CARIS (1 mM)
0 30 100
+ + +

Figure II-3. Effects of flumazenil on carisoprodol activity at $GABA_A$ receptors. A, representative traces demonstrating carisoprodol-mediated currents and the potentiation of GABA-gated currents by diazepam and carisoprodol in the presence and absence of flumazenil. B, coapplication of diazepam potentiated GABA-gated currents to 238.3 ± 28.7% of control values. The actions of diazepam were blocked by a saturating concentration of flumazenil. Coapplication with carisoprodol potentiated GABA-gated currents to 210.7 ± 14.9% of control values. The allosteric actions of carisoprodol were not significantly different in the presence of flumazenil (218.6 ± 17.1% of control). Likewise, carisoprodol-mediated currents were 113.8 ± 4.9% of control values in the presence of flumazenil (N.S., $p > 0.05$). Each bar represents the mean ± S.E. of data collected from four cells.

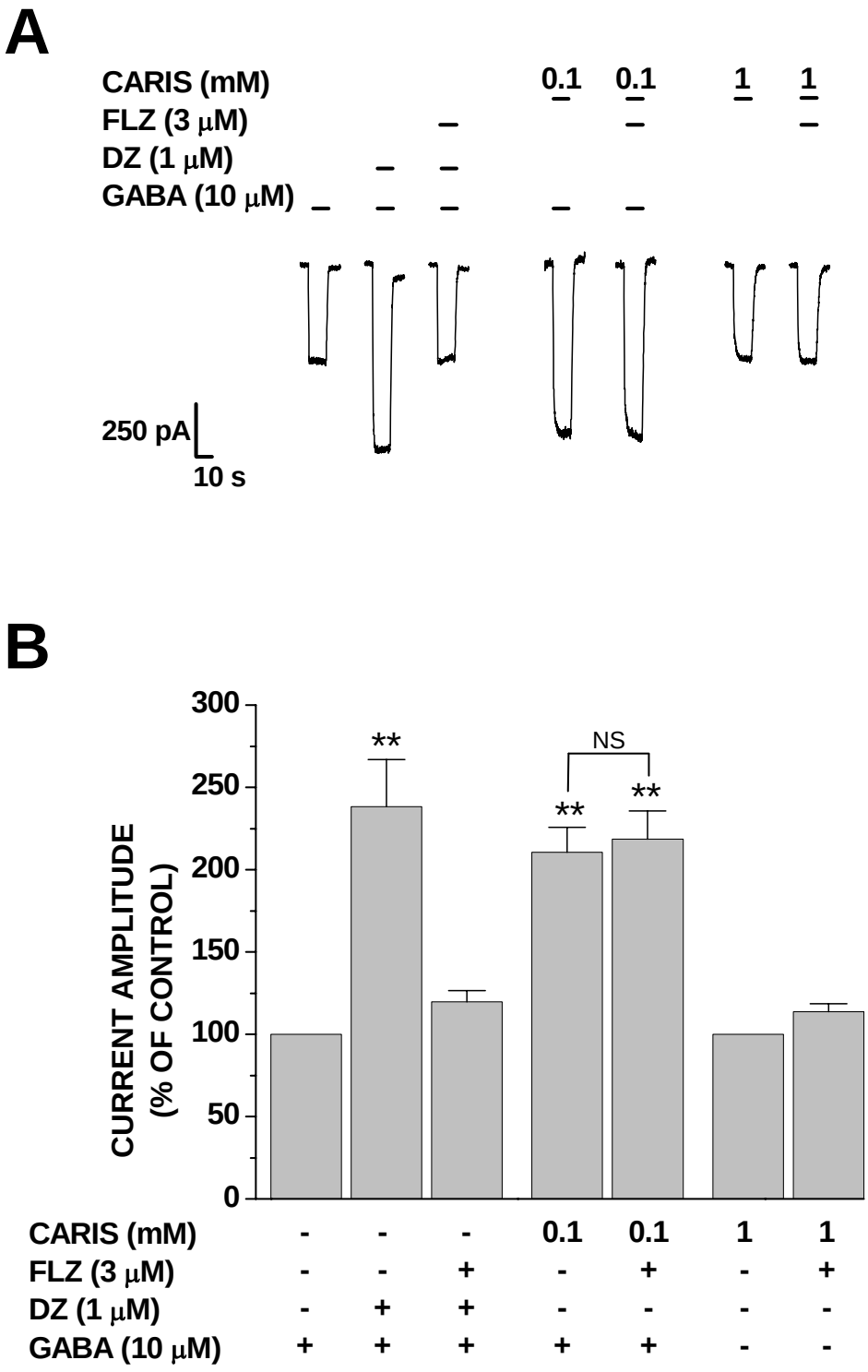

A
CARIS (mM)
0.1
0.1
1
1
FLZ (3 μM)
DZ (1 μM)
GABA (10 μM)
250 pA
10 s
B
CURRENT AMPLITUDE
(% OF CONTROL)
300
250
200
150
100
50
0
**
**
**
NS
CARIS (mM) - - - 0.1 0.1 1 1
FLZ (3 μM) - - + - + - +
DZ (1 μM) - + + - - - -
GABA (10 μM) + + + + + - -

Figure II-4. Antagonism of carisoprodol-mediated currents by bemegride. A, representative traces demonstrating carisoprodol-activated currents are reduced following bemegride incubation. These experiments were conducted using the stable human $\alpha 1\beta 2\gamma 2$ cell line. B, subsequent to incubation with bemegride for 2 min, peak current amplitude was reduced to 24.4 ± 5.0% of control ($n = 4$; *, $p < 0.001$). Recovery was not significantly different from control ($p > 0.05$).

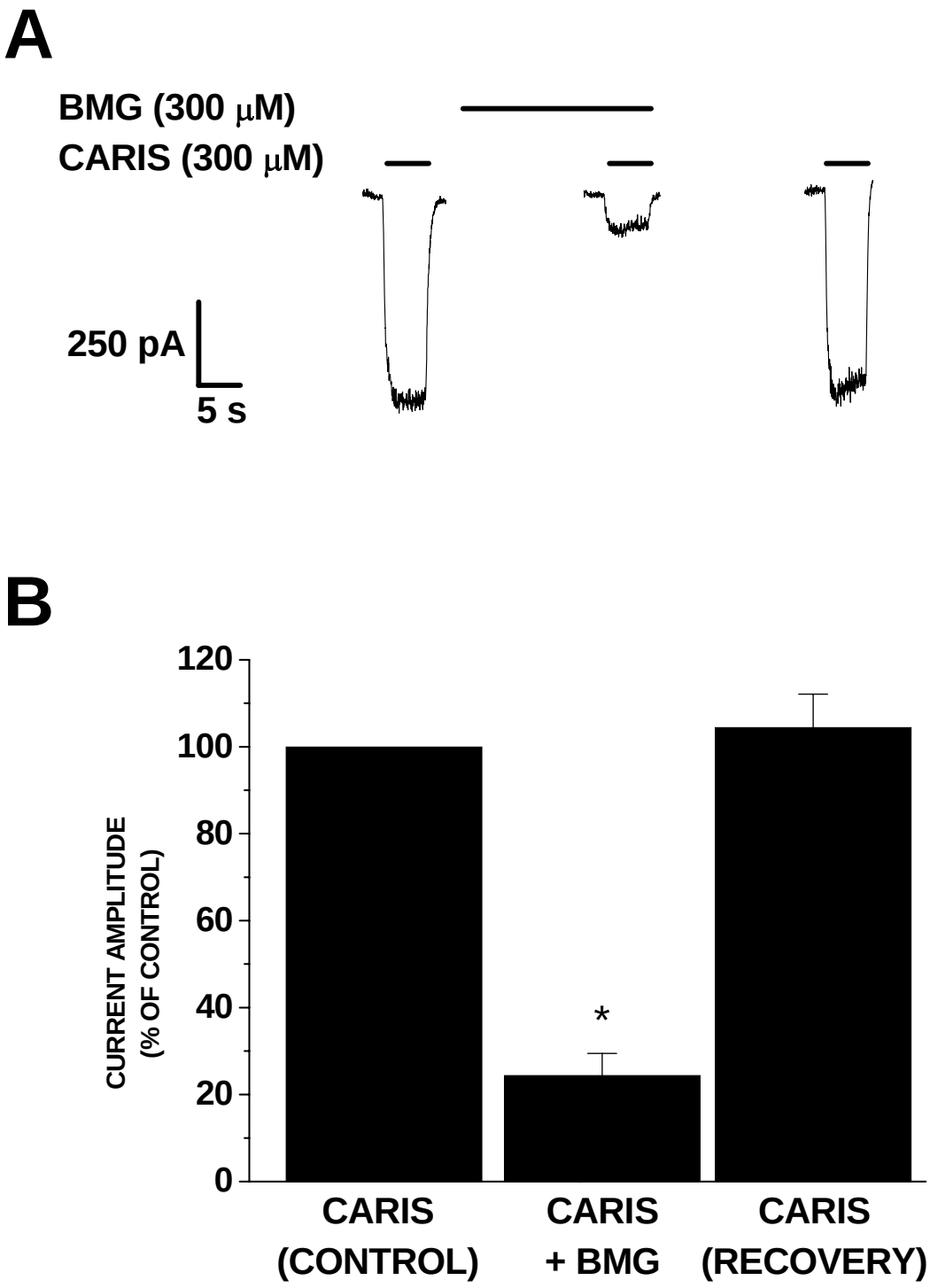

A
BMG (300 μM)
CARIS (300 μM)
250 pA
5 s
B
CURRENT AMPLITUDE
(% OF CONTROL)
120
100
80
60
40
20
0
*
CARIS
(CONTROL)
CARIS
+ BMG
CARIS
(RECOVERY)

Figure II-5. Effects of carisoprodol on homomeric ρ1 GABA and homomeric α1 glycine receptors. A, representative traces demonstrating inward currents evoked by GABA alone or in the presence of 300 μM carisoprodol. B, summary graph of experiments described in A. Peak amplitude of GABA-gated current in ρ1 receptors was unaffected by 300 μM carisoprodol. Each bar represents the mean ± S.E. of three cells tested. C, representative traces demonstrating inward currents evoked by glycine in the absence or presence of carisoprodol. D, summary concentration-response curve of studies depicted in C. Carisoprodol did not have a stimulatory effect on glycine-gated currents, whereas modest inhibitory effects were observed at 1 mM carisoprodol. Each data point represents the mean ± S.E. of a minimum of three cells tested.

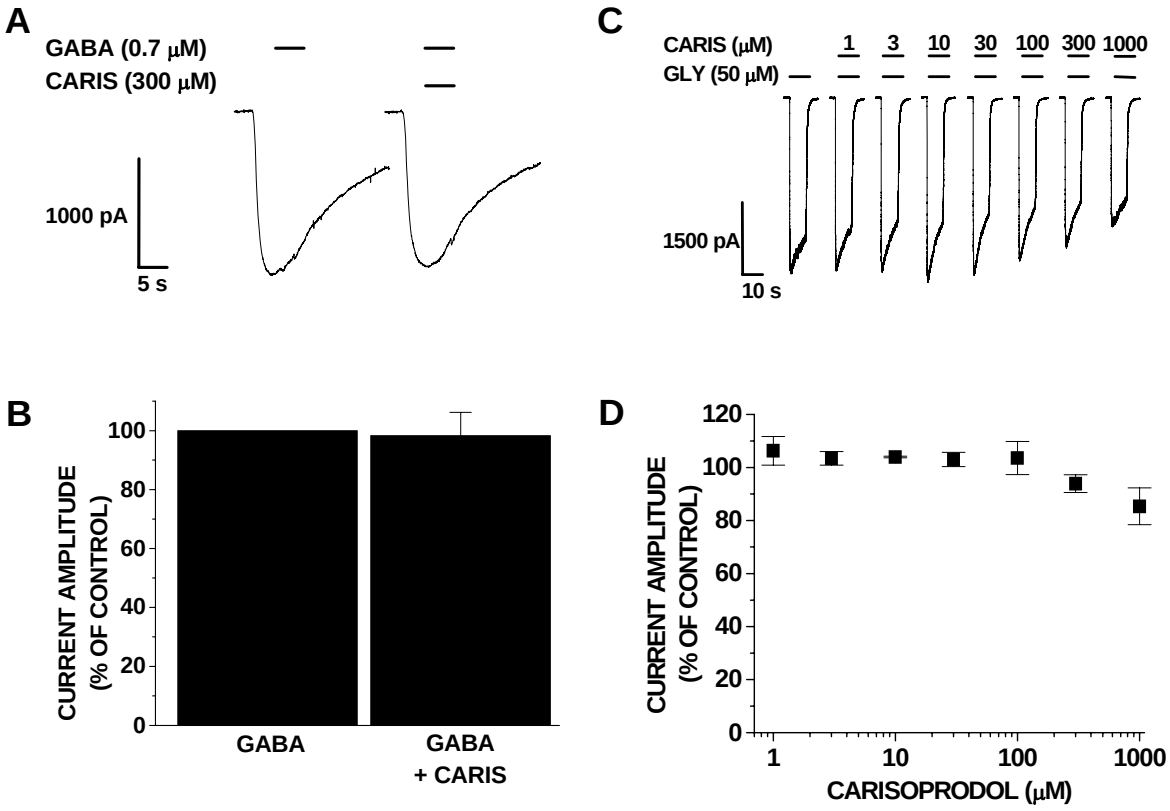
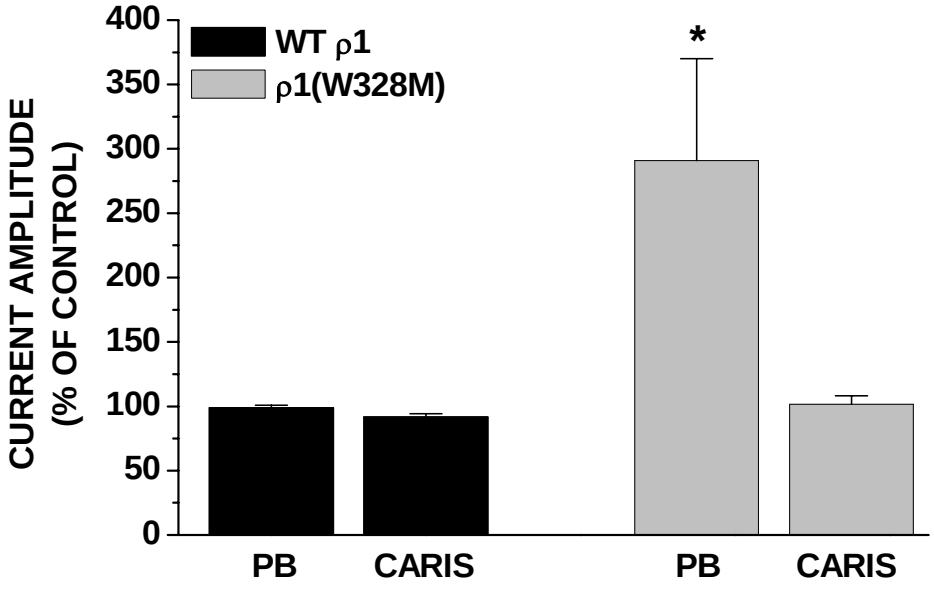

A
GABA (0.7 μM)
CARIS (300 μM)
1000 pA
5 s
C
CARIS (μM)
1
3
10
30
100
300
1000
GLY (50 μM)
1500 pA
10 s
B
CURRENT AMPLITUDE
(% OF CONTROL)
100
80
60
40
20
0
GABA
GABA
+ CARIS
D
CURRENT AMPLITUDE
(% OF CONTROL)
120
100
80
60
40
20
0
1
10
100
1000
CARISOPRODOL (μM)

Figure II-6. W328M confers sensitivity to pentobarbital but not carisoprodol. A, in wild-type homomeric ρ1 receptors, neither pentobarbital (300 μM) nor carisoprodol (1 mM) enhanced GABA (EC_{20})-gated current. In W328M mutant receptors, pentobarbital, but not carisoprodol, could enhance GABA-activated currents. The GABA EC_{20} is denoted as control current amplitude. B, similar phenomenon existed with regard to direct-gating effects. In the W328M mutant receptors, pentobarbital could directly gate the channel to approximately 15% of the maximal current amplitude gated by GABA. In contrast, carisoprodol was ineffective in direct gating in either wild-type or W328M mutant receptors. Each data point represents the mean ± S.E. of four cells. Maximal GABA-gated current is denoted as 100%. *, significantly different from the wild-type response ($p < 0.05$).

A


400
350
300
250
200
150
100
50
0
CURRENT AMPLITUDE
(% OF CONTROL)
WT ρ1
ρ1(W328M)
*
PB
CARIS
PB
CARIS

B

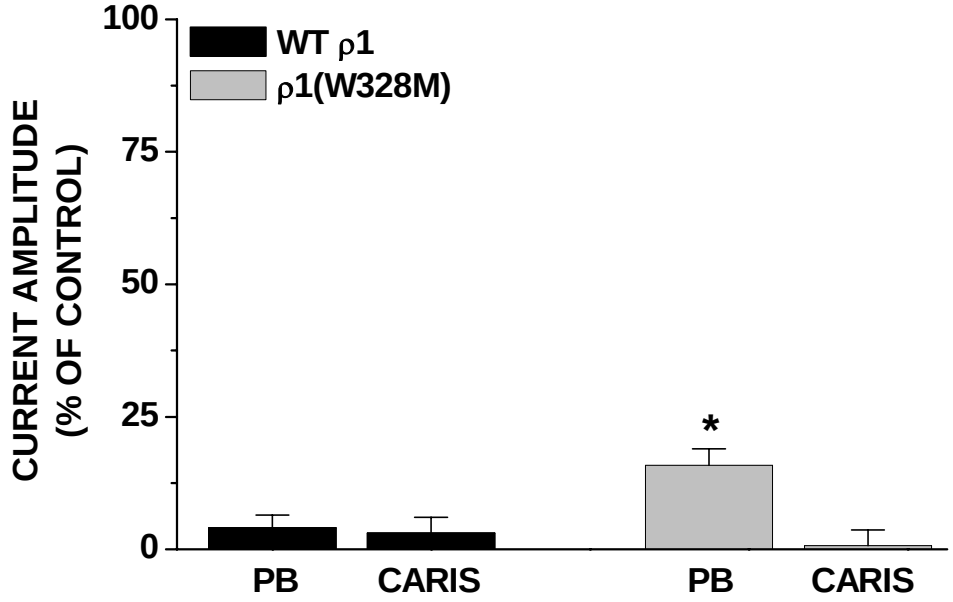

100
75
50
25
0
CURRENT AMPLITUDE
(% OF CONTROL)
WT ρ1
ρ1(W328M)
*
PB
CARIS
PB
CARIS

Figure II-7. Time course of carisoprodol- and meprobamate-induced locomotor depression. Each panel shows mean ambulation counts per 10-min interval for one dose of the test compound in comparison to the saline control in mice. Treatment with carisoprodol (left, $n = 16$) resulted in depression of locomotor activity after 300 or 560 mg/kg p.o. Maximal depressant effects for these doses were evident after 10 min and ended 40 or 110 min, respectively, following administration. Treatment with meprobamate (right, $n = 8$) resulted in dose-dependent depression of locomotor activity after 100 or 300 mg/kg i.p. Depressant effects of these doses were evident within 10 min and ended 50 or 150 min, respectively, following injection. *, doses significantly different ($p < 0.05$) from vehicle for the period 10 to 20 min after injection.

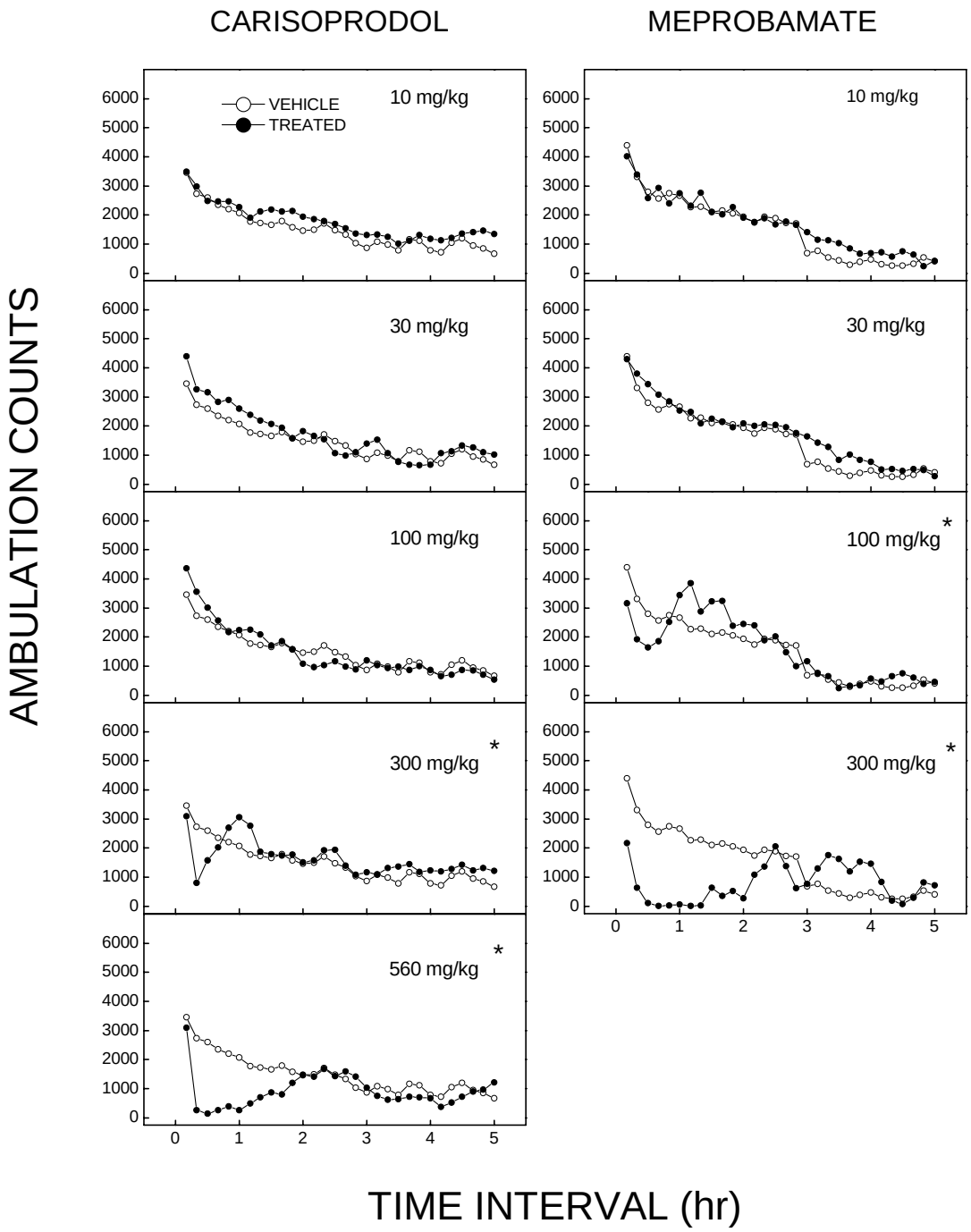

CARISOPRODOL
MEPROBAMATE
AMBULATION COUNTS
TIME INTERVAL (hr)
VEHICLE
TREATED
10 mg/kg
30 mg/kg
100 mg/kg
300 mg/kg
560 mg/kg
*

Figure II-8. Rate of onset for behavioral depression following carisoprodol or meprobamate. Mean ambulation counts as a function of 4-min time periods for separate groups of eight mice receiving carisoprodol orally, carisoprodol intraperitoneally, meprobamate intraperitoneally, or the vehicle administered intraperitoneally or orally. The dose administered to each drug group was 300 mg/kg. No difference in the rate of onset of behavioral depression was evident after any of the drug treatments.

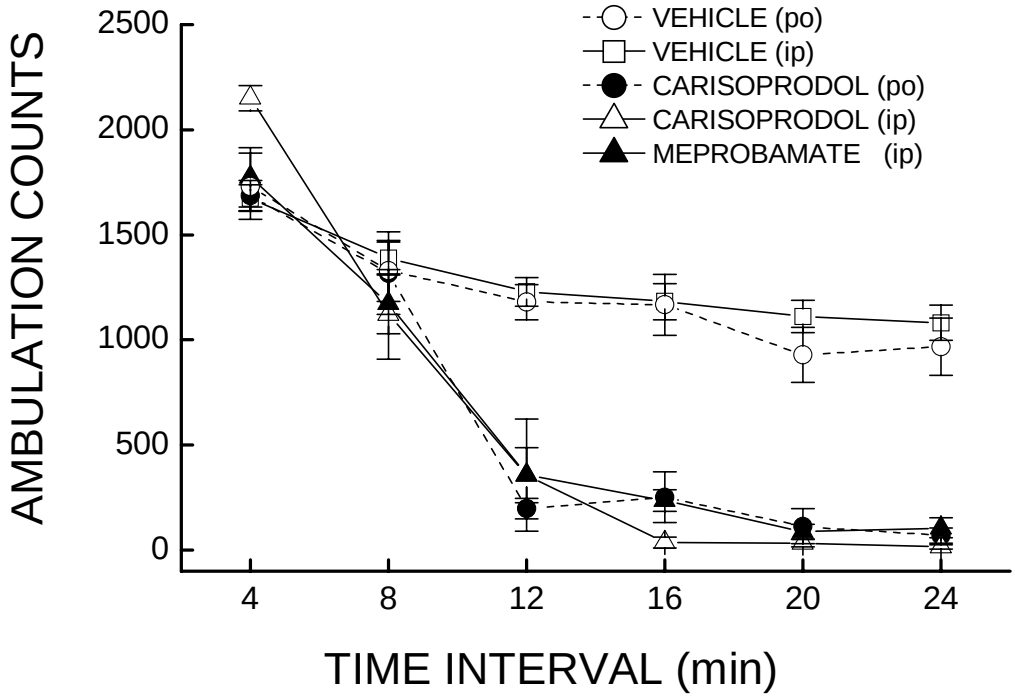

AMBULATION COUNTS
2500
2000
1500
1000
500
0
4
8
12
16
20
24
TIME INTERVAL (min)
VEHICLE (po)
VEHICLE (ip)
CARISOPRODOL (po)
CARISOPRODOL (ip)
MEPROBAMATE (ip)

Figure II-9. Substitution for the discriminative stimulus effects of carisoprodol. Top, percentage of total responses made on the carisoprodol-appropriate lever. Bottom, rate of responding in responses per second (r/s). Carisoprodol, pentobarbital, meprobamate, and chlordiazepoxide fully substituted for the discriminative stimulus effects of 100 mg/kg carisoprodol. Carisoprodol produced a modest increase in response rate, whereas chlordiazepoxide produced no effect, and pentobarbital markedly reduced rates at the highest doses tested (n = 10 rats).

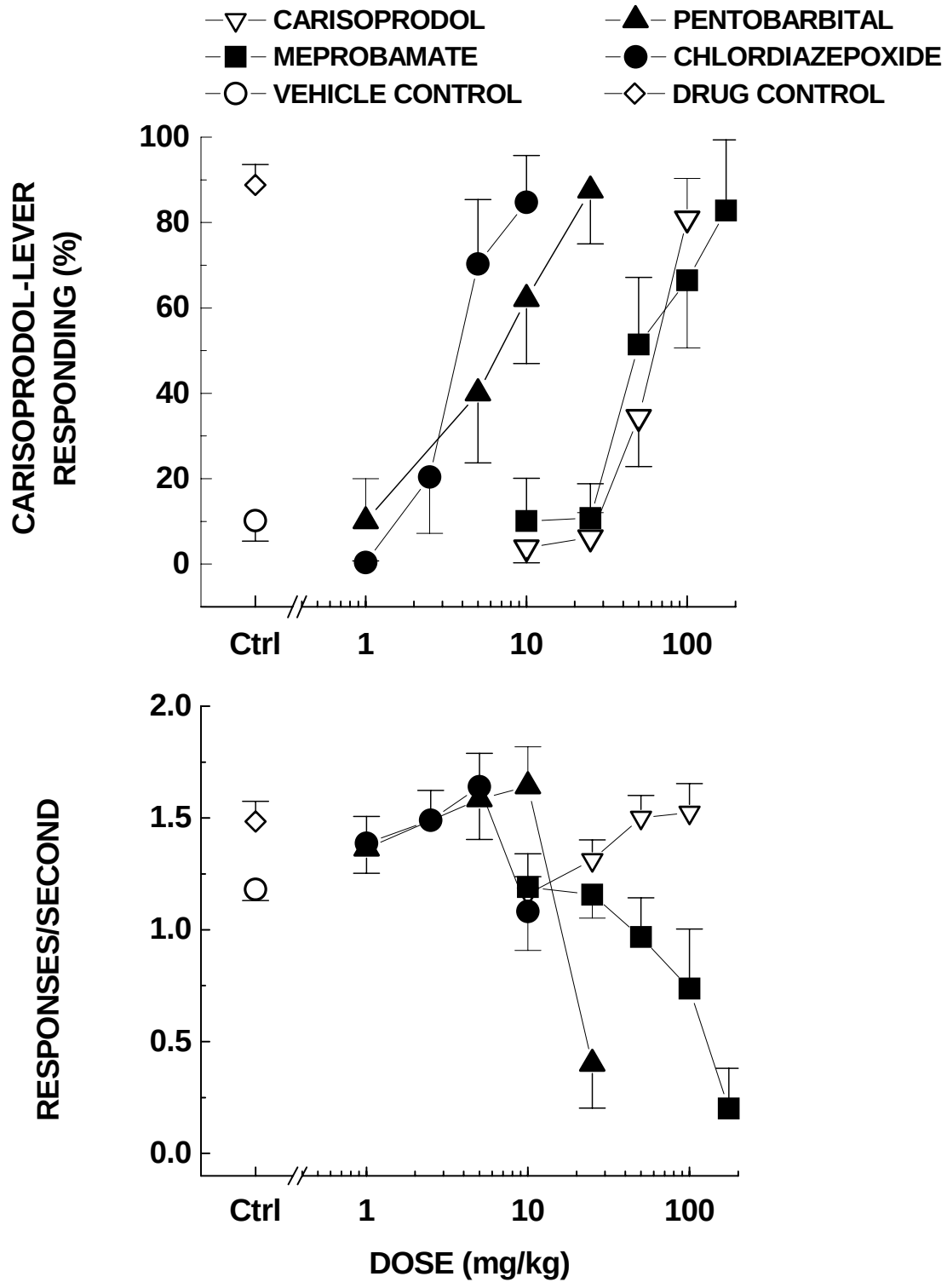
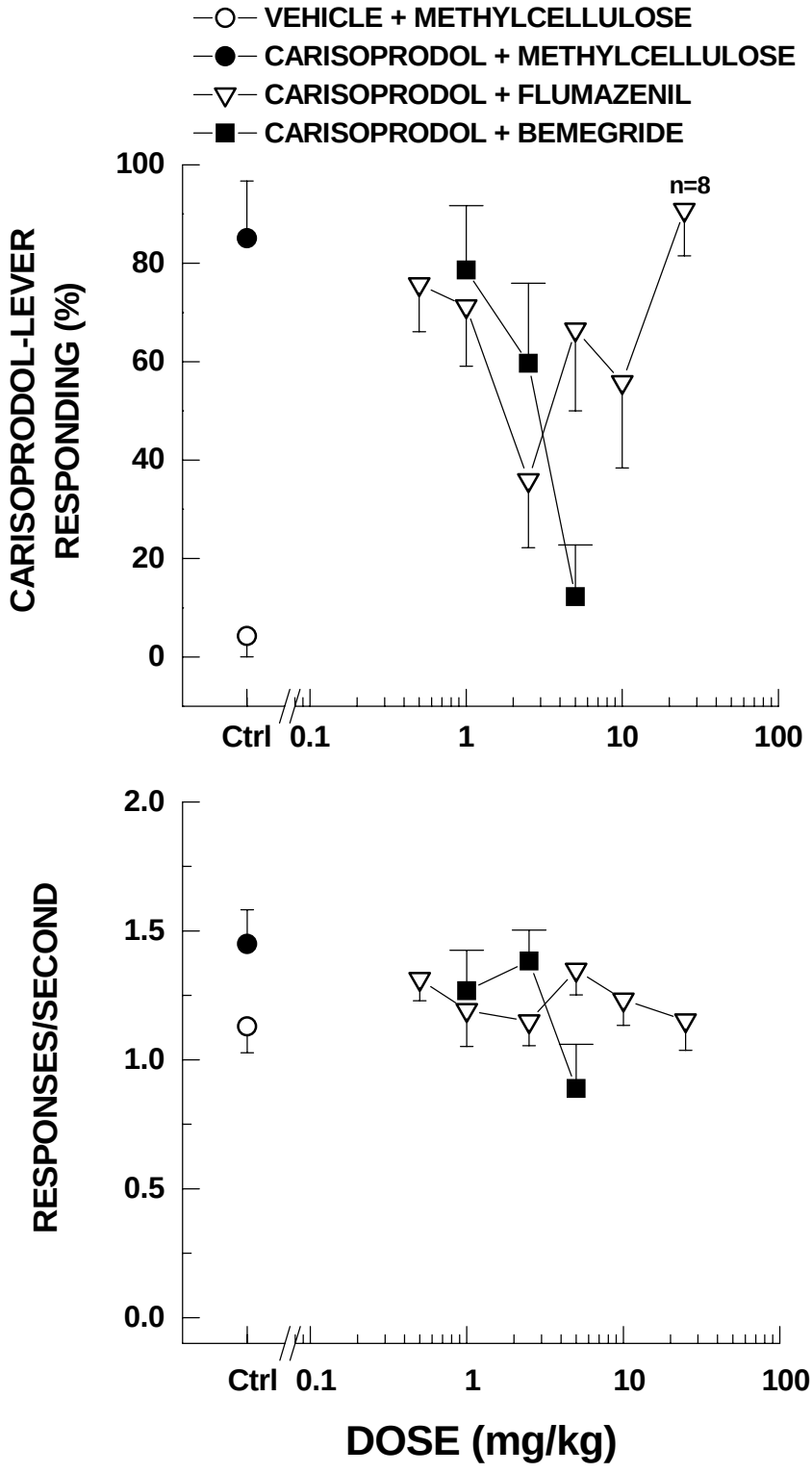

CARISOPRODOL
PENTOBARBITAL
MEPROBAMATE
CHLORDIAZEPOXIDE
VEHICLE CONTROL
DRUG CONTROL
CARISOPRODOL-LEVER RESPONDING (%)
100
80
60
40
20
0
Ctrl
1
10
100
RESPONSES/SECOND
2.0
1.5
1.0
0.5
0.0
Ctrl
1
10
100
DOSE (mg/kg)

Figure II-10. Blockade of the discriminative stimulus effects of the training dose of carisoprodol (100 mg/kg p.o.). Top, percentage carisoprodol-lever responding. Bottom, rate of responding (r/s). The barbiturate antagonist bemegride fully antagonized the discriminative stimulus effects of carisoprodol, whereas the benzodiazepine antagonist flumazenil had little or no effect. Response rates were not significantly affected by either compound. (n = 10 rats for bemegride and n = 9 for flumazenil, except where shown).


VEHICLE + METHYLCELLULOSE
CARISOPRODOL + METHYLCELLULOSE
CARISOPRODOL + FLUMAZENIL
CARISOPRODOL + BEMEGRIDE
CARISOPRODOL-LEVER RESPONDING (%)
n=8
100
80
60
40
20
0
Ctrl
0.1
1
10
100
RESPONSES/SECOND
2.0
1.5
1.0
0.5
0.0
DOSE (mg/kg)

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As demonstrated in the previous chapter, carisoprodol has the capacity to allosterically modulate and directly gate $GABA_A$ receptors ($GABA_ARs$). Its effects in vitro and in vivo cannot be fully explained by its metabolism to meprobamate, suggesting the actions of carisoprodol, itself, are pharmacologically and physiologically relevant in their own right. To this point, our studies focused on the most physiologically abundant $GABA_AR$ configuration, $\alpha1\beta2\gamma2$. However, subunit diversity allows for a wealth of possible receptor configurations, potentially contributing to the overall effects of carisoprodol. In addition, while the interaction of carisoprodol with $GABA_ARs$ is evident in the previous studies, the site(s) of action for the drug on the receptor remain unclear. To address these issues, subunit-dependence and potential sites of action for carisoprodol were investigated and will be discussed in the following chapter.

CHAPTER III

SUBUNIT-DEPENDENT ACTIVITY OF CARISOPRODOL AT $GABA_A$ RECEPTORS

Lorie A. González, Cathy L. Bell-Horner, and Glenn H. Dillon

ABSTRACT

Carisoprodol is a centrally-acting muscle relaxant with well-documented abuse potential. Its sedative effects, which underlie its therapeutic and recreational use, are attributed to interaction of its primary metabolite, meprobamate, with $GABA_A$ receptors ($GABA_ARs$). Previously, we demonstrated carisoprodol, itself, directly activates and allosterically modulates human $\alpha1\beta2\gamma2$ $GABA_ARs$ via sites distinct from barbiturate and benzodiazepine sites of the receptor. In this study, we examine if carisoprodol preferentially interacts with specific subunit configurations of $GABA_ARs$, and we identify domains of the $GABA_AR$ $\alpha1$ subunit involved in mediating carisoprodol's actions. Whole-cell patch clamp recordings were obtained from HEK293 cells expressing human $\alpha1\beta2$ and $\alpha x\beta z\gamma2$ (where x = 1-4 and z = 1-2) $GABA_ARs$. Potentiation of GABA-gated currents was observed for all configurations with carisoprodol being more efficacious at $\alpha1\beta2\gamma2$ receptors; potency was not subunit-dependent. The rank order of efficacy for direct activation by carisoprodol was $\alpha1\beta1\gamma2 > \alpha1\beta2 = \alpha1\beta2\gamma2 =$

$\alpha2\beta2\gamma2 = \alpha4\beta2\gamma2 >> \alpha3\beta2\gamma2$. To identify domains of the α subunit involved in mediating carisoprodol activity, we generated a chimeric subunit using carisoprodol-insensitive GABA $\rho1$ and carisoprodol-sensitive GABA $\alpha1$ subunits. Chimeric subunits retained insensitivity to direct activation by carisoprodol, but gained sensitivity to the modulatory effects of carisoprodol. Our findings indicate carisoprodol modulates $GABA_ARs$ in a subunit-dependent manner, with α and β subunits contributing to the pharmacological profile of carisoprodol and possibly its abuse potential. Partial restoration of the modulatory, but not the direct gating effect of carisoprodol suggests this drug may mediate its effects via multiple sites on $GABA_ARs$.

INTRODUCTION

γ-Aminobutyric acid type A receptors ($GABA_ARs$) are ion channel-coupled, multi-subunit proteins that serve as the primary mediators of inhibitory neurotransmission in the adult central nervous system (CNS). Functional receptors are composed of individual subunits arranged in a pentameric manner. In mammals, the various subunits and their isoforms have been divided into the following classes: α(1-6), β(1-3), γ(1-3), ρ, δ, ε, π, and θ (Huang et al., 2006). Subunit architecture is highly conserved among $GABA_ARs$ with each subunit composed of an extracellular amino-terminal, four transmembrane (TM) domains, a large intracellular loop, and an extracellular carboxyl-terminal. Subunit composition determines channel conductance, kinetics, and gating properties of the receptor (Verdoorn et al., 1990; Mathers, 1991) in addition to its pharmacological profile (Sigel et al., 1990). Given their vital role in maintaining inhibitory tone in the CNS, $GABA_ARs$ are the targets of several clinically relevant compounds. These compounds include benzodiazepines, barbiturates, general and inhalational anesthetics, and certain centrally-acting muscle relaxants.

Carisoprodol is a centrally-acting muscle relaxant indicated in the alleviation of acute musculoskeletal conditions. However, recreational use of carisoprodol is an increasing problem. The dangers associated with carisoprodol abuse, including severe withdrawal leading to seizures and death, are well-documented (Adams et al., 1975; Elder, 1991; Littrell et al., 1993; Rust et al., 1993; Reeves and Parker, 2003). Its illicit

effects are generally attributed to the actions of its primary metabolite, meprobamate—a controlled substance with barbiturate-like activity at $GABA_A$Rs (Rho et al., 1997). While conversion to meprobamate likely contributes to the therapeutic and illicit effects of carisoprodol, the pharmacological and physiological profiles of carisoprodol are not entirely consistent with that of its metabolite, suggesting carisoprodol may have effects independent of meprobamate.

In addition, we previously demonstrated carisoprodol allosterically modulates and directly activates human $\alpha1\beta2\gamma2$ $GABA_A$Rs, and its actions are not mediated via reported sites of action for benzodiazepines or barbiturates (Gonzalez et al., 2009). Although receptors of $\alpha1\beta2\gamma2$ subunit composition are the prevalent configuration in the brain, a vast array of $GABA_A$R configurations have been shown to exist throughout the CNS, with each configuration contributing to specific physiological and pharmacological responses (Olsen and Sieghart, 2008). To gain a better understanding of the pharmacological profile of carisoprodol at $GABA_A$Rs, we assessed potential subunit-dependent interactions of the effects of carisoprodol at these receptors with the overall goal of identifying critical domains involved in mediating the drug's effects. Despite having structural differences, other compounds with allosteric and agonistic actions at $GABA_A$Rs exert their effects via common regions of the receptor. Most notably, amino acids within the transmembrane domains—specifically TM2 and TM3—of the α and β subunits have been implicated in binding of or gating by these compounds (Korpi et al., 2002). Given the similarities in the actions of these compounds and carisoprodol, we assessed whether the transmembrane domains play an equally significant role in

mediating the effects of carisoprodol. As demonstrated in the present study, carisoprodol acts in a subunit-dependent manner at $GABA_A$Rs with potential sites of action located in the transmembrane domains of the α subunit.

MATERIALS AND METHODS

Cell Culture and Transfection. Both stably- and transiently-transfected cells were used in the present study. Human embryonic kidney 293 (HEK293) cells were transiently-transfected with human $GABA_A$ α1, α3, and α4; human β1-2; and human γ2s (short isoform) cDNA in a 1:1:5 ratio using *Trans*IT®-293 (Mirus Bio, Madison, WI) 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 (Weill Cornell Medical College). HEK293 stably expressing human α1β2γ2 or α2β2γ2 $GABA_A$Rs were also used. A complete description of the preparation and maintenance of these stable cell lines has been published previously (Hawkinson et al., 1996). Cells were plated on glass coverslips coated with poly-L-lysine in 35-mm culture dishes. Cells were incubated and maintained at 37°C in a humidified incubator with an atmosphere of 5% CO_2.

Subcloning of the Human $GABA_A$ α1 Subunit. The human $GABA_A$ α1 subunit (pCIS2) was subcloned into the vector pcDNA3.1/V5-His C (Invitrogen, Carlsbad, CA) using *NotI* and *XhoI* restriction sites. Briefly, the following primers were synthesized and used to introduce a new and silence an existing XhoI site, respectively:

1) 5'-GATCCCCGGGGGCTCGAGCGCGAATTAAC-3', corresponding to bases 2418-2446 in the noncoding region of the human $GABA_A$ α1 cDNA sequence and

2) 5'-GGTTCTATCGATTCTAGACCCGAGGTCCGCG-3', corresponding to bases 906-936 in the polylinker region of the pCIS2 vector.

The point mutations were introduced with the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Incorporation of each mutation was verified by DNA sequencing. Human $GABA_A$ α1 cDNA was amplified with the following primers:

3) 5'-GAGGTCCGCGGCCGCGTTCGC-3', corresponding to bases 927-944 of the pCIS2 vector and bases 1-3 of the human $GABA_A$ α1 cDNA sequence and

4) 5'-GTTAATTCGCGCTCGAGCCCCCGG-3', corresponding to bases 1473-1493 of human $GABA_A$ α1 cDNA sequence and bases 945-947 of the pCIS2 vector.

The $GABA_A$ α1 amplification product was isolated via agarose gel electrophoresis and eluted from the gel using Freeze 'N Squeeze™ DNA Gel Extraction Spin Columns (Bio-Rad®, Hercules, CA) according to the manufacturer's specifications. The eluted DNA was purified and concentrated via ethanol precipitation. Purified DNA was digested with *NotI* and *XhoI*. The fragment of interest was isolated via agarose gel electrophoresis and eluted from the gel as described previously.

The pcDNA3.1/V5-His C vector was digested with *NotI* and *XhoI* and subsequently dephosphorylated with calf intestinal alkaline phosphatase (Invitrogen™, Carlsbad, CA). The fragment of interest was isolated via agarose gel electrophoresis and eluted from the gel in the manner described for the $GABA_A$ α1 subunit. The *NotI-XhoI-*

digested α1 and pcDNA3.1/V5-His C fragments were ligated using T4 DNA ligase. Subcloned products were confirmed by DNA sequencing.

Generation of the ρ1/α1 Chimera. A chimeric receptor was generated using wild-type human GABA ρ1 cDNA (in pcDNA3.1) and human $GABA_A$ α1 cDNA (in pcDNA3.1/V5-His C). The wild-type human GABA ρ1 subunit was generously provided by David Weiss (University of Texas Health Science Center at San Antonio).

The following oligonucleotides were synthesized and were used to introduce *KpnI* restriction sites in homologous regions at the start of TM2:

5) 5'-CGACCGCAGAGCGGTACCTGCCAGAGTCCCC-3', corresponding to bases 933-963 of the human GABA ρ1 cDNA sequence and
6) 5'-GGCTCAACAGAGAGTCGGTACCAGCAAGAAC-3', corresponding to bases 914-944 of the human $GABA_A$ α1 cDNA sequence.

The silent mutations were introduced with the QuikChange® Site-Directed Mutagenesis Kit. Incorporation of each mutation was verified by DNA sequencing. The subunits were subsequently digested with *KpnI*, and the linearized DNA was isolated via agarose gel electrophoresis. The fragments of interest were eluted from the gel as described previously for the $GABA_A$ α1 cDNA. Following ethanol precipitation, the linearized DNA was digested with *XhoI*. The fragments of interest were eluted from the gel, purified, and concentrated via ethanol precipitation. The *KpnI*-*XhoI*-digested α1 and ρ1 fragments were ligated using T4 DNA ligase, and the ligation reaction was used to transform XL10-Gold® ultracompetent cells (Stratagene, La Jolla, CA). Plasmid DNA

from resulting colonies was purified using the QIAGEN® Plasmid Mini Kit (QIAGEN. Valencia, CA). Chimeric products were verified via DNA sequencing.

All restriction enzymes and T4 DNA ligase were obtained from Promega (Madison, WI). Oligonucleotide synthesis and DNA sequencing were performed by Eurofins MWG Operon (Huntsville, AL).

Electrophysiology. Whole-cell patch clamp electrophysiology was used to assess GABA- or carisoprodol-activated Cl^- currents. All electrophysiology 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 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 (Olympus IX71; Olympus, Tokyo, Japan) 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 7.3. Agonist-induced Cl^- currents were obtained with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) equipped with a CV-203BU headstage. 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 solution and applied to each cell by gravity flow using a Y-shaped tube positioned adjacent to the cell. The modulatory effects of carisoprodol on GABA-gated currents were assessed using an EC_{20} gating concentration of GABA as the control. This gating concentration was selected to ensure there was a sufficient range to observe the full potential of carisoprodol. To ensure the gating concentration was approximately an EC_{20}, control responses were compared to the maximal GABA-gated current. Carisoprodol was tested only if the gating concentration was within the $EC_{15\text{-}25}$ range. Control responses were established by observing two consecutive agonist-activated currents that varied in amplitude by no more than ±10%. For αβγ configurations, GABA-gated control currents were recorded in the presence of diazepam to confirm incorporation of the γ2 subunit. After establishing the control response, effects of the test drug were determined. For studies investigating carisoprodol-mediated currents, carisoprodol was dissolved in external solution and applied in the manner described above.

Data Analysis. Concentration-response profiles for the positive modulatory actions of carisoprodol were generated (Origin 5.0; OriginLab Corp., Northampton, MA) using the equation $I/I_{max} = [\text{carisoprodol}]^n/([\text{carisoprodol}]^n + EC_{50}^n)$, 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. All data are presented as mean values ± S.E. Statistical significance ($p < 0.05$) 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.

RESULTS

Assessment of GABA Sensitivity in HEK293 Cells Transiently-Transfected with $GABA_ARs$. To ensure equipotent concentrations were used for gating, GABA concentration-response data were collected for human α1β2 and αxβzγ2 (where x = 1-4 and z = 1-2) $GABA_ARs$ (Table 1). From these data, EC_{20} and saturating GABA concentrations were calculated for each configuration and used in subsequent investigations of the allosteric and direct effects of carisoprodol, respectively.

Allosteric Modulatory and Direct Gating Effects of Carisoprodol Do Not Require the γ Subunit. To investigate the role of the γ subunit on the allosteric and direct effects of carisoprodol at $GABA_ARs$, HEK293 cells were transiently transfected with human α1β2 and α1β2γ2 receptors. Micromolar concentrations of carisoprodol potentiated the GABA-gated currents of α1β2 $GABA_ARs$ in a concentration-dependent manner (Fig. 1A). At millimolar concentrations, rebound currents were observed upon termination of drug application, and coapplication of 3 mM carisoprodol elicited an inhibitory effect on GABA-gated currents during drug application. The patterns of potentiation and inhibition by carisoprodol at α1β2 $GABA_ARs$ were similar to those observed at α1β2γ2 $GABA_ARs$, shown here (Fig. 1A) and previously using a stable α1β2γ2 cell line (Gonzalez et al., 2009). In our analyses of the modulatory effects of carisoprodol, peak current amplitude was defined as the maximum current elicited by carisoprodol. For recordings where an

inhibitory component of carisoprodol was present, the amplitude of the rebound current amplitude was regarded as the peak current. Using these parameters, the estimated EC_{50} for carisoprodol at $\alpha1\beta2$ $GABA_A$Rs was 121 ± 8 μM compared to 131 ± 21 μM for receptors containing the γ2 subunit, with direct gating by carisoprodol likely contributing to maximal current amplitude elicited by higher concentrations of the drug. Maximum potentiation of control currents occurred with 1 mM carisoprodol for each configuration (Fig. 1B) with efficacies of 488 ± 80% and 572 ± 64% for $\alpha1\beta2$ and $\alpha1\beta2\gamma2$ $GABA_A$Rs, respectively. Thus, the γ subunit did not significantly influence the potency or efficacy of carisoprodol as an allosteric modulator (Table 2). Coapplication of 30 μM carisoprodol yielded modestly enhanced potentiation of currents from $\alpha1\beta2$ receptors ($p < 0.05$).

Moreover, the efficacy of carisoprodol as a direct agonist was unaffected by the γ subunit (Fig. 2B and Table 3). Peak current amplitude of carisoprodol-evoked currents was 45 ± 6% of the maximum GABA-gated current for $\alpha1\beta2$ receptors whereas it was 34 ± 6% for $\alpha1\beta2\gamma2$ receptors. Together with the allosteric studies, these findings suggest the γ subunit is not essential for carisoprodol-mediated regulation of $GABA_A$R function. More importantly, $GABA_A$Rs composed solely of α and β subunits retain the site(s) of action for carisoprodol; thus, the focus was shifted to these subunits.

Carisoprodol-Mediated Activity Is Influenced by the Isoform of the $GABA_A$ β Subunit. The influence of the β subunit was investigated by comparing the modulatory actions of carisoprodol at $\alpha1\beta1\gamma2$ and $\alpha1\beta2\gamma2$ $GABA_A$Rs. Carisoprodol modulated the GABA-gated currents of $\alpha1\beta1\gamma2$ $GABA_A$Rs in a manner previously described for

$\alpha1\beta2\gamma2$ $GABA_A$Rs—potentiation in a concentration-dependent manner accompanied by offshoot currents and inhibition with millimolar concentrations (Fig. 3A). Carisoprodol was equally potent at enhancing the currents of β1- and β2-containing receptors (88 ± 19 μM and 131 ± 21 μM, respectively), yet it was more efficacious at the latter. Maximum efficacy was 357 ± 36% of control values for β1-containing receptors, significantly less than the value reported earlier for β2-containing receptors.

As reported for β2-containing receptors here and previously (Gonzalez et al., 2009), carisoprodol elicited inward currents from $\alpha1\beta1\gamma2$ $GABA_A$Rs in the absence of GABA. Direct activation occurred in a concentration-dependent manner with millimolar concentrations producing significantly greater currents at β1-containing receptors (Fig. 4 and Table 3). This is in contrast to the pattern observed for the allosteric effects of carisoprodol in which the drug was more efficacious at receptors containing the β2 isoform.

Carisoprodol-Mediated Activity Is Influenced by the Isoform of the $GABA_A$ α Subunit. To assess the influence of the α subunit isoform on carisoprodol-mediated activity, HEK293 cells were transiently transfected with $\alpha x\beta2\gamma2$ (where x = 1-4) combinations of $GABA_A$Rs. The pattern of allosteric modulation described previously was observed for each of the combinations tested with one exception. Inhibition of GABA-gated currents was consistently observed at high concentrations of carisoprodol; however, this was not the case for α3-containing receptors (Fig. 5, A and B). At all concentrations tested, the allosteric effects of carisoprodol were significantly greater at

α1-containing receptors (Fig. 5B and Table 2). However, carisoprodol potency was not dependent upon the α subunit isoform.

Moreover, carisoprodol directly activated each of the configurations tested, evoking inward currents in the absence of GABA. Significant differences were not observed with lower concentrations of carisoprodol (≤ 100 μM). In addition, responses from α1-, α2-, and α4-containing receptors were comparable at each concentration tested, with maximal efficacies ranging from approximately 31-34% (Table 3). In contrast, carisoprodol was significantly less efficacious at α3-containing receptors, eliciting only 8 ± 3% of the maximum GABA-gated current.

Chimeric ρ1/α1 Subunits Assemble Functional Homomeric Receptors. The fact that subunit-dependent influences vary for allosteric and direct effects suggest the functional domains for the effects may be distinct. To gain a better understanding of the critical domains involved in mediating the two effects of this drug, we used a chimeric approach in which domains were exchanged between carisoprodol-sensitive and -insensitive subunits. Because homomeric ρ1 GABA receptors are insensitive to the positive modulatory effects of carisoprodol and its agonistic actions (Figs. 8 and 9) (Gonzalez et al., 2009), a chimeric subunit was generated by exchanging the regions encoding TM2 through the carboxyl-termini of $GABA_A$ ρ1 and α1 subunits (Fig. 7) as these domains of the α and β subunits have been implicated in mediating the effects of other compounds at $GABA_A$Rs (Korpi et al., 2002; Rudolph and Antkowiak, 2004; Hosie et al., 2006).

When expressed in HEK293 cells, chimeric subunits assembled homomeric receptors that were kinetically distinct from either of its wild-type counterparts. Although

the GABA sensitivity of the homomeric ρ1/α1 receptor was not significantly different from that of wild-type ρ1 receptors (Table 1), moderate concentrations of GABA elicited rapidly desensitizing currents from chimeric receptors—a property not associated with wild-type ρ1 receptors (Polenzani et al., 1991; Qian and Dowling, 1993). The functional properties of the chimeric receptor generated in the current study, with respect to GABA sensitivity and desensitization, were consistent with a previous report in which a similar chimera was expressed in *Xenopus* oocytes (Martinez-Torres et al., 2000). Desensitization, which could confound interpretation of the results, was not observed with application of an EC_{20} concentration of GABA—the gating concentration used in the investigation of the allosteric effects of carisoprodol.

Chimeric ρ1/α1 Receptors Are Insensitive to Direct Activation by Carisoprodol But Sensitive to the Allosteric Effects of Carisoprodol. As seen in wild-type ρ1 receptors, ρ1/α1 receptors were insensitive to the agonistic actions of carisoprodol (Fig. 8, A and B). Thus, the site(s) of action underlying direct activation of $GABA_A$Rs by carisoprodol are either located within or require interaction with regions that were not exchanged between the subunits.

Calculations of the modulatory effects of carisoprodol at chimeric ρ1/α1 receptors were conducted in the manner previously described for other configurations. Coapplication of lower concentrations of carisoprodol had no significant effect on GABA-gated currents whereas higher concentrations elicited an inhibitory effect accompanied by rebound currents that were potentiated compared to control currents. Despite significant inhibition during drug application, rebound currents demonstrated

enhancement of control currents in a manner described for each of the other configurations tested (Fig. 9A). The efficacy of carisoprodol was 201 ± 17% of control at ρ1/α1 chimeric receptors, indicating partial restoration of the allosteric effects elicited at αβ and αβγ $GABA_A$Rs. These findings suggest critical domains for the allosteric actions of carisoprodol and its inhibitory effects at millimolar concentrations are located within TM2 through the carboxyl-terminal of the α1 subunit.

The Modulatory Effects of Carisoprodol Are Not Mediated via the Large Intracellular Loop of $GABA_A$Rs. Given the lipophilicity of carisoprodol, this drug may potentially act at an intracellular site of action as has been reported for neurosteroids (Akk et al., 2005). The region that was exchanged between ρ1 and α1 subunits contained a single intracellular domain—the large intracellular loop located between TM3 and TM4. To assess whether allosteric modulation by carisoprodol requires interaction with this domain, carisoprodol, at a concentration known to elicit robust potentiation, was included in the pipette solution. Thus, dialysis of the intracellular contents with the whole-cell patch clamp configuration facilitates access of the drug to its potential site of action. Experiments were conducted with cells stably expressing human α1β2γ2 $GABA_A$Rs. When carisoprodol was not included in the pipette solution, carisoprodol potentiated GABA-gated currents by 201 ± 21%, a value not significantly different from the enhancement of GABA-gated currents (203 ± 14%) when carisoprodol was included in the pipette solution. These findings suggest the large intracellular loop of $GABA_A$Rs is not likely to mediate the allosteric actions of carisoprodol.

DISCUSSION

Carisoprodol is a muscle relaxant that is frequently prescribed for the treatment of acute musculoskeletal conditions. Despite its clinical merit, the use of this drug is hampered by its abuse potential. Carisoprodol abuse, tolerance, and withdrawal are well-documented in the literature (Adams et al., 1975; Elder, 1991; Littrell et al., 1993; Rust et al., 1993; Reeves and Parker, 2003). The abuse liability of carisoprodol is often attributed to meprobamate—the primary metabolite of carisoprodol and a controlled substance at the federal level. However, we have demonstrated carisoprodol, itself, acts at $GABA_AR$s in a manner described for drugs of abuse that act via the GABAergic system (Gonzalez et al., 2009). Interestingly, the abuse and dependence potential of these drugs are related to their subunit-selective interactions with $GABA_AR$s (Ito et al., 1996; Ator, 2005; Wafford, 2005; Licata and Rowlett, 2008). Thus, we assessed whether the actions of carisoprodol are subunit-dependent, potentially underlying its physiological effects and abuse liability. Using different configurations of $GABA_AR$s, we demonstrated carisoprodol acts in a subunit-dependent manner with α and β subunits mediating the allosteric and direct effects of the drug. Based upon subunit-dependent assessments and chimeric studies, we identified domains within the $\alpha1$ subunit that contribute to the modulation of $GABA_AR$ function by carisoprodol.

The role of the γ subunit has been established for several modulators of $GABA_AR$ function. The most prominent example is the benzodiazepine class of drugs which require the presence of a γ subunit to potentiate GABA-gated currents (Pritchett et al., 1989). In

contrast, our results suggest the γ subunit does not play an essential role in mediating the actions of carisoprodol at $GABA_A$Rs because the modulatory and agonistic effects of the drug were not significantly different between α1β2 and α1β2γ2 configurations. These findings support our assertion that carisoprodol does not act at the benzodiazepine site of the receptor (Gonzalez et al., 2009). More importantly, $GABA_A$Rs composed solely of α and β subunits retained the site(s) of action for carisoprodol, highlighting the contribution of these subunits to the pharmacological profile of carisoprodol.

In the current study, the influence of β subunit isoforms was examined via comparison of effects elicited from β1- and β2-containing receptors. Whereas the actions of GABAergic compounds such as etomidate, loreclezole, and furosemide are dependent upon incorporation of β2 or β3 subunits (Korpi et al., 1995; Korpi et al., 2002), carisoprodol-mediated effects were observed at α1β1γ2 $GABA_A$Rs, suggesting critical domains for carisoprodol activity are located within regions conserved between the β1 and β2 isoforms. Comparison at the amino acid level reveals 78% homology between β1 and β2 with highest similarity between transmembrane regions and greatest variation in the signal peptide and large intracellular loop (Hadingham et al., 1993). Although the large intracellular loop has been identified as a site of action for neurosteroids (Akk et al., 2005), the likelihood that carisoprodol acts at the transmembrane domains is greater given our findings with the ρ1/α1 chimera (discussed below). Also, the lipophilicity of carisoprodol may prompt its accumulation in the membrane, fostering its interactions with sites located in the transmembrane domains. In further support of this hypothesis,

sites within TM1, TM2, TM3, and TM4 have been implicated in mediating the effects of other lipophilic, GABAergic compounds (Korpi et al., 2002; Huang et al., 2006).

Furthermore, the β subunit isoform influenced the efficacy of carisoprodol. For allosteric modulation, carisoprodol was significantly more efficacious at β2-containing receptors. Expression of the β2 isoform in the brain is virtually ubiquitous (Hevers and Luddens, 1998), suggesting the majority of receptor configurations are susceptible to modulation by carisoprodol. In contrast, direct activation by carisoprodol was significantly greater at β1-containing receptors. This disparity suggests allosteric modulation and direct gating by carisoprodol may be mediated by distinct sites of the β subunit.

Moreover, previous reports have implicated the α subunit isoform as a determinant of $GABA_AR$ pharmacology. For instance, the efficacy of pentobarbital as an agonist is greater than that of GABA only at α6-containing receptors (Drafts and Fisher, 2006). In addition, $GABA_ARs$ containing α4 or α6 subunits are insensitive to allosteric modulation by diazepam (Knoflach et al., 1996). Because carisoprodol has both allosteric and direct effects, we investigated whether the α subunit isoform played a similar role in determining carisoprodol-mediated activity at $GABA_ARs$. We also sought to determine if the functional domains for allosteric modulation and direct gating were comparable.

All α subunit isoforms were sensitive to the agonistic actions of carisoprodol with significant differences observed at moderate to high concentrations (≥100 μM). According to case reports, blood or plasma concentrations of carisoprodol as low as 140

μM have proven to be fatal (Robertson and Marinetti, 2003). Interestingly, this concentration correlates to the onset of direct activation. Receptors containing the α3 isoform were virtually insensitive to direct activation by carisoprodol with appreciable currents elicited only with 3 mM concentrations; this suggests the α3 isoform may impair receptor sensitivity to the agonistic activity of the drug.

For allosteric modulation, carisoprodol, at all concentrations, was more efficacious at α1-containing receptors. Our findings indicate a preferential interaction of carisoprodol with α1-containing $GABA_AR$s at concentrations that span the therapeutic and toxic effects of the drug. Because the α1 isoform mediates the sedative properties of benzodiazepines (Rudolph and Mohler, 2006), it is likely carisoprodol mediates its sedative effects via interaction with α1-containing $GABA_AR$s, contributing to its therapeutic and illicit effects. In addition, recent studies suggest the efficacy of benzodiazepine-type compounds at α1-containing $GABA_AR$s may predict their abuse potential (Licata and Rowlett, 2008). In support of this, preferential agonist activity at these receptors is adequate for a drug to promote reinforcement in self-administration studies, indicating drugs acting at these configurations have the potential for abuse (Ator, 2005). Carisoprodol elicited robust potentiation at $GABA_AR$s containing α2, α3, and α4 subunits. Coupled with the widespread distribution of α1 subunits, this general interaction of carisoprodol with $GABA_AR$s suggests a universal mode of action in the brain. Licata and Rowlett (2008) reported physical dependence is more likely to develop with drugs that interact with all $GABA_AR$ subtypes as opposed to drugs exhibiting

subtype-selectivity. Taken together, the pharmacological profile of carisoprodol is consistent with its clinical effects and its potential for abuse.

Subunit-dependent studies suggested carisoprodol-mediated activity at $GABA_ARs$ is dependent upon α and β subunits. Ideally, the effects of carisoprodol would be assessed using homomeric receptors consisting solely of α or β subunits, allowing assessment of the contributions of each subunit. However, recombinant expression of homomeric α or β receptors has been inconsistent and controversial at best (Sieghart et al., 1999). In recombinant systems, ρ1 subunits assemble functional heteromeric receptors with other ρ isoforms or with γ2 subunits, but not α or β (Olsen and Sieghart, 2008). These options would not further our knowledge of carisoprodol's mechanism of action since other ρ subunits may also be carisoprodol-insensitive, and carisoprodol activity is unaffected by the γ2 subunit. To circumvent these issues, chimeric strategies were utilized to identify sites of action for other GABAergic compounds. Previous chimeric strategies have included exchange of domains between different isoforms of the same subunit class (Bianchi et al., 2002; Drafts and Fisher, 2006), between subunit classes (Serafini et al., 2000; Jones-Davis et al., 2005), and between members of the ligand-gated ion channel superfamily (Koltchine et al., 1996; Martinez-Torres et al., 2000). Because physiologically relevant concentrations of carisoprodol elicited significantly greater effects at α1-containing $GABA_ARs$, we chose to generate a chimeric ρ1/α1 subunit in an attempt to identify domains that confer the direct gating and allosteric modulatory effects of carisoprodol.

Although the agonistic activity of carisoprodol was not restored, allosteric modulation by carisoprodol was partially restored. Allosteric modulation was not significantly different with intracellular application of carisoprodol, indicating sites of action for this drug are not located within the large intracellular loop (Belelli et al., 1997). This finding is consistent with the notion that lipophilic compounds mediate their effects at $GABA_AR$s by acting at hydrophobic regions of membrane-embedded proteins.

Perhaps, the sites of action required for direct activation by carisoprodol are located in regions not exchanged between the subunits. Given the complexity of $GABA_AR$ pharmacology, however, this assertion is probably overly simplistic. Although our subunit-dependence studies suggest α and β subunits influence the effects of carisoprodol, they do not reveal whether the subunits contribute equally. It is possible the agonistic actions of carisoprodol may be mediated, to a greater extent, by the β subunit, prohibiting us from restoring carisoprodol-sensitivity. In a recent study, activating concentrations of anesthetics induced conformational changes associated with channel opening; the structural changes leading to opening of the channel by anesthetics were dependent upon the β subunit (Rosen et al., 2007). This may be the case for carisoprodol as well. Moreover, general $GABA_AR$ function is dependent upon intra- and intersubunit interactions. Thus, a scenario that seems more consistent with our findings is that the allosteric and direct effects of carisoprodol require interactions between and within α and β subunits—interactions that are not fostered by homomeric ρ1/α1 receptors. The importance of intersubunit interactions in the allosteric modulation of $GABA_AR$s has been described for pentobarbital. Homomeric β2 receptors are sensitive to the allosteric

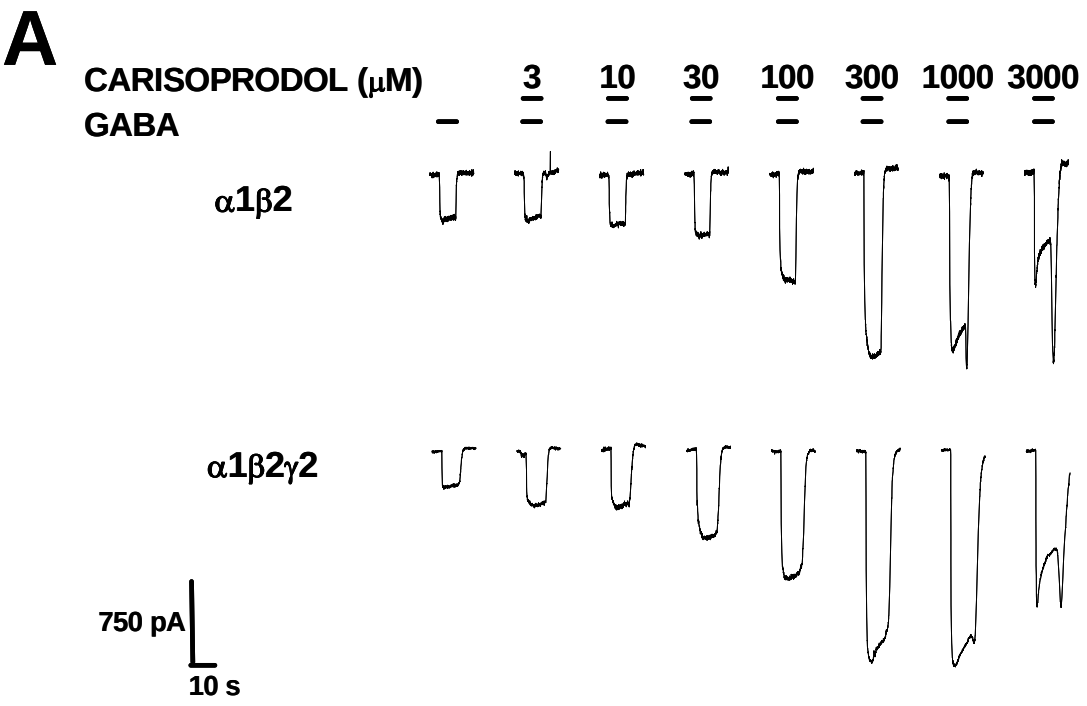
effects of pentobarbital whereas β2W328M receptors are not; however, coexpression of α1 with β2W328M restores pentobarbital-sensitivity (Amin, 1999). Thus, addition of an α subunit provides an interaction that is absent in homomeric receptors. More importantly, some drugs allosterically modulate and directly activate $GABA_A$Rs via a single site while others act as multiple sites (Muroi et al., 2009). In a recent analysis of pentobarbital-induced tail currents, it was postulated pentobarbital—a drug with effects similar to those of carisoprodol—may have as many as five sites of action on $GABA_A$Rs (Gingrich et al., 2009). Although our findings do not definitively identify the number of carisoprodol sites on the receptor, the fact that we were able to partially restore allosteric modulation by carisoprodol without affecting direct activation suggests carisoprodol may be acting at multiple sites on $GABA_A$Rs.

In the current study, we demonstrated carisoprodol preferentially interacts with selective $GABA_A$R subunits. Based upon our findings, the pharmacological profile of carisoprodol at $GABA_A$Rs is consistent with the therapeutic effects of the drug, and its subunit-dependence may underlie its potential for abuse. Using a chimeric approach, we identified functional domains of the α subunit that underlie the modulatory effects of carisoprodol at $GABA_A$Rs. Of equal importance, similar domains were not sufficient to restore the direct gating effects of carisoprodol, suggesting the complex interactions of carisoprodol may require multiple sites on the receptor.

Table III-1. GABA sensitivity of different $GABA_AR$ subunit configurations. GABA EC_{50} values and Hill coefficients were calculated from concentration-response data for each configuration.

Receptor Configuration	EC_{50} (μM)	Hill Coefficient	Sample Size
α1β2	14.0 ± 1.01	1.32 ± 0.11	3-4
α1β2γ2	35.5 ± 0.64	1.32 ± 0.03	5
α2β2γ2	48.4 ± 5.71	1.09 ± 0.12	3-9
α3β2γ2	34.8 ± 2.09	1.04 ± 0.06	4-8
α4β2γ2	4.48 ± 0.29	1.36 ± 0.11	4-6
α1β1γ2	16.6 ± 1.07	1.17 ± 0.09	5-6
ρ1	1.07 ± 0.04	1.79 ± 0.13	3-8
ρ1/α1	1.40 ± 0.28	0.73 ± 0.11	3-6

Figure III-1. Influence of the γ subunit on allosteric modulation by carisoprodol. A, representative traces demonstrating the potentiation of GABA-gated (EC_{20}) currents from human α1β2 and α1β2γ2 $GABA_A$Rs by carisoprodol. At high concentrations, offshoot currents were observed upon termination of drug application, and currents were inhibited. B, concentration-response curves for the allosteric modulation of GABA-gated currents from hα1β2 and hα1β2γ2 $GABA_A$Rs by carisoprodol. Each data point represents the mean ± S.E. from a minimum of three cells. *, $p < 0.05$.


A
CARISOPRODOL (μM)
3
10
30
100
300
1000
3000
GABA
α1β2
α1β2γ2
750 pA
10 s

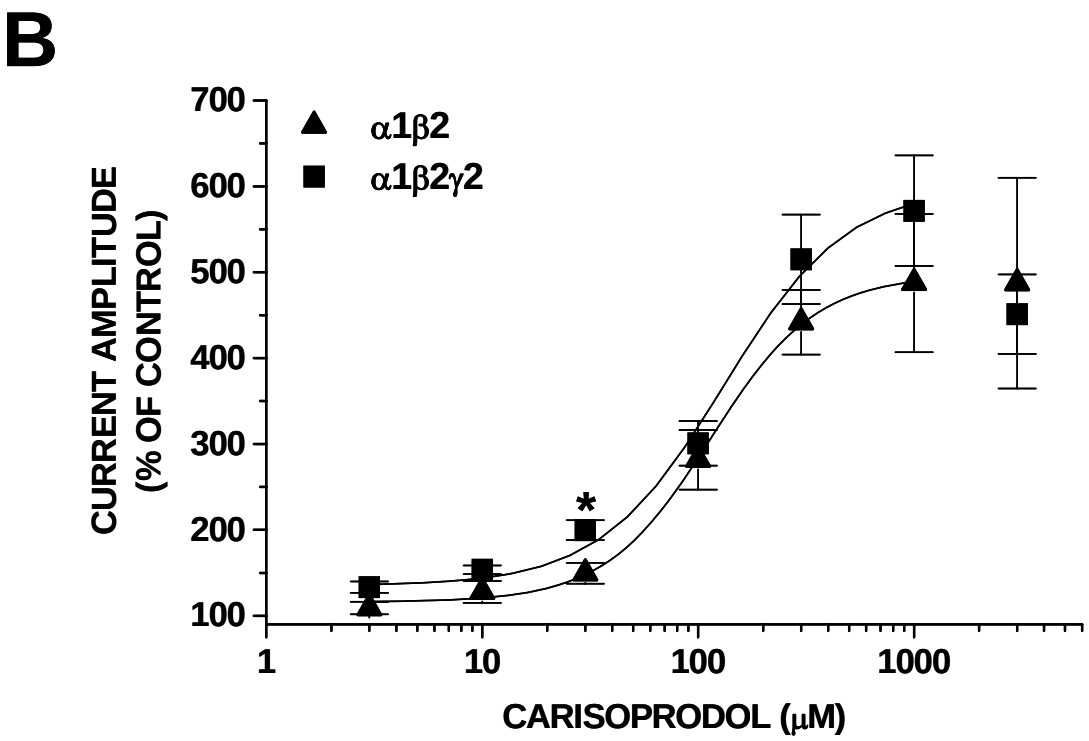

B
700
600
500
400
300
200
100
α1β2
α1β2γ2
CURRENT AMPLITUDE
(% OF CONTROL)
*
1
10
100
1000
CARISOPRODOL (μM)

Figure III-2. Influence of the γ subunit on direct activation by carisoprodol. A, representative traces demonstrating carisoprodol activates human α1β2 and α1β2γ2 $GABA_A$Rs in a concentration-dependent manner. Carisoprodol-activated currents are presented relative to the maximum current elicited by GABA (100 μM and 1 mM GABA for α1β2 and α1β2γ2, respectively). B, concentration-response curves for carisoprodol-mediated currents for hα1β2 and hα1β2γ2 $GABA_A$Rs. There were no significant differences between the two configurations at each concentration tested ($p > 0.05$). Each data point represents the mean ± S.E. of a minimum of four cells.

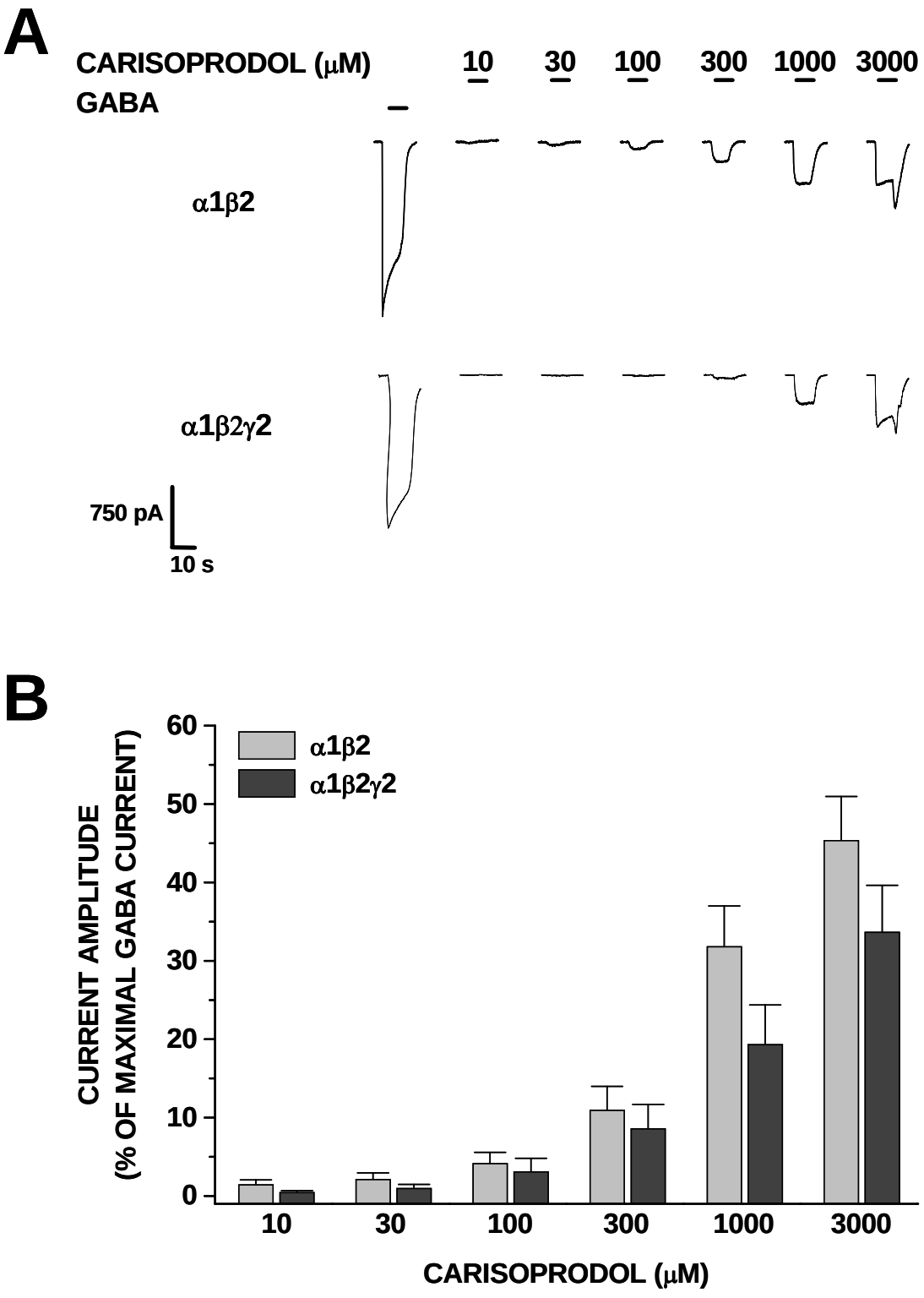
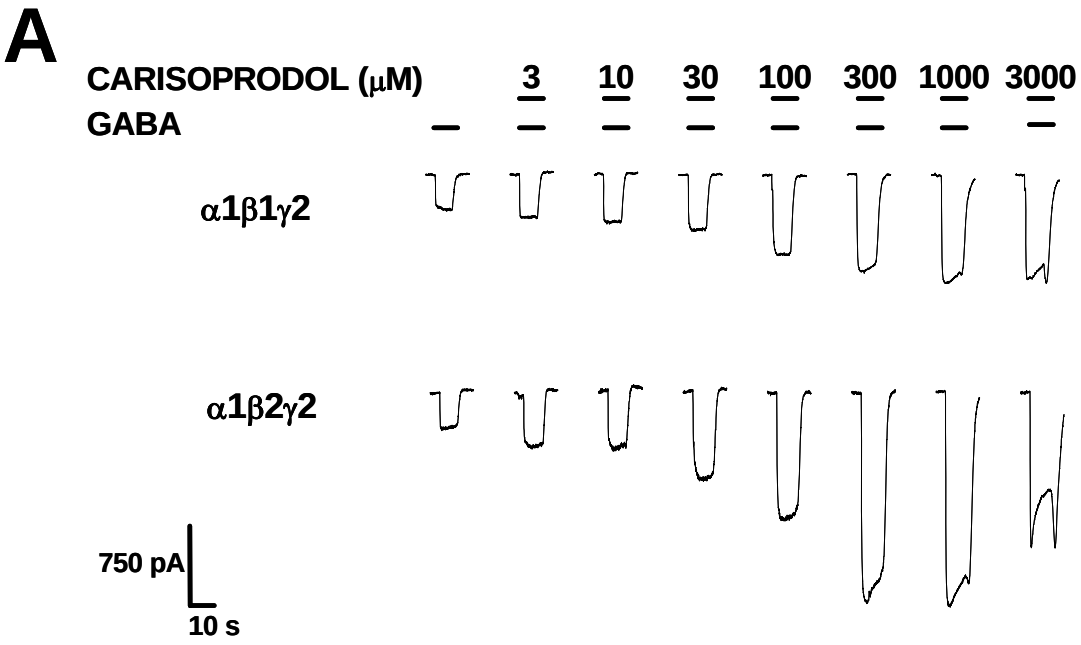

A
CARISOPRODOL (μM)
10
30
100
300
1000
3000
GABA
α1β2
α1β2γ2
750 pA
10 s
B
α1β2
α1β2γ2
CURRENT AMPLITUDE
(% OF MAXIMAL GABA CURRENT)
60
50
40
30
20
10
0
10
30
100
300
1000
3000
CARISOPRODOL (μM)

Figure III-3. Influence of the β subunit on allosteric modulation by carisoprodol. A, representative traces demonstrating the potentiation of GABA-gated (EC_{20}) currents from human α1β1γ2 and α1β2γ2 $GABA_A$Rs by carisoprodol. Traces for α1β2γ2 receptors are replotted from Fig. 1A. At high concentrations, offshoot currents were observed upon termination of drug application, and currents were inhibited. B, concentration-response curves for the allosteric modulation of GABA-gated currents from hα1β1γ2 and hα1β2γ2 $GABA_A$Rs by carisoprodol. Carisoprodol was significantly more efficacious at β2-containing receptors. The values for hα1β2γ2 $GABA_A$Rs were replotted from Fig. 1B. Each data point represents the mean ± S.E. of a minimum of four cells. *, $p < 0.05$; ***, $p < 0.0001$.


A
CARISOPRODOL (μM)
3
10
30
100
300
1000
3000
GABA
α1β1γ2
α1β2γ2
750 pA
10 s

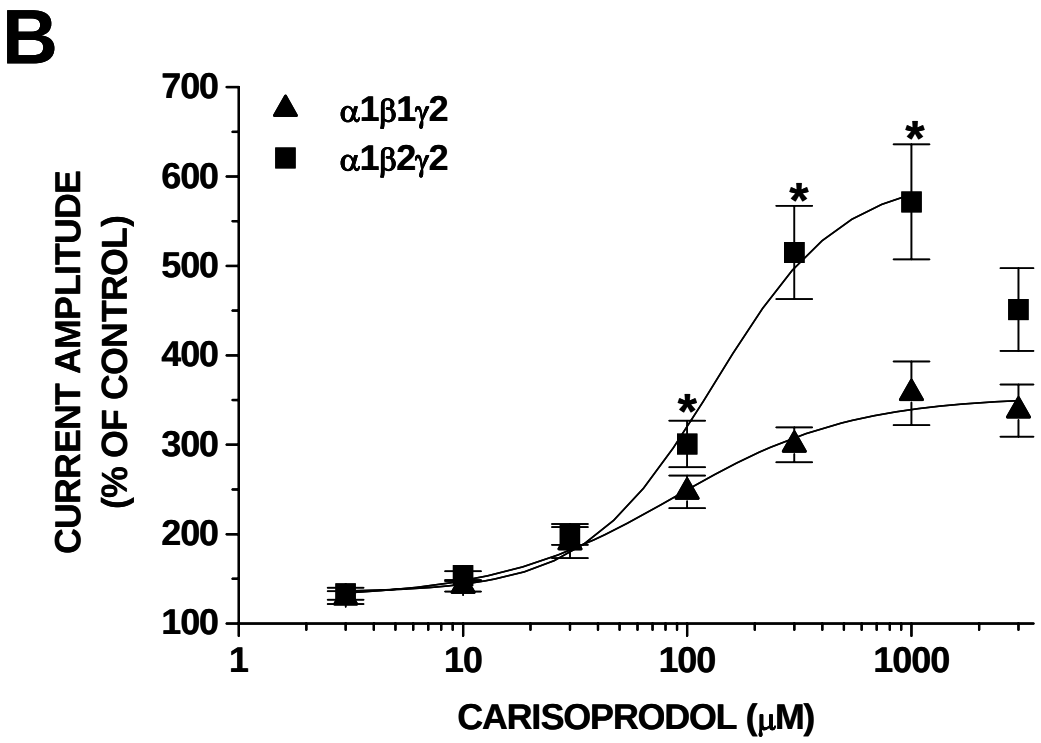
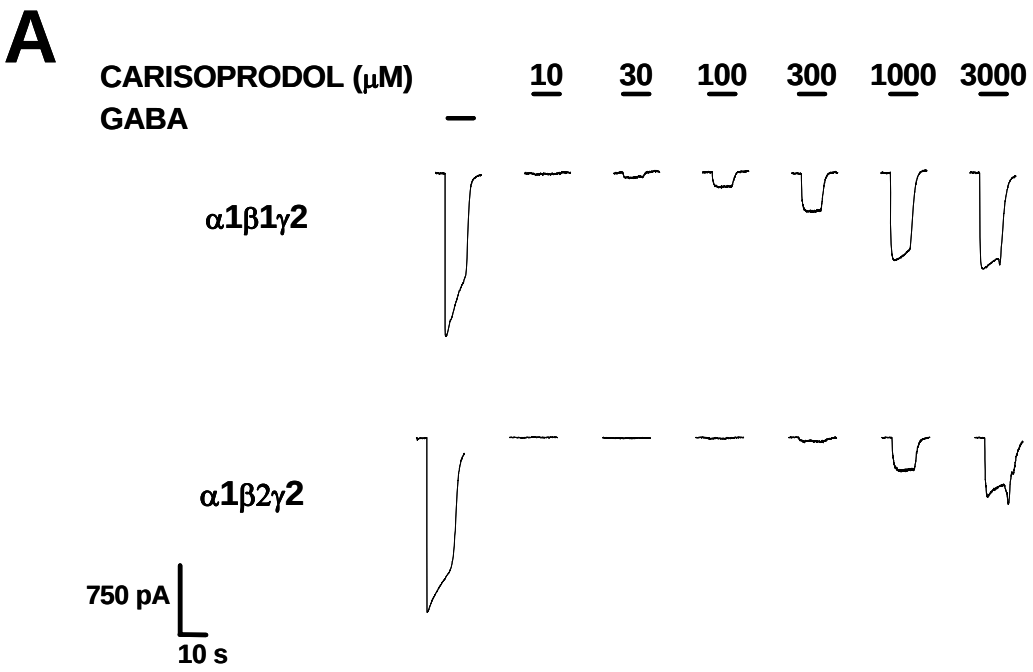

B
700
600
500
400
300
200
100
α1β1γ2
α1β2γ2
CURRENT AMPLITUDE
(% OF CONTROL)
1
10
100
1000
CARISOPRODOL (μM)
*
*
*

Figure III-4. Influence of the β subunit on direct activation by carisoprodol. A, representative traces demonstrating direct activation of human α1β1γ2 and α1β2γ2 $GABA_A$Rs by carisoprodol. Traces for α1β2γ2 receptors are replotted from Fig. 2A. Carisoprodol-mediated currents are shown relative to the current elicited by a saturating concentration of GABA (1 mM). B, concentration-response curves for carisoprodol-mediated currents from hα1β2γ2 and hα1β1γ2 $GABA_A$Rs. Carisoprodol was significantly more efficacious at β1-containing receptors. The values for hα1β2γ2 $GABA_A$Rs were replotted from Fig. 2B. Each data point represents the mean ± S.E. of a minimum of four cells. *, $p < 0.05$; **, $p < 0.01$.


A
CARISOPRODOL (μM)
10
30
100
300
1000
3000
GABA
α1β1γ2
α1β2γ2
750 pA
10 s

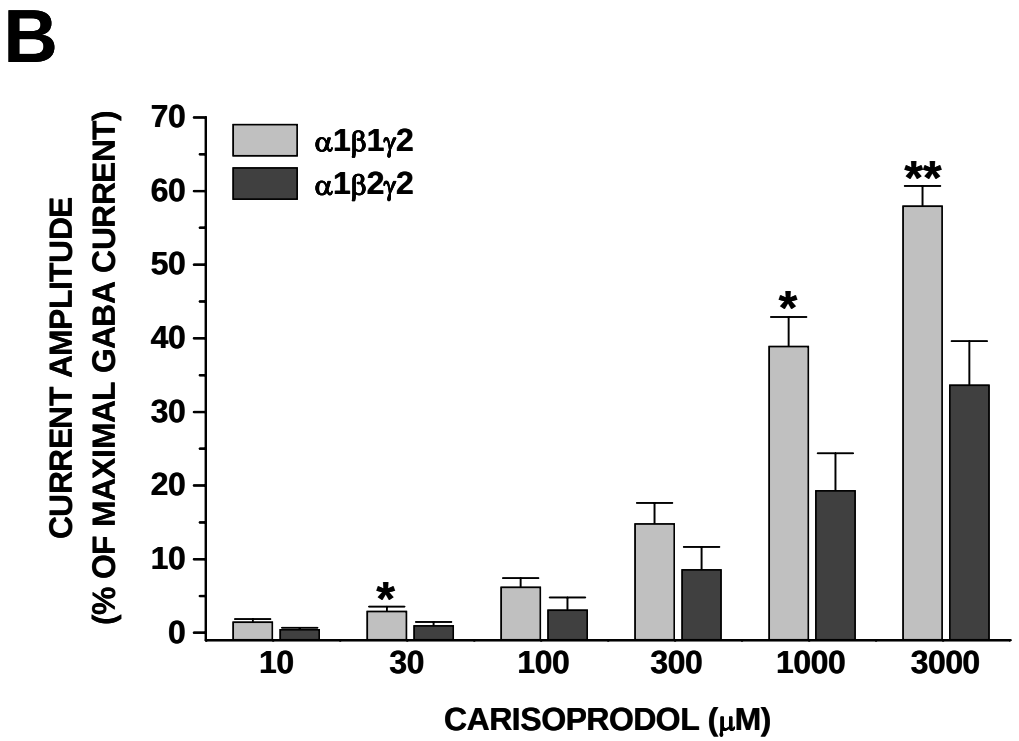

B
CURRENT AMPLITUDE
(% OF MAXIMAL GABA CURRENT)
α1β1γ2
α1β2γ2
70
60
50
40
30
20
10
0
*
*
**
10
30
100
300
1000
3000
CARISOPRODOL (μM)

Figure III-5. Influence of the α subunit on allosteric modulation by carisoprodol. A, representative traces demonstrating the potentiation of GABA-gated (EC_{20}) currents from human α3β2γ2 and α1β2γ2 $GABA_A$Rs by carisoprodol. For α3-containing receptors, concentrations of carisoprodol above 1 mM continued to potentiate GABA-gated currents; the currents of other configurations were inhibited at these concentrations. Traces for α1β2γ2 receptors are replotted from Fig. 1A. B, concentration-response curves for the allosteric modulation of GABA-gated currents from human α1-, α2-, α3-, and α4β2γ2 $GABA_A$Rs by carisoprodol. The values for hα1β2γ2 $GABA_A$Rs were replotted. Each data point represents the mean ± S.E. from a minimum of three cells. *, $p < 0.05$.

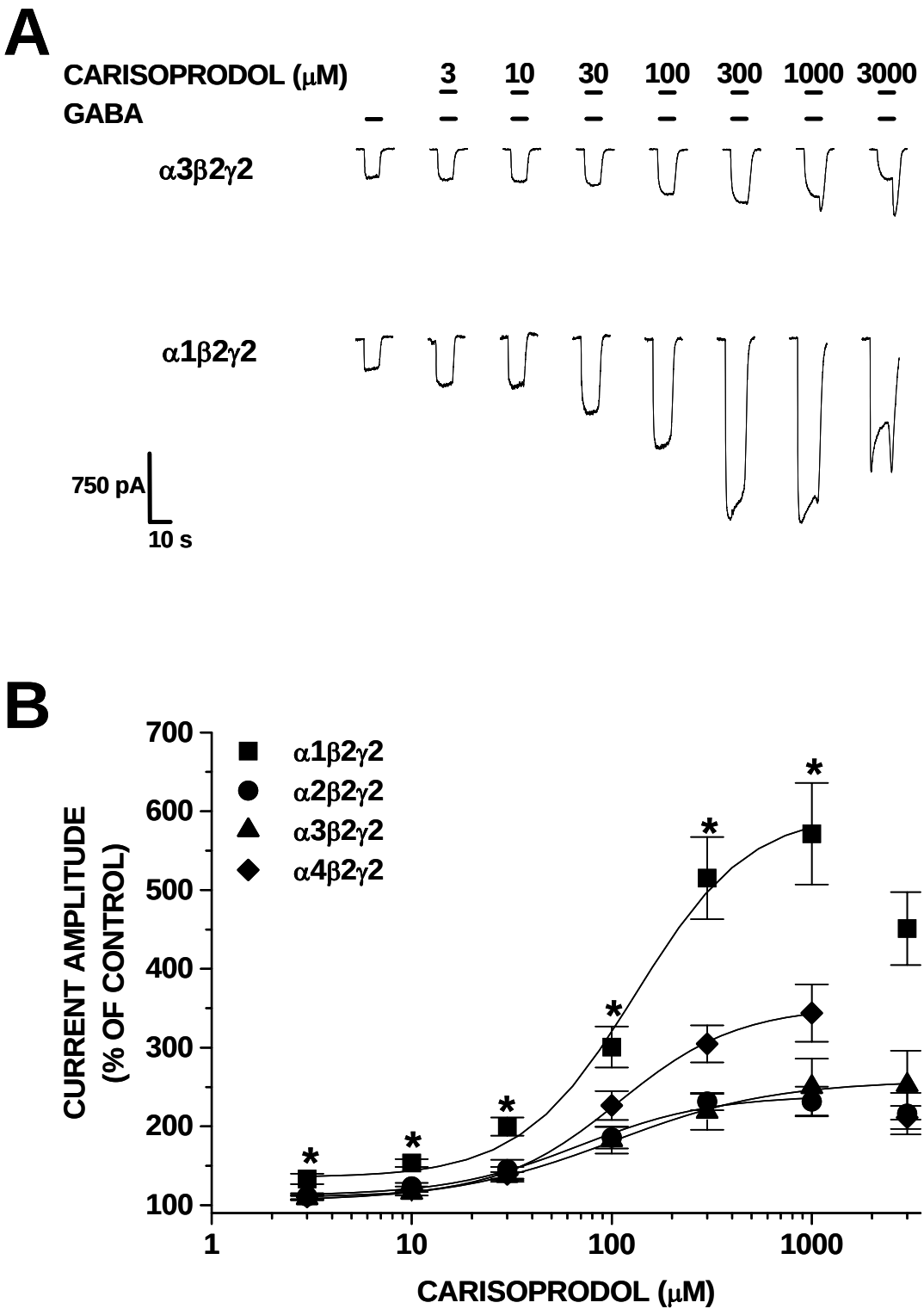
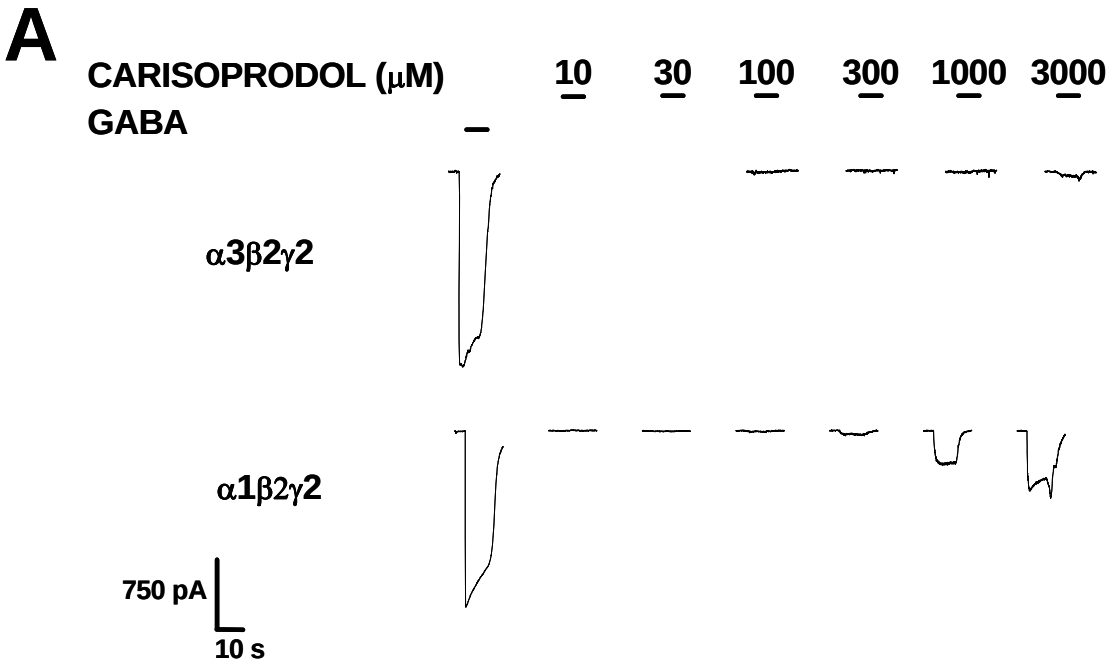

A
CARISOPRODOL (μM)
3
10
30
100
300
1000
3000
GABA
α3β2γ2
α1β2γ2
750 pA
10 s
B
α1β2γ2
α2β2γ2
α3β2γ2
α4β2γ2
CURRENT AMPLITUDE
(% OF CONTROL)
700
600
500
400
300
200
100
1
10
100
1000
CARISOPRODOL (μM)

Figure III-6. Influence of the α subunit on direct activation by carisoprodol. A, representative traces demonstrating carisoprodol activates human α3β2γ2 and α1β2γ2 $GABA_A$Rs in a concentration-dependent manner. Concentrations below 100 μM were not tested on α3β2γ2 receptors as moderate to high concentrations did not yield inward currents. Traces for α1β2γ2 receptors are replotted from Fig. 2A. Carisoprodol-mediated currents are shown relative to the current elicited by a saturating concentration of GABA (1 mM). B, concentration-response curves for carisoprodol-mediated currents for human α1-, α2-, α3-, and α4β2γ2 $GABA_A$Rs. The values for hα1β2γ2 $GABA_A$Rs were replotted from Fig. 2B. Each data point represents the mean ± S.E. of a minimum of three cells. *, $p < 0.05$.


A
CARISOPRODOL (μM)
10
30
100
300
1000
3000
GABA
α3β2γ2
α1β2γ2
750 pA
10 s

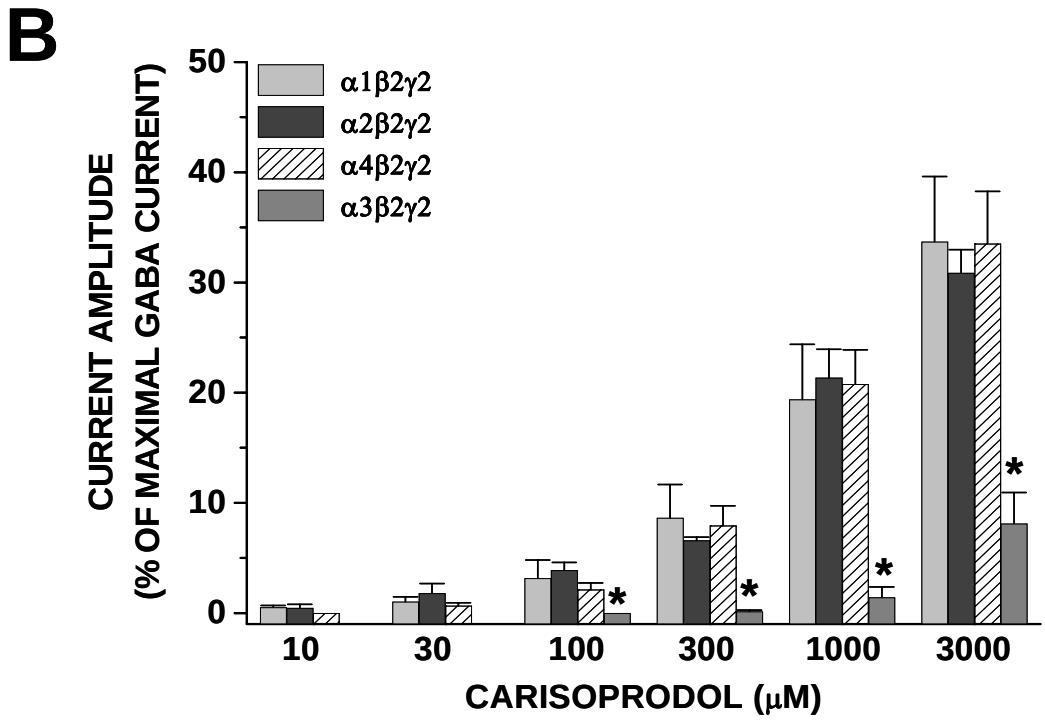

B
CURRENT AMPLITUDE
(% OF MAXIMAL GABA CURRENT)
α1β2γ2
α2β2γ2
α4β2γ2
α3β2γ2
50
40
30
20
10
0
10
30
100
300
1000
3000
CARISOPRODOL (μM)

Table III-2. Comparison of the potency and efficacy of carisoprodol at various $GABA_AR$ subunit configurations. There were no significant differences in the potency of carisoprodol at each of the configurations tested ($p > 0.05$). Carisoprodol was significantly more efficacious at $\alpha1\beta2\gamma2$ $GABA_ARs$ ($p < 0.05$ relative to $\alpha4$; $p < 0.01$ relative to $\alpha2$ and $\alpha3$). Significant differences in efficacy relative to $\alpha1\beta2\gamma2$ are denoted by *, $p < 0.05$.

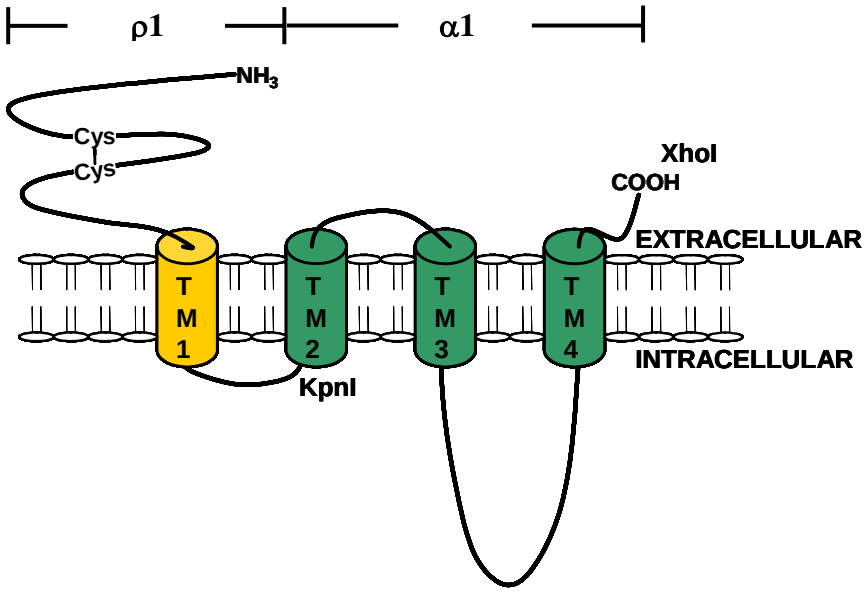
Receptor Configuration	EC_{50} (μM)	Hill Coefficient	Maximal Efficacy (%)	Sample Size
α1β2	120.9 ± 8.3	1.58 ± 0.17	487.4 ± 80.4	3
α1β2γ2	131.2 ± 20.5	1.53 ± 0.28	571.6 ± 64.4	6-7
α2β2γ2	69.1 ± 16.7	1.32 ± 0.41	231.5 ± 18.8*	4-6
α3β2γ2	102.1 ± 9.5	1.07 ± 0.11	252.4 ± 43.8*	3-4
α4β2γ2	108.3 ± 6.6	1.45 ± 0.12	343.9 ± 36.5*	6-11
α1β1γ2	87.6 ± 18.5	0.68 ± 0.19	357.4 ± 35.7*	4-6
ρ1/α1	244.5 ± 32.2	2.06 ± 0.54	200.7 ± 34.5	3-4

Table III-3. Comparison of the efficacy of carisoprodol as a direct agonist at various $GABA_AR$ subunit configurations. The efficacy of carisoprodol was significantly less at α3-containing receptors relative to $GABA_ARs$ containing α1, α2, or α4 subunit isoforms. *, $p < 0.05$.

Receptor Configuration	Maximal Efficacy (%)	Sample Size
$\alpha 1\beta 2$	45.4 ± 5.6	4-5
$\alpha 1\beta 2\gamma 2$	33.7 ± 5.9	4-8
$\alpha 2\beta 2\gamma 2$	30.9 ± 2.1	3-5
$\alpha 3\beta 2\gamma 2$	8.11 ± 2.8*	3-5
$\alpha 4\beta 2\gamma 2$	33.5 ± 4.7	4-6
$\alpha 1\beta 1\gamma 2$	58.0 ± 2.7	4-9

Figure III-7. Description of GABA ρ1/α1 chimeric subunits. A, schematic depicting the chimeric subunit generated via domain exchange between GABA ρ1 and α1 subunits using *KpnI* and *XhoI* restriction sites. The chimeric ρ1/α1 subunit is composed of ρ1 domains from the amino-terminal to the *KpnI* restriction site located at the start of transmembrane 2 (TM2); the remaining domains—TM2 through the *XhoI* restriction site—are derived from α1. B, amino acid alignment of wild-type GABA α1, ρ1, and chimeric ρ1/α1. Putative TM1 and TM2 domains are underlined and shown in boldface. The location of the *KpnI* restriction site, in the context of the amino acid sequence, is denoted by an arrow.

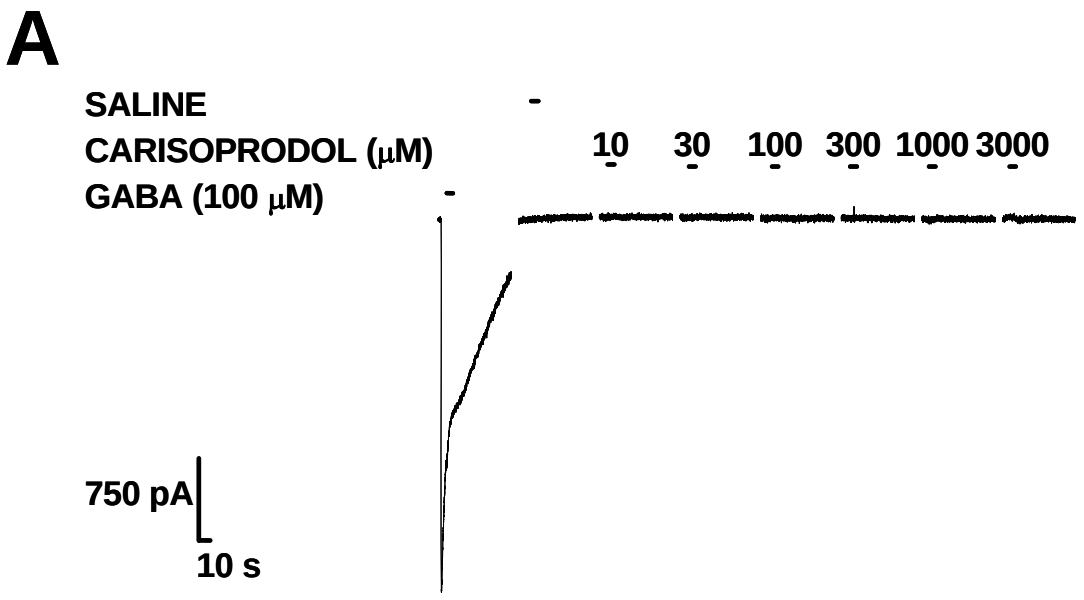
A



B

hGABA ρ1	RRHIF**FFLLQTYFPATLMVMLSWVSFWI**DRRA**VPARVPLGITTVLTMSTIITGV**
hGABA α1	KRKIGYF**VIQTYLPCIMTVILSQVSFWL**NRES**VPARTVFGVTTVLTMTTLSISA**
ρ1/α1	RRHIF**FFLLQTYFPATLMVMLSWVSFWI**DRRA**VPARTVFGVTTVLTMTTLSISA**

Figure III-8. Direct activation of homomeric ρ1/α1 GABA receptors by carisoprodol. A, representative traces demonstrating the agonistic actions of carisoprodol at homomeric ρ1/α1 chimeric receptors. Carisoprodol did not elicit inward currents at the concentrations tested. B, concentration-response data for direct activation of α1β2, wild-type ρ1 and chimeric ρ1/α1 receptors by carisoprodol. Each data point represents the mean ± S.E. of at least three cells (n = 3-6). Data points for α1β2 receptors were replotted for comparison. The direct gating effects of carisoprodol at chimeric receptors were not significantly different from those of wild-type ρ1 receptors ($p > 0.05$).


A
SALINE
CARISOPRODOL (μM)
GABA (100 μM)
10
30
100
300
1000
3000
750 pA
10 s

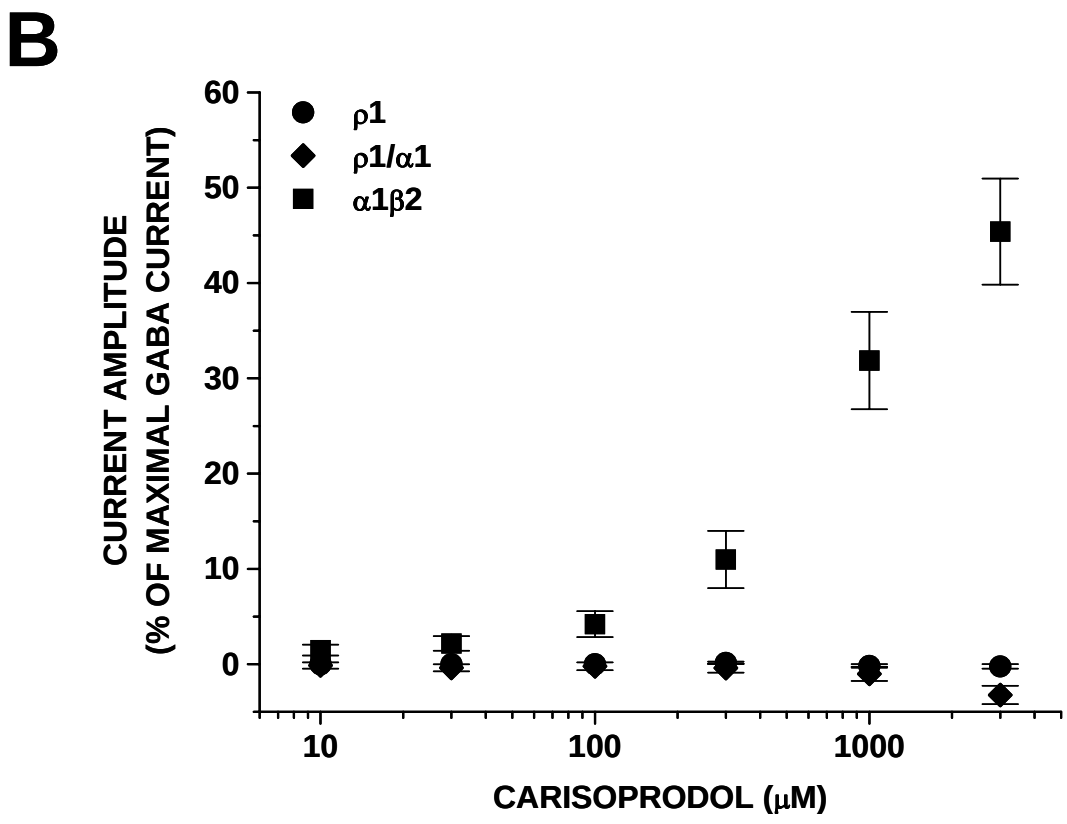
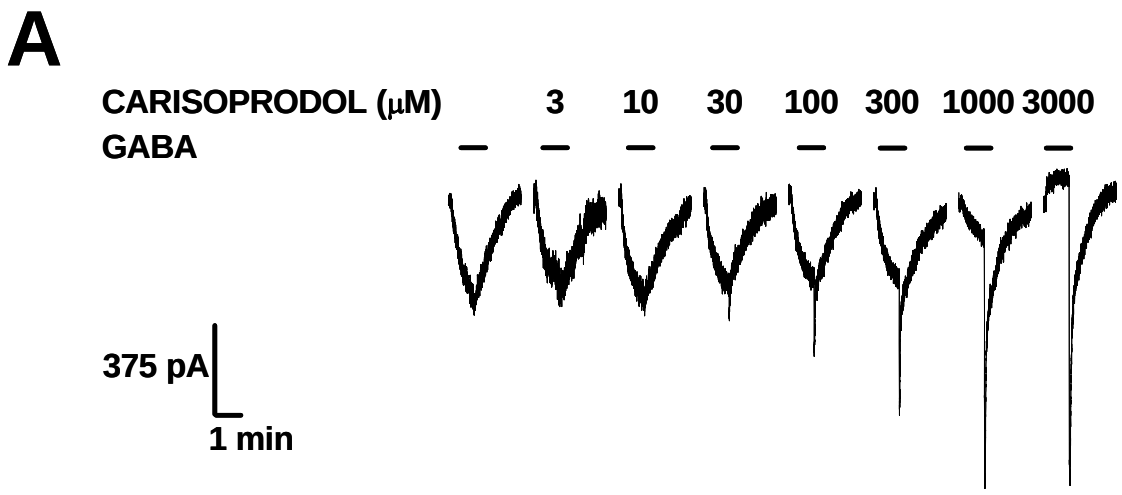

B
ρ1
ρ1/α1
α1β2
CURRENT AMPLITUDE
(% OF MAXIMAL GABA CURRENT)
60
50
40
30
20
10
0
10
100
1000
CARISOPRODOL (μM)

Figure III-9. Allosteric modulation of homomeric ρ1/α1 GABA receptors by carisoprodol. A, representative traces demonstrating the potentiation of GABA-gated currents from homomeric ρ1/α1 chimeric receptors by increasing concentrations of carisoprodol. Coapplication of carisoprodol concentrations (≥ 30 μM) inhibited currents relative to control (GABA EC_{20}). Upon termination of coapplication, offshoot currents were observed; these currents were used to determine the modulatory effects of carisoprodol. B, concentration-response curves for the allosteric effects of carisoprodol at α1β2, wild-type ρ1, and ρ1/α1 receptors. Each data point represents the mean ± S.E. of at least three cells (n = 3-7). Data for α1β2 receptors were replotted for comparison. Carisoprodol had no significant effect on GABA-gated currents from wild-type ρ1 receptors; however, millimolar concentrations of the drug had an inhibitory effect. Potentiation by carisoprodol was significantly greater for chimeric receptors than for wild-type ρ1 receptors. The EC_{50} for carisoprodol at chimeric receptors was 245 ± 32 μM. *, $p < 0.05$; **, $p < 0.01$ relative to wild-type ρ1 receptors.


A
CARISOPRODOL (μM)
3
10
30
100
300
1000
3000
GABA
375 pA
1 min

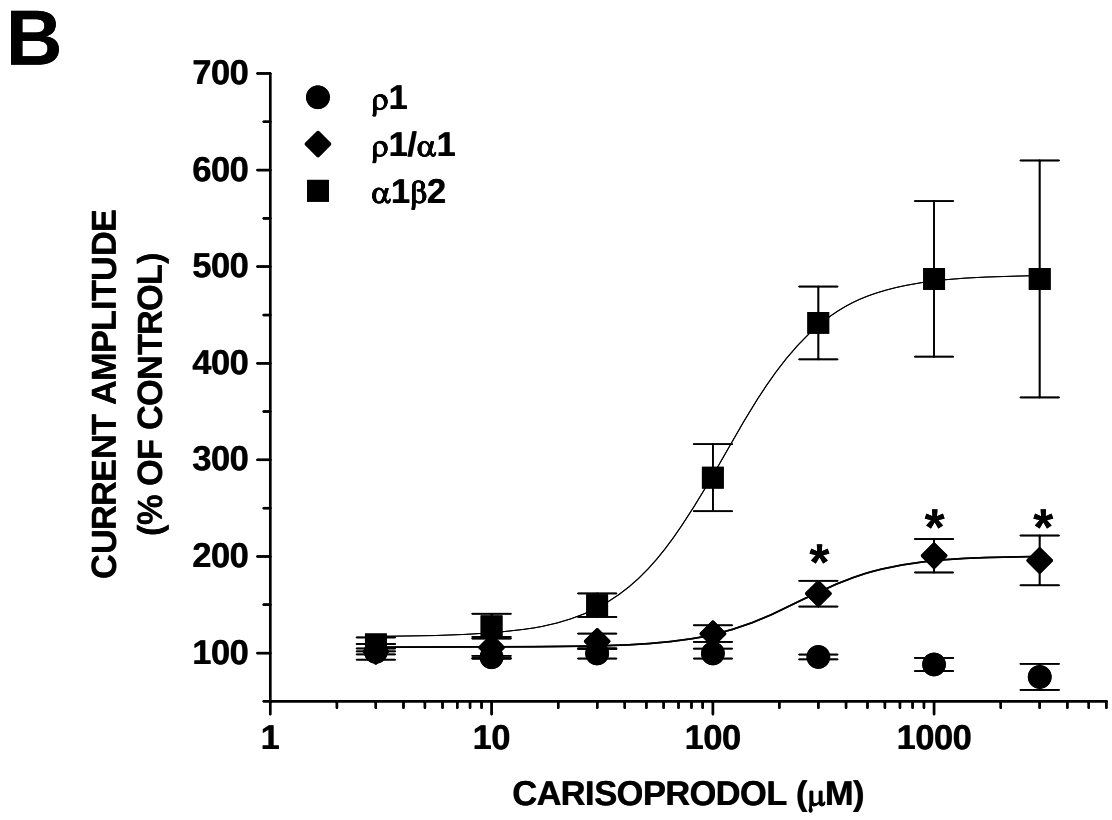
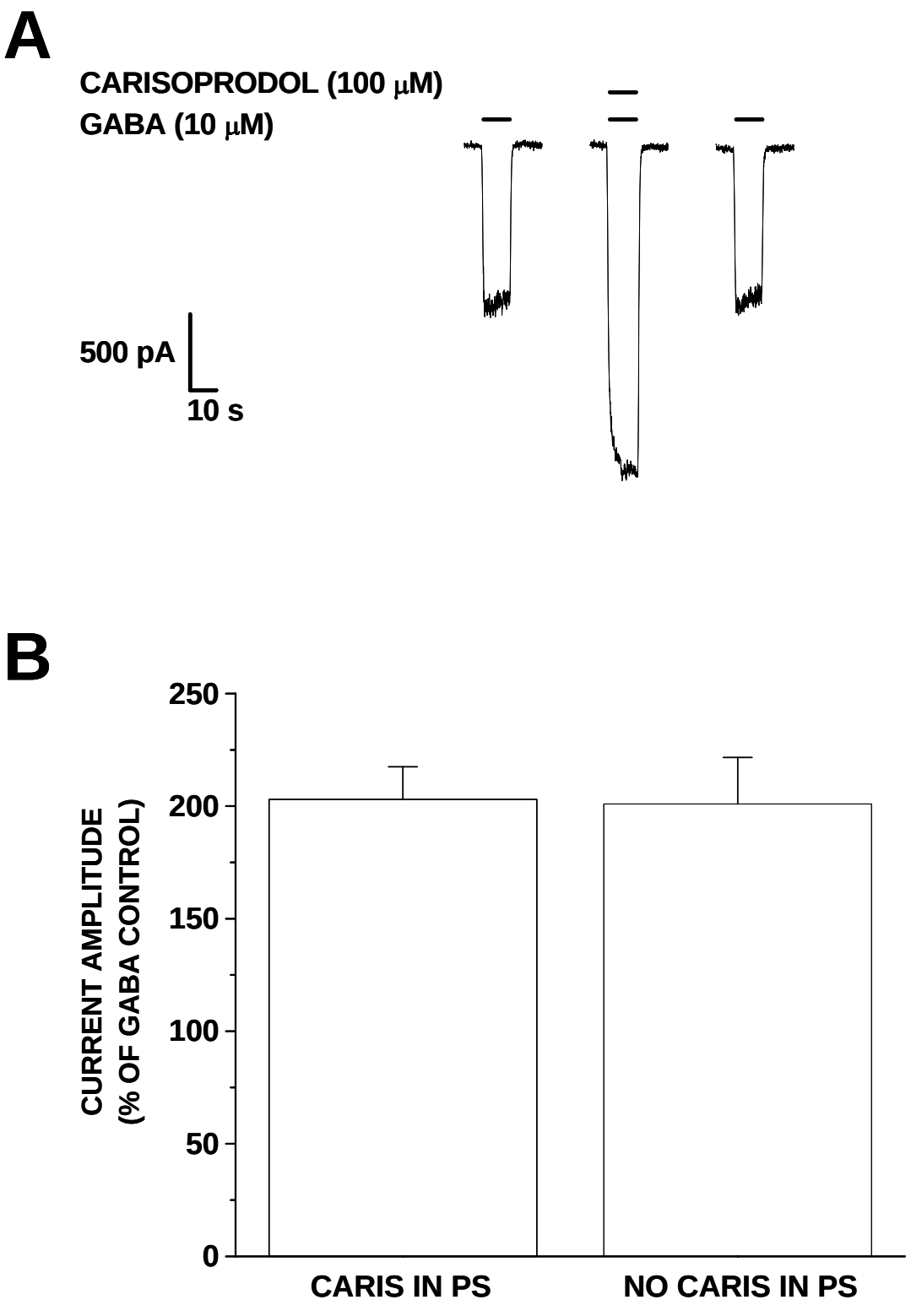

B
ρ1
ρ1/α1
α1β2
CURRENT AMPLITUDE
(% OF CONTROL)
700
600
500
400
300
200
100
1
10
100
1000
CARISOPRODOL (μM)
*
*
*

Figure III-10. Effects of intracellular application of carisoprodol on GABA-gated currents recorded from human α1β2γ2 $GABA_A$ receptors. A, representative traces obtained from a single cell stably expressing human α1β2γ2 $GABA_A$Rs with carisoprodol included in the pipette solution prior to coapplication. Enhancement of GABA-gated currents by carisoprodol was observed upon coapplication of GABA (EC_{20}) and carisoprodol despite the drug's access to intracellular domains of $GABA_A$ receptors. B, comparison of relative current amplitude recorded in the presence and absence of carisoprodol in the pipette solution. GABA-gated currents were potentiated equally with carisoprodol in the pipette solution (203.0 ± 14.4%, $n = 4$) or without inclusion of the drug (201.0 ± 20.6%, $n = 17$) ($p > 0.05$).


A
CARISOPRODOL (100 μM)
GABA (10 μM)
500 pA
10 s
B
CURRENT AMPLITUDE
(% OF GABA CONTROL)
250
200
150
100
50
0
CARIS IN PS
NO CARIS IN PS

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CHAPTER IV

SUMMARY AND DISCUSSION

Carisoprodol is a centrally-acting muscle relaxant introduced in 1959. To date, it remains one of the most frequently prescribed drugs in its class with approximately 10 million prescriptions issued in 2006 (United States Department of Justice Drug Enforcement Administration Office of Diversion Control, 2008). Although it has been in clinical use for decades, its true mechanism of action remains unclear. In light of numerous reports highlighting its increasing abuse, it is of crucial importance to determine the mechanisms underlying the therapeutic and illicit effects of this drug. Its primary metabolite, meprobamate, acts at $GABA_A$ receptors ($GABA_ARs$) in a barbiturate-like manner (Rho et al., 1997); however, the pharmacological profile of carisoprodol cannot be fully explained by its conversion to meprobamate. Given the structural similarities between carisoprodol and meprobamate, we hypothesized carisoprodol, too, may mediate similar effects via $GABA_ARs$.

Initial studies were conducted at the whole-animal level to assess the likelihood that carisoprodol acts via the GABAergic system. The discriminative stimulus effects of carisoprodol were comparable to those of the GABAergic ligands pentobarbital, chlordiazepoxide, and meprobamate, suggesting carisoprodol is mediating its effects, at least in part, via $GABA_ARs$. Although both benzodiazepines and barbiturates substituted for carisoprodol, its effects were more consistent with those of barbiturates since its

discriminative stimulus effects were antagonized by a barbiturate antagonist, but not an antagonist at the benzodiazepine site of the receptor. The questions remained: is pentobarbital substituting for carisoprodol because of the barbiturate-like actions of meprobamate, or is carisoprodol mediating its own barbiturate-like effects? At the whole-animal level, however, it is difficult to distinguish the effects of the parent drug from its metabolite because metabolism begins virtually instantaneously.

To circumvent the issues of metabolism, the effects of carisoprodol were examined using a simpler model system. Using stably- and transiently-transfected human embryonic kidney 293 (HEK293) cells expressing various configurations of $GABA_A$Rs, we demonstrated carisoprodol, like its metabolite, acts in a barbiturate-like manner at these receptors. The barbiturate binding site remains elusive, preventing identification of a true barbiturate site antagonist. In the absence of such an important pharmacological tool, carisoprodol-sensitivity was assessed at barbiturate-sensitive ρ1W328M receptors. Although the effects of carisoprodol were antagonized by a barbiturate antagonist, these receptors were carisoprodol-insensitive, suggesting distinct sites of action exist for carisoprodol and barbiturates on $GABA_A$Rs. Interestingly, reverse mutations in the β subunit (βM286W) reduce or abolish the effects of menthol (Watt et al., 2008), propofol (Korpi et al., 2002), and etomidate (Stewart et al., 2008). This suggests the domains involved in carisoprodol-mediated activity are not identical to domains utilized by these compounds as well.

Based upon subunit-dependence studies, potential sites of action for carisoprodol are located on α and/or β subunits. Using a chimeric strategy, transmembrane domains 2-

4 of the α subunit were identified as critical domains for the allosteric effects of the drug. The role of the β subunit was not investigated; however, insensitivity of ρ1/α1 subunits to direct gating by carisoprodol suggests the β subunit may be a large determinant in mediating the agonistic effects of this drug. More importantly, we were able to partially restore the modulation by carisoprodol independently of its direct gating effects. This indicates there may be multiple sites for carisoprodol on $GABA_A$Rs. Inhibitory effects, indicated by rebound currents, were restored as well. Rebound currents were observed with micromolar concentrations at ρ1/α1 receptors, but only with millimolar concentrations at αβ and αβγ configurations. It is unclear as to why sensitivity to the inhibitory effect was shifted, but increased sensitivity to desensitization observed with the chimeric receptors may contribute to the shift.

Carisoprodol abuse has been associated with dependence, tolerance, and withdrawal (Heacock and Bauer, 2004; Reeves et al., 2007). The central nervous system is constantly adapting to its environment; thus, it comes as no surprise that prolonged exposure to compounds elicits compensatory changes at the receptor level. Although we did not explore changes associated with chronic carisoprodol exposure, carisoprodol abuse is likely to elicit fundamental changes in the GABAergic system. With chronic opiate administration, $GABA_A$Rs in the ventral tegmental area transition from inhibitory to excitatory signaling, acting as a switch for the dopaminergic reward pathway and contributing to opiate dependence (Laviolette et al., 2004). Although opiates are not GABAergic compounds, these findings serve as precedence for the involvement of $GABA_A$Rs in the development of drug dependence.

Moreover, chronic use of drugs that act at $GABA_ARs$ modifies transmission in the GABAergic system and may lead to tolerance; the extent of modification depends upon the dose and duration of drug use (Korpi et al., 2002; Wafford, 2005). In general, $GABA_ARs$ are less sensitive to acute challenge following chronic exposure to a drug. This phenomenon may be due to uncoupling of allosteric sites (Ito et al., 1996), alterations in receptor turnover (Kumar et al., 2003; Pericic et al., 2003), or desensitization. Whether expression of a subunit is upregulated or downregulated in response to chronic use varies with its location in the brain (Wafford, 2005). In some studies, downregulation of the α1 subunit has been observed (Ito et al., 1996; Wafford, 2005). It is usually accompanied by compensatory upregulation of other subunits. In our studies, carisoprodol was most efficacious at α1-containing receptors. Preferential interaction with α1-containing receptors is significant in its own right as similar subunit-dependence of other drugs has been implicated in their abuse liability (Ator, 2005). Moreover, since carisoprodol is less efficacious at other receptor configurations, replacing α1 subunits with other isoforms may diminish the physiological effects of carisoprodol. The shift towards configurations that are less sensitive to carisoprodol's effects may contribute to tolerance because higher doses are needed to achieve the same effect. In addition, even subtle changes in inhibitory neurotransmission can have dire consequences. Such compensatory mechanisms associated with chronic activation of the GABAergic system are analogous to inhibitory dysregulation. Thus, abrupt removal of the drug is likely to precipitate withdrawal symptoms as the central nervous system attempts to restore normal inhibitory function.

As mentioned previously, the abuse liability of carisoprodol is often attributed to its primary metabolite. However, our findings demonstrate carisoprodol can mediate effects similar to those of its metabolite, and it does so with greater efficacy and potency. Furthermore, its pharmacological profile likely contributes to its abuse potential. Interestingly, meprobamate is a controlled substance at the federal level, but its parent drug is not. The United States Food and Drug Administration uses an eight-factor analysis to determine whether a drug warrants legal scheduling (Balster and Bigelow, 2003). Factors include actual or relative abuse potential; the historical and current pattern of abuse; the scope, duration, and significance of abuse; its risk to public health; its potential for dependence liability; whether the substance is a precursor of a controlled substance; the state of current knowledge concerning the substance; and scientific evidence of pharmacological effects. Whereas abuse potential, dependence, and potential health risks are well-documented, scientific evidence regarding carisoprodol's pharmacological effects is lacking in the literature. Thus, the findings reported herein provide much needed information regarding carisoprodol. In these studies, the pharmacological effects of carisoprodol were characterized in vivo and in vitro. Our findings provide insight into the mechanisms underlying the therapeutic and illicit effects of carisoprodol, and they suggest the nonscheduled status of carisoprodol should be reevaluated.

FUTURE DIRECTIONS

As mentioned previously, the pharmacological profile of carisoprodol is not identical to that of meprobamate. These differences may be explained by distinct subunit-dependent effects of the drugs or possibly distinct sites of action. Given the structural similarities between carisoprodol and meprobamate, these reasons may not seem likely. However, felbamate, a propanediol dicarbamate structurally similar to meprobamate and carisoprodol, potentiates GABA-gated currents, but has no agonistic activity at these receptors (Rho et al., 1997), suggesting slight differences in structure can lead to drastic changes in drug-receptor interactions. To elucidate potential differences between the parent drug and its metabolite, subunit-dependence and potential sites of action should be assessed for meprobamate in the manner used for carisoprodol.

In the current studies, we investigated potential interactions between carisoprodol and sites of action reported for other compounds. Although we focused on sites described for compounds in clinical use, endogenous neurosteroids are potent modulators of $GABA_AR$ function. Similar to other compounds, endogenous neurosteroids allosterically modulate and directly activate $GABA_ARs$ (Korpi et al., 2002). Recently, a series of point mutations was used to identify two sites of action for endogenous neurosteroids at $GABA_ARs$ (Hosie et al., 2006). These sites are located within the transmembrane domains of α and β subunits. Interestingly, the potentiating effects of neurosteroids are mediated by the α subunit whereas direct activation is dependent upon residues at the α/β interface (Hosie et al., 2006). This pattern is similar to what we predict for carisoprodol,

so it would be interesting to determine whether these sites are involved in mediating the effects of carisoprodol.

Moreover, we demonstrated carisoprodol acts in a subunit-dependent manner. These studies were conducted using synaptic configurations of $GABA_AR$s. However, the importance of tonic inhibition and its pharmacological modulation should not be overlooked. Although our data suggest α and β subunits are sufficient to mediate the allosteric and agonistic effects of carisoprodol, we cannot conclude that inclusion of a δ subunit will not affect the actions of this drug. Furthermore, the δ subunit preferentially assembles with α4 and α6 subunits in forebrain areas and cerebellar granule cells, respectively (Olsen and Sieghart, 2008). Thus, studying the effects of carisoprodol at native $GABA_AR$ subtypes will allow us to gain full appreciation of the regional effects of carisoprodol in the brain that potentially contribute to its clinical use and abuse liability.

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