Inhibition of Recombinant L-Type Voltage-Gated Calcium Channels by Positive Allosteric Modulators of GABA_A Receptors

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ABSTRACT

Benzodiazepines (BDZs) depress neuronal excitability via positive allosteric modulation of inhibitory GABA_A receptors (GABA_AR). BDZs and other positive GABA_AR modulators, including barbiturates, ethanol, and neurosteroids, can also inhibit L-type voltage-gated calcium channels (L-VGCCs), which could contribute to reduced neuronal excitability. Because neuronal L-VGCC function is up-regulated after long-term GABA_AR modulator exposure, an interaction with L-VGCCs may also play a role in physical dependence. The current studies assessed the effects of BDZs (diazepam, flurazepam, and desalkylflurazepam), allopregnanolone, pentobarbital, and ethanol on whole-cell Ba²⁺ currents through recombinant neuronal Ca_v1.2 and Ca_v1.3 L-VGCCs expressed with β_3 and $\alpha_2\delta$ -1 in HEK293T cells. Allopregnanolone was the most potent inhibitor (IC₅₀, ~10 μ M), followed by BDZs (IC₅₀, ~50 μ M), pentobarbital (IC₅₀, 0.3–1 mM), and ethanol (IC₅₀, ~300 mM). Ca_v1.3

channels were less sensitive to pentobarbital inhibition than Ca_v1.2 channels, similar to dihydropyridine (DHP) L-VGCC antagonists. All GABA_AR modulators induced a negative shift in the steady-state inactivation curve of Ca_v1.3 channels, but only BDZs and pentobarbital induced a negative shift in Ca_v1.2 channel inactivation. Mutation of the high-affinity DHP binding site (T1039Y and Q1043M) in Ca_v1.2 channels reduced pentobarbital potency. Despite the structural similarity between benzothiazepines and BDZs, mutation of an amino acid important for diltiazem potency (I1150A) did not affect diazepam potency. Although L-VGCC inhibition by BDZs occurred at concentrations that are possibly too high to be clinically relevant and is not likely to play a role in the up-regulation of L-VGCCs during long-term treatment, pentobarbital and ethanol inhibited L-VGCCs at clinically relevant concentrations.

Introduction

Benzodiazepines (BDZs) are clinically useful anxiolytics, sedatives, and anticonvulsants. The clinical effects of BDZs are attributed to their ability to allosterically enhance the inhibitory action of GABA_A receptors (GABA_AR). However,

there is also evidence that BDZs directly inhibit L-type voltage-gated calcium channels (L-VGCCs) in muscle and nerve cells at low micromolar concentrations (Yamakage et al., 1999; Xiang et al., 2008). Other GABA_AR modulators, such as the neurosteroid allopregnanolone (3 α -hydroxy-5 α -pregnan-20-one), the barbiturate pentobarbital, and ethanol, can also inhibit neuronal L-VGCCs (Messing et al., 1986; French-Mullen et al., 1993; Hu et al., 2007).

The interaction of GABA_AR modulators with L-VGCCs could play a role in their short-term effects to reduce neuronal excitability and may also contribute to the neuronal hyperexcitability seen after withdrawal from long-term drug exposure. It is noteworthy that long-term treatment with BDZs, barbiturates, and ethanol increases neuronal Ca²⁺ influx in vitro and in vivo, possibly through L-VGCCs (Messing et al., 1986; Rabbani and Little, 1999; Katsura et al., 2006, 2007; Xiang et al., 2008). An up-regulation of L-VGCC function during long-term treat-

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ABBREVIATIONS: BDZ, benzodiazepine; ANOVA, analysis of variance; BTZ, benzothiazepine; DMSO, dimethyl sulfoxide; DHP, dihydropyridine; L-VGCC, L-type voltage-gated calcium channel; PAA, phenylalkylamine; TEA, tetraethylammonium; WT, wild type; LB, Luria-Bertani; HEK, human embryonic kidney.

ment with the various GABA_AR modulators is implicated in contributing to withdrawal hyperexcitability (Dolin et al., 1987; Rabbani and Little, 1999; Xiang and Tietz, 2007; Xiang et al., 2008).

Although it is known that GABA_AR modulators inhibit L-VGCCs, there have been no studies to assess the selective effects of these drugs on the neuronal L-VGCC α_1 subtypes Ca_v1.2 and Ca_v1.3. By use of the whole-cell voltage-clamp technique, the current studies investigated the effects of BDZs (diazepam, flurazepam, and desalkylflurazepam), allopregnanolone, pentobarbital, and ethanol on recombinant L-VGCCs containing the neuronal L-type α_1 subunit, Ca_v1.2 or Ca_v1.3, along with β_3 and $\alpha_2\delta$ -1 subunits. We found that positive allosteric GABA_AR modulators reversibly inhibited both Ca_v1.2- and Ca_v1.3-containing L-VGCCs in a state-dependent manner. The order of potency was allopregnanolone > BDZs > pentobarbital > ethanol, with BDZs displaying 2- to 6-fold less potency than the benzothiazepine (BTZ) diltiazem. Previous studies reported that Ca_v1.3 channels were less sensitive to inhibition by dihydropyridines (DHPs), phenylalkylamines (PAAs), and BTZs than Ca_v1.2 channels (Koschak et al., 2001; Xu and Lipscombe, 2001; Tarabova et al., 2007). The structural similarities among BDZs and BTZs (see Fig. 1) suggested the possibility that L-VGCCs might also display differential sensitivity to GABA_AR modulators. Compared with Ca_v1.2, recombinant Ca_v1.3 channels were 1.5-fold less sensitive to inhibition by flurazepam, 3-fold less sensitive to inhibition by pentobarbital, and 3-fold more sensitive to inhibition by diltiazem. Studies using Ca_v1.2 mutants revealed that pentobarbital potency was reduced 3.5-fold by mutation of the high-affinity DHP binding site (T1039Y and Q1043M). Despite the aforementioned structural similarities, diltiazem did not compete with diazepam inhibition, and mutation of an amino acid within the BTZ binding site (I1150A) had no effect on diazepam potency. Based on their concentration-dependent effects, inhibition of L-VGCCs by pentobarbital and ethanol may contribute to their acute clinical actions. A direct interaction of pentobarbital and ethanol with L-VGCCs may also contribute to the adaptive up-regulation of L-VGCC function after

long-term use of these nonselective central nervous system depressants.

Materials and Methods

Cell Culture and Transient Transfection. SV40 large T-antigen stably transfected HEK293 (HEK293T) cells were used for transient expression of L-VGCCs. Cells were maintained at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium with GlutaMAX-I (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cells were grown to approximately 80% confluence and transfected with α_1 (Ca_v1.2 or Ca_v1.3), β_3 , $\alpha_2\delta$ -1, and monomeric red fluorescent protein plasmids (1:1:1:0.5 μ g, respectively) using Lipofectamine 2000 reagent (Invitrogen). One day after transfection, cells were trypsinized and replated on 35-mm culture dishes before electrophysiological recording 2 to 3 days after transfection. With the exception of experiments with mutated Ca_v1.2, all experiments used mouse neuronal Ca_v1.2 (Helton et al., 2005), rat neuronal Ca_v1.3 (+exon11, Δ exon32, +exon42a; Xu and Lipscombe, 2001), rat neuronal β_3 , and rat neuronal $\alpha_2\delta$ -1 (Lin et al., 2004) subunit plasmids, which were generously provided by Dr. Diane Lipscombe (Brown University, Providence, RI). The monomeric red fluorescent protein plasmid was generously provided by Dr. Zi-Jian Xie (University of Toledo College of Medicine, Toledo, OH). A DHP binding site (T1039Y and Q1043M) mutant Ca_v1.2 (Hockerman et al., 2000) and its wild-type form from rat brain (Snutch et al., 1991) were generously provided by Dr. Gregory Hockerman (Purdue University, West Lafayette, IN).

Site-Directed Mutagenesis. A mutation previously shown to be important for diltiazem potency, I1150A (Hockerman et al., 2000), was inserted into rat brain wild-type Ca_v1.2 using the QuikChange II XL site-directed mutagenesis kit (Stratagene Cloning Systems, La Jolla, CA) and the following primers: 5'-GTGGAGATCTCCATCTTCTTCGCGATCTACATCATCATCATTGCC-3' and 5'-GGCAATGATGATGATGTAGATCGCGAAGAAGATGAGATCTC-3' (I1150A mutation in bold). The manufacturer's protocol was followed with a few modifications. In brief, 50 ng of Ca_v1.2 plasmid template was used in polymerase chain reaction with the following parameters: one cycle: 95°C, 30 s; 18 cycles: 95°C, 50 s, 60°C, 50 s, 68°C, 18 min (1.5 min/kb); one cycle: 68°C, 7 min. After DpnI treatment, the entire polymerase chain reaction product was ethanol-precipitated, resuspended in 5 μ l of ddH₂O, and transformed into XL10-Gold ultracompetent cells. Transformed cells were plated on LB agar dishes containing 100 μ g/ml

Fig. 1. Chemical structures of the compounds used in the current studies (with the exception of ethanol). Note the similarity in the core structure of the BTZ, diltiazem to the BDZs. Structures were created using CS ChemDraw software, version 5.0 (CambridgeSoft Corporation, Cambridge, MA).

ampicillin for >16 h at 37°C. Single colonies were grown for >16 h in LB broth containing 100 µg/ml ampicillin; plasmid DNA then was extracted using phenol/chloroform/isoamyl alcohol (25:24:1). Mutation was confirmed by sequencing (Eurofins MWG Operon, Huntsville, AL). A clone positive for the mutation was grown in LB broth containing 100 µg/ml ampicillin, and the plasmid DNA was purified using QIAGEN Plasmid Maxi Kit (QIAGEN Inc., Valencia, CA).

Recording Solutions. The external solution (300 mOsm) contained 135 mM choline Cl, 1 mM MgCl₂, 5 mM BaCl₂, and 10 mM HEPES, adjusted to pH 7.4 with TEOH. As indicated in the results, a number of experiments used 2 mM Ca²⁺ as the charge carrier instead of 5 mM Ba²⁺. Glass-recording pipettes were pulled from micro-hematocrit capillary tubes (Thermo Fisher Scientific, Pittsburgh, PA) to a resistance of 2 to 5 MΩ. To facilitate seal formation, recording pipette tips were filled with a lower osmolarity internal solution (270 mOsm) lacking ATP. Pipettes were then back-filled with internal solution (285 mOsm) containing 135 mM CsCl, 4 mM MgCl₂, 4 mM ATP, 10 mM HEPES, 10 mM EGTA, and 1 mM EDTA, adjusted to pH 7.2 with TEOH.

Whole-Cell Voltage-Clamp Electrophysiology. Red-fluorescing cells were visualized on an Olympus 1×51 fluorescent microscope (Olympus, Center Valley, PA). Whole-cell currents were recorded with an Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA). Data were sampled at 2 to 10 kHz, low pass-filtered at 1 kHz, and analyzed using pClamp v9.2 (Molecular Devices). After pipette and membrane capacitance compensation, the series resistance was corrected by 80 to 90% with a 20- to 100-µs lag time. Cells with a series resistance greater than 20 MΩ were not used for analysis. After whole-cell break-in, Ba²⁺ currents were elicited in voltage-clamp mode by stepping from a holding potential (V_h) of -80 mV to test potentials (V_t) of 0 (Ca_v1.2) or -25 mV (Ca_v1.3) for 200 ms. Current rundown, plotted as total charge transfer elicited at 10-s intervals (or higher frequencies, as indicated), was fitted with a one- or two-phase exponential decay curve. Recordings using this protocol were not leak-subtracted.

Drugs were applied by gravity flow and washed out rapidly by external solution applied with a separate pipette. Percentage inhibition was calculated with the following equation: % inhibition = $(1 - [I_{\text{actual}}/I_{\text{expected}}]) \times 100$, where I_{actual} is the actual current remaining during steady-state inhibition by the drug, and I_{expected} is the expected current remaining at the same time point based on the exponential decay fit of the current rundown. Concentration-response curves were fitted with a four-parameter logistic equation: % inhibition = % min + (% max - % min) / $[1 + 10^{(\log IC_{50} - \log[\text{drug}]) \times n_H}]$, where % min represents percentage inhibition by vehicle, % max represents maximal percentage inhibition by the drug, IC_{50} was the drug concentration that yielded half-maximal inhibition, and n_H was the Hill slope. % min was constrained to zero because average inhibition by vehicle was not significantly different from zero. Current decay was measured as the ratio of current remaining after a 200-ms depolarization relative to peak current, which was defined as the r200 value, similar to previous studies (Cai et al., 1997).

For voltage-dependent activation and inactivation protocols, data obtained from each voltage step was leak-subtracted online by removing the sum of the current obtained from four consecutive waveforms of 1/4 amplitude and opposite polarity to the test potentials (a P/4 protocol). Voltage dependence of activation was determined using either 5 mM Ba²⁺ or 2 mM Ca²⁺ as charge carriers. Channels were activated with 200-ms voltage steps from $V_h = -80$ mV to various test potentials, $V_t = -75$ to +40 mV (Ca_v1.3) or $V_t = -50$ to +65 mV (Ca_v1.2) in 5-mV steps. Peak current plotted against the test potential was fitted with the following equation: $I = G_{\text{max}}(V_t - V_{\text{rev}}) / [1 + \exp[(V_{50} - V_t)/k]]$, where G_{max} is the maximal conductance of the cell, V_{rev} is the reversal potential, V_{50} is the potential for half-maximal activation, and k is the slope factor of activation. As the test potential approached V_{rev} , currents deviated from the linear relationship predicted by this equation. Therefore, currents obtained

at potentials that were more positive than 25 mV were excluded from fitting analysis.

The voltage dependence of L-VGCC steady-state inactivation was determined by stepping to various conditioning pulse potentials for 1.5 s, preceded by a control pulse to -25 (Ca_v1.3) or 0 mV (Ca_v1.2) and followed by a test pulse to -25 (Ca_v1.3) or 0 mV (Ca_v1.2). The current elicited by the control pulse was used to control for current rundown. Inactivation curves were plotted as normalized peak currents (test peak/control peak) versus the conditioning pulse potential and fit with the Boltzmann equation: $I_n = I_{n(\text{min})} + (I_{n(\text{max})} - I_{n(\text{min})}) / [1 + \exp[(V_{50} - V_t)/k]]$, where I_n is the normalized peak current, $I_{n(\text{min})}$ is minimal normalized current, $I_{n(\text{max})}$ is maximal normalized current, V_{50} is the potential for half-maximal inactivation, and k is the slope factor of inactivation. Drug effects on steady inactivation were tested by directly incubating cells in external solution containing the drug for at least 5 min before running the inactivation protocol.

Drugs. The three BDZs tested were the prototype diazepam, the relatively water-soluble flurazepam, and its major bioactive metabolite in rat and man, desalkylflurazepam (Lau et al., 1987). Flumazenil, a competitive BDZ antagonist, was also tested. Stocks of nimodipine, diazepam, desalkylflurazepam, flumazenil, and allopregnanolone were made in dimethyl sulfoxide (DMSO). Stocks of (+)-*cis*-diltiazem, flurazepam, pH 5.8, and racemic pentobarbital were made in dH₂O. Drug stocks were dissolved into external solution by vortexing. Drugs dissolved in DMSO were diluted at least 1:1000, with the exception that 300 mM diazepam and desalkylflurazepam stocks were diluted 1:300 to achieve the final concentration of 1 mM. DMSO diluted 1:1000 or 1:300 in external solution was used as a vehicle control for some experiments and resulted in negligible inhibition of currents (mean inhibition typically <5% in each experiment). Absolute ethanol (empirical density of 0.75 g/ml) was dissolved directly into external solution. Flurazepam was generously provided by the Drug Supply Program of the National Institutes of Health National Institute on Drug Abuse. Flumazenil and desalkylflurazepam were obtained from Roche (Nutley, NJ). Allopregnanolone was obtained from Tocris Bioscience (Ellisville, MO). Ethanol was obtained from Pharmco-AAPER (Shelbyville, KY). All other drugs were obtained from Sigma-Aldrich (St. Louis, MO).

Statistical Analyses. All curve fits were made using Prism version 5 (GraphPad Software, Inc., La Jolla, CA). With the exception of curve-fit parameters, all data are reported as mean ± S.E.M. Curve-fit parameters (i.e., IC_{50} , Hill slope, V_{50} , and slope factor k) are reported as the best-fit value to the experimental data set with the associated 95% confidence interval from a Levenberg-Marquardt least-squares fitting algorithm. Nonoverlapping confidence intervals between values were accepted as significant differences ($p < 0.05$). A two-tailed Student's *t* test was used to compare percentage inhibition by select drug concentrations between two experimental conditions. Threshold drug concentrations were defined as the lowest concentration to yield significant inhibition measured by repeated measures one-way ANOVA with post hoc comparison by Dunnett's test. Mean r200 values were analyzed by repeated measures one-way ANOVA with post hoc comparison by Dunnett's test or by a paired *t* test. A *p* value of 0.05 or less was considered significant.

Results

Functional Characterization of Recombinant L-VGCCs.

As a confirmation that the transient transfection of HEK293T cells resulted in functional L-VGCCs, the voltage dependence of activation was evaluated for both Ca_v1.2- and Ca_v1.3-containing L-VGCCs and was similar to previous reports for these channels (Koschak et al., 2001; Xu and Lipscombe, 2001). Figure 2A shows Ca_v1.3 and Ca_v1.2 L-VGCC current-voltage responses, with peak 5 mM Ba²⁺ or 2 mM Ca²⁺ currents (picoamperes) normalized to cell capacitance (picofarads). It is noteworthy that activation of Ca_v1.3 channels occurred at more negative potentials than Ca_v1.2 chan-

Fig. 2. Functional expression of recombinant L-VGCCs in HEK293T cells. **A**, the average current density (picoamperes/picofarads) of recombinant $\text{Ca}_v1.3$ (squares) and $\text{Ca}_v1.2$ (circles) L-VGCCs expressed with β_3 and $\alpha_2\delta-1$ subunits in HEK293T cells is shown plotted against the membrane test potential using either 5 mM Ba^{2+} (open symbols) or 2 mM Ca^{2+} (filled symbols) as the charge carrier. Using Ba^{2+} as the charge carrier, activation V_{50} values were -41.3 ± 2.1 ($n = 15$) and -12.9 ± 3.8 mV ($n = 6$) for $\text{Ca}_v1.3$ and $\text{Ca}_v1.2$ channels, respectively. Using Ca^{2+} as the charge carrier, activation V_{50} values were -35.3 ± 4.3 ($n = 6$) and -12.5 ± 5.1 mV ($n = 8$) for $\text{Ca}_v1.3$ and $\text{Ca}_v1.2$ channels, respectively. **B**, average peak Ba^{2+} currents through $\text{Ca}_v1.3$ channels in the absence (open squares, $n = 3$) and presence of 1 μM (half-filled squares, $n = 3$) or 10 μM nimodipine (filled squares, $n = 3$) and $\text{Ca}_v1.2$ channels in the absence (open circles, $n = 2$) and presence of 0.1 μM (half-filled circles, $n = 2$) or 1 μM nimodipine (filled circles, $n = 2$).

nels. Half-maximal inactivation also occurred at more negative potentials in $\text{Ca}_v1.3$ compared with $\text{Ca}_v1.2$ channels (see Fig. 6 and Table 3). As an additional test of channel integrity and appropriate pharmacologic response, channels were inhibited by the DHP, nimodipine, in a concentration-dependent manner. As shown in Fig. 2B, $\text{Ca}_v1.3$ channels were less sensitive to inhibition by nimodipine than $\text{Ca}_v1.2$ channels, consistent with previous reports of recombinant L-VGCC inhibition by DHPs (Koschak et al., 2001; Xu and Lipscombe, 2001).

GABA_A Modulators Reversibly Inhibit Recombinant L-VGCCs. In control cells transfected with $\text{Ca}_v1.3$ L-VGCCs ($n = 4$) in which no drug was applied, the average percentage current remaining 2, 5, and 10 min from the start of recording was 71 ± 11 , 57 ± 13 , and $42 \pm 9\%$, respectively. This extent of rundown was similar to that of $\text{Ca}_v1.2$ L-VGCCs in this study and as reported previously (Kepplinger et al., 2000). There was no apparent correlation between the extent of rundown in different cells and drug concentration-response, suggesting that the mechanisms causing the rundown phenomenon were not a significant factor in modifying L-VGCC inhibition by GABA_A modulators. As seen in Fig. 3A, 5 mM Ba^{2+} currents through $\text{Ca}_v1.3$ L-VGCCs were inhibited in a concentration-dependent manner by diazepam. Inhibition by all drugs tested showed at least partial current

Fig. 3. **A**, concentration-dependent inhibition of $\text{Ca}_v1.3$ Ba^{2+} currents by diazepam. $\text{Ca}_v1.3$ L-VGCCs were activated by 200-ms test pulses to -25 mV every 10 s from a holding potential of -80 mV. The total charge transfer is normalized to its initial value and plotted as a function of time post break-in. Diazepam inhibited $\text{Ca}_v1.3$ currents in a concentration-dependent manner and was reversible upon washout. VEH represents 1:1000 DMSO. Exemplar current traces in the presence and absence of diazepam are shown above. Normalized, rundown-corrected time-courses of $\text{Ca}_v1.2$ (**B**) and $\text{Ca}_v1.3$ (**C**) L-VGCC inhibition by diltiazem (filled circles, 30 and 10 μM for $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$, respectively), flurazepam (open triangles, 30 μM), pentobarbital (half-filled diamonds, 0.3 and 1 mM for $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$, respectively), ethanol (half-filled circles, 300 mM), and allopregnanolone (half-filled squares, 10 μM). Time point zero represents current just before drug application. Because the time courses were taken from the middle of a concentration-response, the current at time point zero had not in all cases completely recovered from the prior concentration of the drug applied. Thus, inhibition that occurs during the first pulse after drug application (10 sec) does not accurately represent closed-channel block. Numbers of cells tested are the same as shown in Table 1.

recovery upon washout. Figure 3, B and C, illustrates that inhibition of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ L-VGCCs, respectively, was rapid for most GABA_A modulators tested. For BDZs, pentobarbital, and ethanol, a maximal response was typically obtained by the third activating pulse after drug application. Inhibition was slower for allopregnanolone and diltiazem, generally requiring at least four activation pulses to reach maximal inhibition. For allopregnanolone, inhibition was slower in $\text{Ca}_v1.2$ channels, and for diltiazem, inhibition was

Fig. 4. Concentration-response curves representing inhibition of Ca_v1.2 (A) and Ca_v1.3 (B) L-VGCCs by GABA_AR modulators. Diltiazem (filled circles) is shown for comparison. The rank order of potency among GABA_AR modulators was allopregnanolone (half-filled squares) > BDZs > pentobarbital (half-filled diamonds) > ethanol (half-filled circles). The BDZs tested, diazepam (open circles), flurazepam (open triangles), and desalkylflurazepam (open squares), were approximately 2- to 6-fold less potent than diltiazem. Ca_v1.2 and Ca_v1.3 L-VGCCs had similar sensitivities to inhibition by diazepam, desalkylflurazepam, allopregnanolone, and ethanol. Ca_v1.3 channels were 1.5-fold less sensitive to flurazepam, 3-fold less sensitive to pentobarbital, and 3-fold more sensitive to diltiazem than Ca_v1.2 channels. VEH represents inhibition by vehicle. IC₅₀ values, Hill slopes, and numbers of cells tested are reported in Table 1.

slower in Ca_v1.3 channels. Inhibition of Ca_v1.3 channels by desalkylflurazepam was similar when 2 mM Ca²⁺ was used as the charge carrier (data not shown).

TABLE 1

Parameters for concentration-dependent inhibition of L-VGCCs by diltiazem and GABA_AR modulators

IC₅₀ values can be considered absolute for all drugs with the exception of allopregnanolone, which has a relative IC₅₀ given that maximal L-VGCC inhibition by allopregnanolone was less than 100%.

Drug	Ca _v 1.2					Ca _v 1.3				
	Threshold	IC ₅₀	IC ₅₀ 95% CI	Hill Slope	<i>n</i>	Threshold	IC ₅₀	IC ₅₀ 95% CI	Hill Slope	<i>n</i>
		μM					μM			
Diltiazem	1	26*	21–32	1.0 ± 0.2	3	1	8	6–12	1.1 ± 0.3	4
Diazepam	30	49	38–64	1.5 ± 0.4	4	10	53	20–138	1.3 ± 0.7	3
Flurazepam	10	48*	40–57	1.2 ± 0.2	4	10	72	59–88	1.2 ± 0.2	3
Desalkylflurazepam	10	55	45–68	1.3 ± 0.3	3	10	37	29–48	1.1 ± 0.3	6
Diazepam + 100 μM flumazenil	N.D.	66	56–77	1.5 ± 0.3	2	N.D.	N.D.	N.D.	N.D.	
Diazepam + 300 μM flumazenil	10	56	39–79	1.1 ± 0.4	5	N.D.	N.D.	N.D.	N.D.	
Desalkylflurazepam + 100 μM flumazenil	N.D.	N.D.	N.D.	N.D.		10	65†	51–82	1.3 ± 0.3	8
Allopregnanolone	3	7	3–16	0.8 ± 0.4	5	10	11	4–30	1.0 ± 0.6	3
Pentobarbital	100	336*	266–424	1.0 ± 0.2	3	100	1053	860–1291	1.5 ± 0.4	4
Ethanol	30,000	182,523	46,091–722,802	1.4 ± 0.9	3	30,000	276,452	35,612–2,146,000	1.0 ± 0.5	3

* *P* < 0.05, compared with respective Ca_v1.3 IC₅₀ value.

† *P* < 0.05, compared with Ca_v1.3 desalkylflurazepam IC₅₀ value in the absence of flumazenil.

Figure 4, B and C, shows the concentration-response relationship of diltiazem and all GABA_AR modulators tested in Ca_v1.2 and Ca_v1.3 L-VGCCs, respectively. Threshold concentrations, IC₅₀ values, Hill slopes, and the numbers of cells tested are reported in Table 1. BDZs had relatively similar potencies, with the exception that Ca_v1.3 channels were 1.5-fold less sensitive to flurazepam than Ca_v1.2 channels (*p* < 0.05). The BDZs were approximately 2- to 6-fold less potent than diltiazem, with Ca_v1.3 channels displaying 3-fold greater sensitivity to diltiazem than Ca_v1.2 channels (*p* < 0.05). The BDZ receptor-competitive antagonist flumazenil had no effect to inhibit Ca_v1.2 or Ca_v1.3 channels at concentrations up to 300 μM (data not shown). Flumazenil (100–300 μM) was unable to antagonize diazepam inhibition of Ca_v1.2 channels (Table 1). It is interesting that coapplication with 100 μM flumazenil resulted in a 1.8-fold reduction in desalkylflurazepam potency in Ca_v1.3 channels (Table 1). Although allopregnanolone was the most potent GABA_AR modulator tested (approximately 6-fold more potent than BDZs), it incompletely inhibited L-VGCCs and was less efficacious at inhibiting Ca_v1.3 than Ca_v1.2 channels (29 versus 63% maximal response, respectively). Pentobarbital was approximately 7- to 21-fold less potent than BDZs. Ca_v1.3 channels were 3-fold less sensitive to pentobarbital than Ca_v1.2 channels (*p* < 0.05). Ethanol was the least potent drug tested, approximately 4500-fold less potent than BDZs.

GABA_AR Receptor Modulators Enhance L-VGCC Current Decay. Inhibition by various L-VGCC antagonists is known to depend on the state of the channel (Lee and Tsien, 1983; Bean, 1984; Uehara and Hume, 1985; Cai et al., 1997; Hockerman et al., 1997). In particular, antagonists can stabilize the inactive state or display enhanced binding during channel opening (Hockerman et al., 1997), resulting in increased current decay during prolonged depolarization. Figure 5A illustrates that the decay of Ca_v1.3 Ba²⁺ current is enhanced by diazepam. Figure 5B shows that the ratio of residual current at 200 ms to peak current (r200) was reduced in a concentration-dependent manner by diazepam. Table 2 summarizes L-VGCC r200 values in the presence and absence of diltiazem and GABA_AR modulators at approximately equipotent concentrations. A significant decrease in the r200 value of both L-VGCCs was observed for all GABA_AR modulators, with the exception of flurazepam at

Fig. 5. Diazepam enhances L-VGCC current decay. **A**, current traces from the same cell shown in Fig. 3A are normalized to peak current. Diazepam enhanced $\text{Ca}_v1.3$ Ba^{2+} current decay during prolonged depolarization in a concentration-dependent manner. **B**, the ratio of residual current at 200 ms normalized to peak current (r200 value) is shown for various diazepam concentrations. Diazepam (30 and 100 μM) significantly reduced the r200 value compared with vehicle ($n = 3$). This effect was reversed upon washout. *, $p < 0.05$.

$\text{Ca}_v1.3$ channels and ethanol at $\text{Ca}_v1.2$ channels because of variability and the low numbers of cells tested. By comparison, diltiazem at its IC_{50} concentration resulted in substantially less enhancement of L-VGCC current decay.

GABA_AR Modulators Induce a Negative Shift in L-VGCC Steady-State Inactivation. The voltage dependence of L-VGCC steady-state inactivation was measured as described in the methods in the presence and absence of drug. As illustrated in Fig. 6A, there was a significant shift in the voltage dependence of $\text{Ca}_v1.2$ L-VGCC steady-state inactivation toward more negative potentials in the presence of approximately equipotent concentrations of diazepam (60 μM) and pentobarbital (300 μM) but not allopregnanolone (30 μM) or ethanol (300 mM). The potentials of half-maximal inactivation (V_{50}) in the presence and absence of diltiazem

and GABA_AR modulators are reported in Table 3. It is noteworthy that there was a concentration-dependent effect of diazepam to shift $\text{Ca}_v1.2$ inactivation toward more negative potentials, which was significant at 60 and 100 μM . Desalkylflurazepam (100 μM) caused a significant shift in the $\text{Ca}_v1.2$ inactivation curve but was smaller in magnitude than the shift by 100 μM diazepam. A similar result was obtained with 30 μM diltiazem. Consistent with data obtained from 0.1-Hz activation, flumazenil (300 μM) did not shift $\text{Ca}_v1.2$ inactivation and was unable to antagonize the shift by 100 μM diazepam.

Figure 6B shows a drug-induced shift of $\text{Ca}_v1.3$ steady-state inactivation toward more negative potentials in the presence of approximately equipotent concentrations of diazepam (60 μM), allopregnanolone (30 μM), pentobarbital (1 mM), and ethanol (300 mM), although ethanol was weakly effective relative to the other drugs. As shown in Table 3, 100 μM desalkylflurazepam caused a significant shift in the $\text{Ca}_v1.3$ inactivation curve but was of smaller magnitude than the shift by 100 μM diazepam. Diltiazem at its IC_{50} concentration of 10 μM was completely ineffective to shift the $\text{Ca}_v1.3$ inactivation curve.

Inhibition of $\text{Ca}_v1.2$ Channels by Desalkylflurazepam Is State- and Frequency-Dependent. To test the hypothesis that BDZ potency is enhanced by channel inactivation, the inhibitory action of desalkylflurazepam was measured on channels activated from a more depolarized holding potential resulting in a greater extent of channels in the inactive state. As illustrated in Fig. 7A, desalkylflurazepam inhibited $\text{Ca}_v1.2$ channels 2-fold more potently when channels were activated from a more depolarized holding potential, with a significantly greater degree of inhibition occurring at 30 and 100 μM desalkylflurazepam. For $\text{Ca}_v1.3$ channels, which inactivate at more negative membrane potentials, a holding potential of -60 mV was used. As illustrated in Fig. 7B, desalkylflurazepam was similarly potent when $\text{Ca}_v1.3$ channels were activated from a more depolarized holding potential, and the degree of inhibition was not significantly different at any concentration at or above threshold.

To test whether BDZ potency might be similarly enhanced by the frequency of channel activation, desalkylflurazepam potency was tested on L-VGCCs activated at 1 Hz. As illustrated in Fig. 7C, desalkylflurazepam inhibited $\text{Ca}_v1.2$ L-VGCCs 2-fold more potently when channels were activated at

TABLE 2

GABA_AR modulators enhance L-VGCC current decay

Diltiazem (30 and 10 μM) was analyzed for $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$, respectively. Pentobarbital (0.3 and 1 mM) was analyzed for $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$, respectively. r200 is the ratio of current remaining after a 200-ms depolarization relative to the peak current. Control r200 values were obtained in the same cells in the absence of drug. Per. Dec. represents average percentage drug-induced decrease in the r200 value.

Drug	$\text{Ca}_v1.2$				$\text{Ca}_v1.3$			
	CON r200	Drug r200	Per. Dec.	n	CON r200	Drug r200	Per. Dec.	n
			%				%	
30/10 μM Diltiazem	0.53 $\pm$ 0.03	0.45 $\pm$ 0.06	15 $\pm$ 7	3	0.73 $\pm$ 0.06	0.76 $\pm$ 0.05	-5 $\pm$ 4	4
30 μM Diazepam	0.49 $\pm$ 0.09	0.26 $\pm$ 0.08**	50 $\pm$ 7	4	0.68 $\pm$ 0.07	0.49 $\pm$ 0.07*	28 $\pm$ 5	3
30 μM Flurazepam	0.35 $\pm$ 0.09	0.25 $\pm$ 0.07*	31 $\pm$ 4	4	0.79 $\pm$ 0.07	0.64 $\pm$ 0.10	19 $\pm$ 6	3
30 μM Desalkylflurazepam	0.36 $\pm$ 0.08	0.26 $\pm$ 0.07*	30 $\pm$ 5	3	0.49 $\pm$ 0.07	0.35 $\pm$ 0.08**	32 $\pm$ 7	6
30 μM Allopregnanolone	0.50 $\pm$ 0.08	0.24 $\pm$ 0.06***	56 $\pm$ 5	5	0.66 $\pm$ 0.11	0.57 $\pm$ 0.11*	15 $\pm$ 3	3
0.3/1 mM Pentobarbital	0.44 $\pm$ 0.10	0.19 $\pm$ 0.06*	58 $\pm$ 5	3	0.69 $\pm$ 0.09	0.37 $\pm$ 0.11**	49 $\pm$ 8	4
300 mM Ethanol	0.29 $\pm$ 0.12	0.12 $\pm$ 0.05	62 $\pm$ 4	3	0.54 $\pm$ 0.04	0.45 $\pm$ 0.04**	16 $\pm$ 2	3

* $P < 0.05$, drug versus CON r200, paired t test.

** $P < 0.01$, drug versus CON r200, paired t test.

*** $P < 0.001$, drug versus CON r200, paired t test.

Fig. 6. L-VGCC voltage-dependent steady-state inactivation curves in the presence and absence of GABA_AR modulators. Normalized peak currents are plotted against the membrane potential of a 1.5-s conditioning pulse. **A**, the Ca_v1.2 steady-state inactivation curve was significantly shifted toward more negative potentials by approximately equipotent concentrations of diazepam (open circles, 60 μ M) and pentobarbital (half-filled diamonds, 300 μ M) but not by allopregnanolone (half-filled squares, 30 μ M) or ethanol (half-filled circles, 300 mM). The V_{50} values (potential of half-maximal inactivation) in the presence and absence (filled circles) of each drug are plotted in the inset. Error bars were omitted for clarity in both A and B. *, $p < 0.05$. **B**, the Ca_v1.3 steady-state inactivation curve was significantly shifted toward more negative potentials by approximately equipotent concentrations of diazepam (60 μ M), allopregnanolone (30 μ M), pentobarbital (1 mM), and ethanol (300 mM). The inactivation V_{50} values are plotted in the inset. *, $p < 0.05$ relative to control V_{50} . Inactivation V_{50} values, slope factors, and numbers of cells tested are reported in Table 3.

1 Hz compared with activation at 0.1 Hz, with a significantly greater degree of inhibition occurring at 10, 30, and 100 μ M desalkylflurazepam. As illustrated in Fig. 7D, inhibition of Ca_v1.3 L-VGCCs by desalkylflurazepam was not significantly different when channels were activated at 1 Hz compared with activation at 0.1 Hz and showed no significant difference in inhibition at any concentration at or above threshold.

Two Amino Acids Important for DHP Potency Reduce Pentobarbital Potency. Although the various classes of L-VGCC antagonists have unique binding sites, they also have overlapping amino acids that contribute to binding. Thus, it was of interest to see whether single and double amino acid mutations, already characterized to affect binding

of specific L-VGCC antagonists, might also affect binding of GABA_AR modulators. Previous studies have found two amino acids in domain IIIS5 of Ca_v1.2, Thr1039 and Gln1043, which are critical for DHP affinity (Mitterdorfer et al., 1996; Hockerman et al., 2000). Mutation of these two amino acids thus creates a DHP-insensitive Ca_v1.2 (DHPI). As can be seen in Fig. 8A, half-maximal inhibition of wild-type (WT) Ca_v1.2 L-VGCCs occurred at approximately 0.1 μ M nimodipine and maximal block at 1 μ M (data represent inhibition at 0-mV test potential shown in Fig. 2B). In two cells transfected with DHPI L-VGCCs, application of 1 μ M nimodipine resulted in negligible inhibition ($5 \pm 14\%$). Application of 10 μ M nimodipine resulted in substantial inhibition ($58 \pm 3\%$) but was approximately 100-fold less potent than in WT, confirming that DHPI channels are resistant to DHPs.

As shown in Fig. 8B, DHPI L-VGCCs were significantly less sensitive to inhibition by pentobarbital compared with WT. Although this suggests that Thr1039 and Gln1043 may play a role in pentobarbital binding to Ca_v1.2, the 3.5-fold reduction in potency is quite small compared with the estimated 100-fold reduction in nimodipine potency. As shown in Fig. 8, C and E, inhibition of Ca_v1.2 channels by diazepam and ethanol, respectively, was unaffected by mutation of the DHP binding site. Figure 8D illustrates that DHPI channels were slightly more sensitive to allopregnanolone than WT Ca_v1.2 channels, but only 10 μ M allopregnanolone resulted in significantly more inhibition.

It should be noted that the WT Ca_v1.2 used for comparison of nimodipine, allopregnanolone, and ethanol inhibition was a mouse brain clone, whereas the DHPI mutant was created from a rat brain Ca_v1.2 clone. Thus, the small difference in inhibition by allopregnanolone might be due to species differences between the two channels. Additional controls in rat WT Ca_v1.2 were obtained for diazepam, pentobarbital, and diltiazem (see below).

A Single Amino Mutation That Affects Diltiazem Potency Does Not Affect Diazepam Potency. Based on the structural similarity between BDZs and BTZs, it was hypothesized that they might share a similar binding site at L-VGCCs. A single amino acid mutation, I1150A, was previously shown to affect diltiazem potency but not other classes of L-VGCC antagonists (Hockerman et al., 2000). As shown in Fig. 9A, mutation of Ile1150 in rat Ca_v1.2 resulted in a significant 2-fold decrease in diltiazem potency, with a significantly lower degree of inhibition by 10, 30, and 300 μ M diltiazem. However, as shown in Fig. 9B, diazepam potency was not significantly affected by the mutation, with no difference in the degree of inhibition by any concentration tested. To confirm that diazepam probably does not share a binding site with diltiazem, inhibition of WT Ca_v1.2 channels by diazepam was tested in the presence of an IC₅₀ concentration of diltiazem (30 μ M). As seen in Fig. 9C, the presence of diltiazem did not significantly affect inhibition at any diazepam concentration tested.

Discussion

GABA_AR Modulator Pharmacology at L-VGCCs: Comparison with GABA_ARs. Prior studies investigated inhibition of native L-VGCCs by GABA_AR modulators either in non-neuronal or neuronal cells that also contain GABA_ARs and multiple types of calcium channels, making it difficult to

TABLE 3

Negative shift in L-VGCC steady-state inactivation by GABA_AR modulatorsDiltiazem (30 and 10 μ M) were used for Ca_v1.2 and Ca_v1.3, respectively. Pentobarbital (0.3 and 1 mM) were used for Ca_v1.2 and Ca_v1.3, respectively.

Drug(s)	Ca _v 1.2			Ca _v 1.3		
	V ₅₀	k	n	V ₅₀	k	n
	mV			mV		
Control	-25.7 $\pm$ 1.7	7.6 $\pm$ 1.6	8	-46.1 $\pm$ 1.6	5.4 $\pm$ 1.5	10
30/10 μ M Diltiazem	-29.9 $\pm$ 1.9*	7.7 $\pm$ 1.8	3	-45.4 $\pm$ 3.3	6.8 $\pm$ 3.1	3
30 μ M Diazepam	-28.0 $\pm$ 1.6	6.9 $\pm$ 1.5	5	-50.2 $\pm$ 3.2	5.2 $\pm$ 2.9	4
60 μ M Diazepam	-33.5 $\pm$ 1.1*	7.4 $\pm$ 1.0	6	-59.2 $\pm$ 2.7*	7.1 $\pm$ 2.4	6
100 μ M Diazepam	-36.9 $\pm$ 1.3*	6.7 $\pm$ 1.1	3	-55.1 $\pm$ 0.9*	4.8 $\pm$ 0.8	6
100 μ M Desalkylflurazepam	-30.9 $\pm$ 2.0*	9.0 $\pm$ 2.0	8	-50.9 $\pm$ 1.1*	5.6 $\pm$ 1.1	10
300 μ M Flumazenil	-27.2 $\pm$ 2.9	9.6 $\pm$ 3.0	3	N.D.	N.D.	
100 Diazepam + 300 flumazenil	-37.3 $\pm$ 1.2*	6.6 $\pm$ 1.1	3	N.D.	N.D.	
30 μ M Allopregnanolone	-25.1 $\pm$ 4.2	12.0 $\pm$ 4.6	4	-55.6 $\pm$ 2.4*	6.5 $\pm$ 2.3	5
0.3/1 mM Pentobarbital	-31.2 $\pm$ 1.5*	7.5 $\pm$ 1.4	6	-54.7 $\pm$ 1.3*	5.6 $\pm$ 1.2	4
300 mM Ethanol	-25.2 $\pm$ 1.5	6.7 $\pm$ 1.4	5	-48.8 $\pm$ 0.6*	4.3 $\pm$ 0.5	4

N.D., not determined.

* $P < 0.05$ compared to respective control inactivation V₅₀ in the absence of drug.

Fig. 7. Ca_v1.2 L-VGCCs are inhibited by desalkylflurazepam in a state- (A and B) and frequency-dependent (C and D) manner. A, desalkylflurazepam (30 and 100 μ M) inhibited significantly more current when Ca_v1.2 channels were activated from -45 mV (open circles) compared with activation from -80 mV (filled circles). The inset illustrates a corresponding significant 2-fold decrease in the desalkylflurazepam IC₅₀ value at V_h = -45 mV (28 μ M, 23–34 μ M, n = 4) compared with V_h = -80 mV (55 μ M, 45–68 μ M, n = 3). VEH represents inhibition by vehicle. *, $p < 0.05$, **, $p < 0.01$. B, inhibition by desalkylflurazepam was not significantly different when Ca_v1.3 channels were activated from -60 mV (open circles) compared with -80 mV (filled circles). The inset illustrates the similarity in desalkylflurazepam IC₅₀ values at V_h = -60 mV (32 μ M, 13–80 μ M, n = 4) and V_h = -80 mV (37 μ M, 29–48 μ M, n = 6). C, desalkylflurazepam (10, 30, and 100 μ M) inhibited significantly more current when Ca_v1.2 channels were activated at 1 Hz (open circles) compared with activation at 0.1 Hz (filled circles). The inset illustrates a corresponding significant 2-fold decrease in the desalkylflurazepam IC₅₀ value at 1 Hz (28 μ M, 21–37 μ M, n = 4) compared with 0.1 Hz activation (55 μ M, 45–68 μ M, n = 3). *, $p < 0.05$. D, inhibition by desalkylflurazepam was not significantly different when Ca_v1.3 L-VGCCs were activated at 1 Hz (open circles) compared with 0.1 Hz (filled circles). The inset illustrates the similarity in desalkylflurazepam IC₅₀ values at 1 Hz (35 μ M, 17–70 μ M, n = 3) and 0.1 Hz activation (37 μ M, 29–48 μ M, n = 6).

ascertain the selective effects of these drugs on the neuronal L-VGCC subtypes Ca_v1.2 and Ca_v1.3. The current studies analyzed the inhibitory action of GABA_AR modulators on recombinant neuronal Ca_v1.2 and Ca_v1.3 L-VGCCs assembled with β_3 and $\alpha_2\delta$ subunits. Inhibition by modulators was not due to an indirect action at the GABA_AR, because HEK293T cells do not express functional GABA_ARs (Davies et al., 2000).

The BDZs were approximately 3 orders of magnitude less potent (Thomas et al., 1997), allopregnanolone was approximately 1.5 orders of magnitude less potent (Maitra and Reynolds, 1998), pentobarbital was approximately 1 order of magnitude less potent (Thomas et al., 1997), and ethanol was slightly less than 1 order of magnitude less potent (Wallner et al., 2003) at L-VGCCs than at GABA_ARs. Although diazepam was sever-

alfold more potent at GABA_ARs than flurazepam (Thomas et al., 1997), they displayed similar potency at L-VGCCs. The competitive BDZ antagonist, flumazenil, did not inhibit L-VGCCs, yet it antagonized BDZ inhibition of Ca_v1.3 but not Ca_v1.2 channels. This suggests that the Ca_v1.3 α_1 subunit may have a flumazenil binding site analogous to GABA_ARs.

GABA_AR Modulator Manner of L-VGCC Inhibition: Comparison with L-VGCC Antagonists. Maximal inhibition by L-VGCC antagonists is dependent upon channel use, with PAAs displaying high levels of use dependence, followed by BTZs and then DHPs, which display little use dependence (Lee and Tsien, 1983; Uehara and Hume, 1985). Although the current studies were not designed to assess use dependence, maximal inhibition by BDZs, pentobarbital, and eth-

Fig. 8. DHPI contains mutations of two amino acids important for high-affinity DHP binding, T1039Y and Q1043M. The sensitivity of DHPI L-VGCCs to GABA_AR modulators was tested. **A**, DHPI channels were approximately 100-fold less sensitive to inhibition by nimodipine compared with WT Ca_v1.2 channels. **B**, DHPI channels were significantly less sensitive to inhibition by pentobarbital compared with WT Ca_v1.2 channels. The inset illustrates a significant 3.5-fold increase the pentobarbital IC₅₀ value in DHPI (758 μM, 579–992 μM, *n* = 4) compared with WT Ca_v1.2 channels (215 μM, 167–276 μM, *n* = 4). VEH represents inhibition by vehicle. *, *p* < 0.05, **, *p* < 0.01, ***, *p* < 0.001. **C**, no difference in inhibition by diazepam was observed between WT and DHPI channels. Diazepam IC₅₀ values for WT (41 μM, 31–55 μM, *n* = 4) and DHPI channels (48 μM, 32–73 μM, *n* = 3) are shown in the inset. **D**, DHPI channels were slightly more sensitive to inhibition by allopregnanolone than WT Ca_v1.2 channels, with 10 μM allopregnanolone inhibiting significantly more current through DHPI channels. As shown in the inset, there was no significant difference in allopregnanolone potency to inhibit in DHPI (3 μM, 2–5 μM, *n* = 3) compared with WT channels (7 μM, 3–16 μM, *n* = 5). **E**, no difference in inhibition by ethanol was observed between WT and DHPI channels. Ethanol IC₅₀ values for WT (183 mM, 46–723 mM, *n* = 3) and DHPI channels (157 mM, 117–216 mM, *n* = 3) are shown in the inset.

Fig. 9. A single amino acid mutation in Ca_v1.2, I1150A reduces diltiazem but not diazepam potency. **A**, as reportedly previously (Hockerman et al., 2000), the I1150A mutation reduced diltiazem potency, with significantly less inhibition by 10, 30, and 300 μM diltiazem. The inset illustrates a significant 2-fold increase of the diltiazem IC₅₀ value in I1150A (53 μM, 40–70 μM, *n* = 6) compared with WT channels (26 μM, 21–32 μM, *n* = 4). VEH represents inhibition by vehicle. *, *p* < 0.05, **, *p* < 0.01. **B**, the I1150A mutation had no effect on diazepam potency. Diazepam IC₅₀ values for WT (41 μM, 31–55 μM, *n* = 4) and I1150A channels (50 μM, 32–77 μM, *n* = 4) are shown in the inset. **C**, as a confirmation that diazepam probably does not share a binding site with diltiazem, inhibition of WT Ca_v1.2 channels by diazepam was assessed in the presence of an IC₅₀ concentration of diltiazem (30 μM). There was no significant difference in inhibition by diazepam at any concentration tested compared with inhibition in the absence of diltiazem. The inset illustrates that there was no significant difference in diazepam potency in the presence (38 μM, 21–68 μM, *n* = 3) compared with the absence of diltiazem (49 μM, 38–64 μM, *n* = 4).

anol consistently required fewer activating pulses compared with inhibition by allopregnanolone and diltiazem (Fig. 3, B and C). Although these time course differences may be due to differential kinetics of drug binding and/or drug action, it could also suggest that the former compounds may not be as dependent on channel opening to bind and can better access their L-VGCC binding sites when channels are in a resting confirmation. Alternatively, antagonists that slow recovery from inactivation may result in an accumulation of channels in the inactive state during repeated activation and have greater effect at higher frequencies of activation (Uehara and Hume, 1985). Although inhibition of Ca_v1.2 channels by de-

salkylflurazepam was slightly enhanced in a frequency-dependent manner (Fig. 7), it is possible that these modulators have a limited ability to slow recovery from inactivation relative to BTZs and PAAs (Uehara and Hume, 1985).

GABA_AR modulators enhance L-VGCC current decay, similar to other L-VGCC antagonists (Lee and Tsien, 1983; Cai et al., 1997). This could represent open channel block and/or stabilization of the inactive state. In support of the latter possibility, desalkylflurazepam potency was increased at Ca_v1.2 channels by recording paradigms that enhance channel inactivation (Fig. 7). In addition, GABA_AR modulators induced a significant negative shift in the Ca_v1.3 steady-state inactivation

curve, and BDZs and pentobarbital also induced a significant negative shift in the $\text{Ca}_v1.2$ steady-state inactivation curves (Fig. 6 and Table 3). Altogether, the data provide evidence for state-dependent block by GABA_A modulators, preferentially interacting with inactive and possibly open states.

$\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ L-VGCC Differential Sensitivity to GABA_A Modulators. Recombinant $\text{Ca}_v1.3$ channels have been shown to be less sensitive to inhibition by DHPs than $\text{Ca}_v1.2$ channels (Koschak et al., 2001; Xu and Lipscombe, 2001). Native $\text{Ca}_v1.3$ channels in auditory hair cells were also found to be less sensitive to inhibition by BTZs and PAAs, although this effect may be cell type-specific (Tarabova et al., 2007). Indeed, recombinant $\text{Ca}_v1.3$ channels were 3-fold more sensitive to diltiazem inhibition than $\text{Ca}_v1.2$ channels. For the GABA_A modulators, $\text{Ca}_v1.3$ channels were 1.5-fold less sensitive to flurazepam and 3-fold less sensitive to pentobarbital than $\text{Ca}_v1.2$ channels. Diazepam, desalkylflurazepam, allopregnanolone, and ethanol displayed no significant differential potency at L-VGCCs. However, because desalkylflurazepam inhibition of $\text{Ca}_v1.2$, but not $\text{Ca}_v1.3$ channels, was increased by protocols that enhance inactivation (Fig. 7), it is possible that $\text{Ca}_v1.2$ L-VGCCs may display greater sensitivity to the latter compounds in a state-dependent manner as proposed previously for DHPs (Koschak et al., 2001). Whether this differential state-dependent effect may be related to the greater extent of inactivation observed for $\text{Ca}_v1.2$ than $\text{Ca}_v1.3$ channels (Table 2) or a more specific BDZ interaction with the channels is currently unknown. The differential potency of these drugs at $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ channels may allow them to selectively modify distinct L-VGCC subunit-mediated neuronal functions. In particular, reduced $\text{Ca}_v1.2$ channel function impairs hippocampal spatial memory, whereas reduced $\text{Ca}_v1.3$ channel function affects neuronal firing patterns and may be protective in reducing age-related neuronal degeneration, as occurs in Parkinson's disease (Striessnig and Koschak, 2008).

GABA_A Modulator Site of Action. Organic L-VGCC antagonists have unique binding sites on the $\text{Ca}_v1.2$ subunit that share a number of overlapping amino acids (Hockerman et al., 1997). Thus, it was of interest to see whether amino acid mutations that are known to affect binding of specific L-VGCC antagonists could similarly reduce inhibition by GABA_A modulators. Two $\text{Ca}_v1.2$ mutants were used in the current studies: a double amino acid mutation (T1039Y and Q1043M) that reduces DHP potency (Mitterdorfer et al., 1996; Hockerman et al., 2000), and a single mutation, I1150A, that reduces diltiazem potency (Hockerman et al., 2000). Neither mutation affected diazepam potency, suggesting that BDZs may have an L-VGCC binding site distinct from DHPs and BTZs. Furthermore, diltiazem did not competitively interfere with diazepam inhibition of $\text{Ca}_v1.2$ channels, and these compounds were somewhat dissimilar in their manner of block in terms of state and use dependence. Thus, despite the structural similarity between BDZs and BTZs and their relatively similar potencies, the evidence suggests that these drugs act via distinct sites. It is noteworthy that pentobarbital potency was significantly reduced 3.5-fold by the DHP mutant, suggesting that the pentobarbital binding site on $\text{Ca}_v1.2$ might overlap that of DHPs.

Clinical Relevance of L-VGCC Inhibition by GABA_A Modulators and Implications for Physical Dependence. Calcium influx through postsynaptic L-VGCCs is linked to

neuronal activity and gene transcription, and L-VGCC inhibition has anxiolytic, antidepressant, and anticonvulsant actions (Striessnig et al., 2006). Thus, inhibition of neuronal L-VGCCs could play a role in the acute clinical actions of GABA_A modulators. Furthermore, long-term inhibition of L-VGCCs may lead to an adaptive up-regulation of L-VGCC function during prolonged GABA_A modulator treatment and contribute to physical dependence (Dolin et al., 1987; Rabbani and Little, 1999; Xiang and Tietz, 2007; Xiang et al., 2008).

Free plasma and cerebrospinal fluid concentrations of BDZs, barbiturates, and ethanol exist in an approximate 1:1 ratio (Richards, 1972; Hallstrom and Lader, 1980; Harris et al., 2008). Taking into account extensive binding (>97%) of diazepam and its active metabolite, desmethyldiazepam, to plasma proteins (Hallstrom and Lader, 1980; Divoll and Greenblatt, 1981), free plasma concentrations of approximately 20 to 60 nM are necessary for acute relief of convulsant activity (Rey et al., 1999), and approximately 50 to 100 nM is needed to produce sedation (Lundgren, 1987). Because of rapid tolerance to the sedative effects of diazepam, higher concentrations up to 200 nM are achieved during long-term treatment for psychiatric therapy (Rutherford et al., 1978; Hallstrom and Lader, 1980; Greenblatt et al., 1981). Because the threshold concentration of BDZs to significantly inhibit recombinant L-VGCCs was approximately 10 μM , inhibition of neuronal L-VGCCs is unlikely to contribute to BDZ clinical actions. However, studies in hippocampal cultures revealed that 1 μM flurazepam significantly inhibited VGCC current in a use-dependent manner (Xiang et al., 2008), suggesting that greater potency can be observed in neuronal systems. The threshold concentration of allopregnanolone to inhibit L-VGCCs was 3 μM , which is much greater than the 5 nM concentration estimated to be produced from progesterone in the brain (Uzunova et al., 1998). The threshold concentration of pentobarbital to inhibit L-VGCCs was 100 μM , within the 200 μM range required to induce anesthesia (Richards, 1972). Unlike pentobarbital, phenobarbital has anticonvulsant actions with minimal sedative effects at 20 to 90 μM (Schulz and Macdonald, 1981). It would be of interest to see whether phenobarbital inhibits L-VGCCs at concentrations required for anticonvulsant activity or whether L-VGCC inhibition is more likely to pertain to the anesthetic effects of barbiturates (Ffrench-Mullen et al., 1993). The threshold concentration of ethanol to inhibit L-VGCCs was 30 mM, within range of intoxicating ethanol concentrations (10–100 mM) (Harris et al., 2008). Thus, inhibition of L-VGCCs may attribute to the intoxicating effects of ethanol, including mood alteration and memory impairment.

The enhancement of neuronal L-VGCC-mediated Ca^{2+} influx observed after long-term BDZ exposure is unlikely to be the result of a direct effect at L-VGCCs because this up-regulation occurs with BDZ concentrations as low as 0.3 μM in vitro (Katsura et al., 2007) and ~ 1 μM measured in rat brain homogenates during long-term BDZ treatment in vivo (Xiang et al., 2008). Thus, persistent allosteric enhancement of GABA_A Rs seems the most plausible mechanism associated with L-VGCC regulation during long-term BDZ administration because L-VGCC up-regulation in cortical cultures can be prevented by nanomolar concentrations of the BDZ-competitive antagonist, flumazenil (Katsura et al., 2007). In addition, a bicarbonate-dependent GABA_A -mediated depolarization that arises during BDZ withdrawal (Zeng and Tietz,

2000) may contribute to the hyperexcitable state (Van Sickel et al., 2004), which could exacerbate the downstream Ca²⁺-mediated effects of L-VGCC up-regulation. As with other GABA_AR modulators, withdrawal from long-term exposure to allopregnanolone is associated with a withdrawal syndrome characterized by anxiety and increased seizure susceptibility (Smith, 2002). However, a role for L-VGCC up-regulation in this phenomenon has not been established. Inhibition of L-VGCCs may contribute to the up-regulation of L-VGCC function after long-term exposure to barbiturates (Rabbani and Little, 1999), as well as ethanol (Messing et al., 1986; Katsura et al., 2006). Collectively, these findings may provide a molecular basis for the adaptive changes that contribute to drug withdrawal symptoms and physical dependence on GABA_AR modulators.

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Authorship Contributions

Participated in research design: Earl and Tietz.

Conducted experiments: Earl.

Performed data analysis: Earl.

Wrote or contributed to the writing of the manuscript: Earl and Tietz.

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