BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Biochemistry **2010,** *49,* 4841–4851 4841
DOI: 10.1021/bi100119t

Dihydropyrimidinone Positive Modulation of δ-Subunit-Containing γ-Aminobutyric Acid Type A Receptors, Including an Epilepsy-Linked Mutant Variant†

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Received January 26, 2010; Revised Manuscript Received April 28, 2010

ABSTRACT: γ-Aminobutyric acid type A receptors ($GABA_A$ receptors) are ligand-gated chloride channels that play a central role in signal transmission within the mammalian central nervous system. Compounds that modulate specific $GABA_A$ receptor subtypes containing the δ-subunit are scarce but would be valuable research tools and starting points for potential therapeutic agents. Here we report a class of dihydropyrimidinone (DHPM) heterocycles that preferentially potentiate peak currents of recombinant $GABA_A$ receptor subtypes containing the δ-subunit expressed in HEK293T cells. Using the three-component Biginelli reaction, 13 DHPMs with structural features similar to those of the barbiturate phenobarbital were synthesized; one DHPM used (monastrol) is commercially available. An up to ∼3-fold increase in the current from recombinant α1β2δ receptors was observed with the DHPM compound JM-II-43A or monastrol when co-applied with saturating GABA concentrations, similar to the current potentiation observed with the nonselective potentiating compounds phenobarbital and tracazolate. No agonist activity was observed for the DHPMs at the concentrations tested. A kinetic model was used in conjunction with dose-dependent measurements to calculate apparent dissociation constant values for JM-II-43A (400 μM) and monastrol (200 μM) at saturating GABA concentrations. We examined recombinant receptors composed of combinations of subunits α1, α4, α5, α6, β2, β3, γ2L, and δ with JM-II-43A to demonstrate the preference for potentiation of δ-subunit-containing receptors. Lastly, reduced currents from receptors containing the mutated δ(E177A) subunit, described by Dibbens et al. [(2004) *Hum. Mol. Genet. 13*, 1315−1319] as a heritable susceptibility allele for generalized epilepsy with febrile seizures plus, are also potentiated by these DHPMs.

The neurotransmitter γ-aminobutyric acid (GABA)[1] activates a class of ligand-gated chloride ion channels known as $GABA_A$ receptors that are important for the regulation of neurotransmission in the central nervous system (CNS) (*1*). Heteropentameric combinations of 19 different $GABA_A$ receptor subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π, and ρ1-3) (*2*) determine the localization, pharmacology, biophysical properties, and cellular roles of these receptors (*3*−*6*). In contrast to $GABA_A$ receptor subtypes that are localized at synapses and have a phasic role in signal transmission, receptors containing the δ-subunit are located peri- and/or extrasynaptically and are thought to have an important role in regulating neurotransmission through tonic inhibition (*5*, *7*, *8*). The importance of the δ-subunit for neuronal signaling is exemplified in knockout mice lacking the δ-subunit that are more susceptible to seizures and exhibit attenuated responses to neurosteroids compared to normal mice (*9*, *10*). In addition, point mutations within several different $GABA_A$ receptor subunits have been genetically associated with various forms of epilepsy and seizures (*11*), such as point mutations E177A (glutamate to alanine), R220H (arginine to histidine), and R220C (arginine to cysteine) in the δ-subunit that are genetically linked to general epilepsy with febrile seizures + and idiopathic generalized epilepsy (*12*, *13*). How these individual mutations within the δ-subunit affect the function of $GABA_A$ receptors is not fully understood, but single-channel studies of the α1β2δ receptor indicate that the E177A and R220H mutations alter the equilibrium between the open- and closed-channel states of the receptor (*14*).

Changes in the mechanism of α1β2δ receptors due to the E177A mutation may reduce the channel-opening equilibrium constant (Φ^{-1}) of the receptor, which was determined (*15*) to be the case for the epilepsy-linked γ2L(K289M) mutant subunit originally described by Baulac et al. in 2001 (*16*). On the basis of the proposed mechanism of the $GABA_A$ receptor (*15*) (Figure 3A), we hypothesized and determined (*17*) that phenobarbital, a barbiturate anticonvulsant, potentiates $GABA_A$ receptors by binding to the open-channel conformation of the receptor with an affinity higher (K_{app} = 0.23 ± 0.06 mM) than that for binding to the closed-channel conformation (K_{app} = 1.08±0.32 mM), stabilizing the open-channel state and thus increasing the channel-opening equilibrium constant.

In an effort to identify compounds that potentiate current amplitudes of $GABA_A$ receptors to a greater extent than does phenobarbital, we synthesized several dihydropyrimidinones (DHPMs) with structural characteristics similar to those of phenobarbital and tested them on the epilepsy-linked α1β2γ2L(K289M) and α1β2δ-(E177A) receptors as well as their corresponding wild-type

†R.W.L. was supported by National Institutes of Health Predoctoral Training Grant 5T32GM7273 in Cell and Molecular Biology and a Teaching Assistantship from the Department of Molecular and Cell Biology, Cornell University. K.P.E. was a Robert and Helen Appel Hunter R. Rawlings III Cornell Presidential Research Scholar and a Cornell Hughes Undergraduate Scholar.

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[1]Abbreviations: ATCC, American Type Culture Collection; CNS, central nervous system; DHPM, dihydropyrimidinone; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; GABA, γ-aminobutyric acid; $GABA_A$ receptor, γ-aminobutyric acid type A receptor; NMDA, *N*-methyl-D-aspartic acid; THDOC, 3α,21-dihydroxy-5α-pregnan-20-one.

Published on Web 05/07/2010

α1β2γ2L and α1β2δ receptors. Although DHPMs did not potentiate currents of the γ-subunit-containing wild-type α1β2γ2L or mutated α1β2γ2L(K289M) receptors, they did potentiate (~3-fold) the δ-subunit-containing wild-type α1β2δ and mutated α1β2δ(E177A) receptors. Thus, these DHPMs selectively modulate $GABA_A$ receptor subtypes.

It is known that $GABA_A$ receptors containing the δ-subunit are pharmacologically distinguishable from other receptor subtypes by their higher sensitivity to zinc ion inhibition (*5*, *18*), greater sensitivity to (and efficacy of) potentiation by tracazolate (*8*, *19*) and neurosteroids such as 3α,21-dihydroxy-5α-pregnan-20-one (THDOC) (*8*, *20*), and a greater sensitivity to the agonist gaboxadol [4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol (THIP)] (*18*, *21*, *22*). However, most of these molecules also act on other $GABA_A$ receptor subtypes. Barbiturates and many other drugs, which have been used for the treatment of epilepsy, depression, anxiety, insomnia, and other diseases (*23*−*26*), also indiscriminately affect various $GABA_A$ receptor isoforms (*2*), including, but not limited to, δ-subunit-containing receptors, and often result in undesirable side effects (*26*−*28*). Ketamine (*29*), ethanol (*20*, *30*, *31*), and two benzamides (*32*) have been previously identified as acting specifically on δ-subunit-containing receptor isoforms. Ketamine is a positive allosteric modulator as well as a partial agonist of α6β2δ and α6β3δ $GABA_A$ receptors and an inhibitor of *N*-methyl-D-aspartic acid (NMDA) receptors (*29*). While somewhat controversial (*33*, *34*), ethanol is reported to primarily potentiate δ-subunit-containing receptors at low agonist concentrations that are similar to conditions the receptors may experience extrasynaptically (*30*, *31*, *35*). The two reported benzamide compounds are allosteric modulators of α4β3δ $GABA_A$ receptors, with one of the two benzamide compounds, DS1, also acting as a partial agonist (*32*).

Members of the family of dihydropyrimidinones reported here allosterically and selectively potentiate currents of several $GABA_A$ receptor subtypes, with a preference for potentiating receptors containing the δ-subunit. Various combinations of subunits α1, α4, α5, α6, β2, β3, γ2L, and δ were transiently expressed in HEK293T cells for a total of 12 subtypes that were electrophysiologically tested with the DHPM JM-II-43A (methyl 6-methyl-2-oxo-4-phenyl-3,4-dihydro-1*H*-pyrimidine-5-carboxylate). JM-II-43A potentiated currents from δ-subunit-containing receptors and the α4β2 receptor subtype but did not change the average peak currents corrected for receptor desensitization (*36*) of the other $GABA_A$ receptor subtypes examined. Currents from α1β2δ receptors over the full dose-dependent range of GABA concentrations (saturating and subsaturating) were potentiated anywhere from 2.9- to 4-fold by JM-II-43A, extents similar to, or greater than, those of other reported $GABA_A$ receptor potentiators such as phenobarbital and tracazolate (*8*, *17*, *19*). Thirteen DHPM compounds were generated through use of a single-step multicomponent reaction (*37*), and one, monastrol, was purchased. All 14 compounds were tested on α1β2δ receptors, yielding a range of potentiation activities. No agonist activity was observed for any of the DHPMs tested. Lastly, these DHPMs potentiated currents of α1β2δ(E177A) mutated receptors genetically linked to epilepsy to an extent similar to those of wild-type α1β2δ receptors.

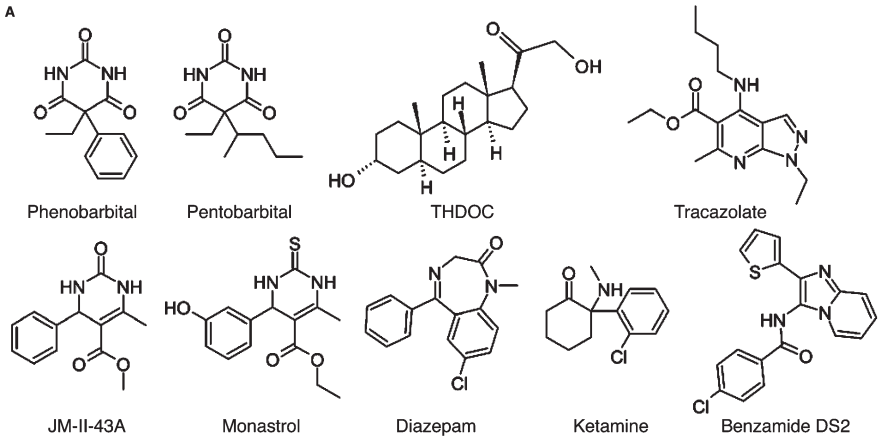
MATERIALS AND METHODS

Reagents, Synthesis, and Preparation of Dihydropyrimidinones. Monastrol was purchased from Tocris (Ellisville, MO). Other reagents were obtained from Sigma Aldrich, Fisher Scientific, or EM Science. GABA solutions were serially diluted from a frozen stock of 100 mM GABA stored at −20 °C and made weekly. DHPMs were synthesized as described previously (*37*). Monastrol and DHPMs were dissolved in pure anhydrous DMSO before being diluted 1:200 with extracellular buffer at 65 °C for a final concentration of either 4 or 2 mM compound in 0.5% DMSO. The 0.5% DMSO aided in compound solubility, with no observed effect on $GABA_A$ receptor currents. The DHPM solutions were cooled to room temperature and serially diluted with extracellular buffer as needed.

Cell Culture and Transient Transfection. HEK293T cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 0.1 mg/mL streptomycin. Cells were maintained at 37 °C with 5% CO_2. cDNAs encoding subunits α1, α4, α5, α6, β2, β3, and γ2L of the rat $GABA_A$ receptor in mammalian pRK-5 expression vectors were kindly provided by H. Lüddens (Johannes Gutenberg-Universität, Mainz, Germany) and P. H. Seeburg (Max-Plank-Institut für medizinische Forschung, Heidelberg, Germany). cDNA encoding the δ-subunit of the rat $GABA_A$ receptor in the pExpress-1 vector was obtained from ATCC. The pGreen Lantern plasmid (Life Technologies Inc., Gaithersburg, MD) was used as a cell transfection marker. A total of 4 μg of plasmid DNA was transfected, with the α:β:pGreen Lantern plasmid ratio being 1:1:0.1 and the α:β:γ2L:pGreen Lantern and α:β:δ:pGreen Lantern plasmid ratios both being 1:1:10:0.1. These ratios were used to strongly bias the expression of a subunit of interest (i.e., γ2L or δ) relative to the expression levels of α- and β-subunits to ensure inclusion of the modulatory subunit in the active receptors. For δ-subunit-containing receptors, this was clearly identifiable by the characteristic lack of, or limited degree of, receptor desensitization. Currents measured from cells presumed to be expressing αβδ receptors that exhibited greater degrees of desensitization were very infrequent, but if they were observed, the cell was discarded and the data were not used. PolyFect transfection reagent (Qiagen, Valencia, CA) or polyethylenimine (PEI) (Polysciences Inc., Warrington, PA) was used for the transient transfection of cells. The Qiagen protocol for transient transfection with PolyFect was followed, the exception being that the DMEM/reagent mixture was replaced after 5−7 h with a fresh DMEM/10% FBS mixture. PEI was used as reported previously (*38*) with a PEI:DNA ratio of 4:4 (micrograms to micrograms) and replacement of medium with a fresh DMEM/10% FBS mixture after 4−7 h. When PEI was used, the HEK293T cells had greater transfection efficiencies and were healthier; no differences in receptor function were observed.

Molecular Biology. Mutations were introduced into the δ-subunit by the QuikChange site-directed mutagenesis technique (Stratagene, La Jolla, CA) and verified by DNA sequencing. The E177A mutation of the δ-subunit was introduced through polymerase chain reaction amplification with the primer 5′-GGA CAG GCA GGC GTG CAT GCT GGA CCT GGA GAG C-3′ and the reverse complement (Integrated DNA Technologies, Coralville, IA).

Whole-Cell Current Recordings. Cells were bathed in an extracellular buffer composed of 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] brought to pH 7.4 with 5 N NaOH. Individual cells in a whole-cell configuration were lifted from the dish with a borosilicate glass pipet and suction. Borosilicate glass pipettes were made from capillaries with an



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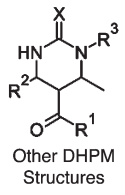

	Compound	R1	R2	R3	X	Fold Potentialtion	Solubility *
1	JM-II-43A	OMe	Ph	H	O	2.9	Sol
2	JGP-I-80A	OEt	Ph	H	O	1.2	PSol
3	JM-III-3	Me	Ph	H	O	1.3	Sol
4	JGP-I-142C	OH	Ph	H	O	1	Sol †
5	JGP-II-17F	NH2	Ph	H	O	1	Sol
6	JGP-II-28K	OMe	Ph	Me	O	1	Sol
7	JGP-I-151B	OMePh	Ph	H	O	NA	NSol
8	JM-III-4B	OMe	4-Br-C6H4	H	O	1.3	Sol
9	JM-III-6A	OMe	3-OMe-C6H4	H	O	1.4	Sol
10	JM-III-3	OMe	4-NO2-C6H4	H	O	1.3	Sol
11	JM-III-12A	OMe	3-OMe-4-OH-C6H3	H	O	1.5	Sol
12	JM-III-14A	OMe	C3H7	H	O	2.1	Sol
13	JGP-I-150B	OMe	Ph	H	S	1	Sol
14	monastrol	OEt	3-OH-C6H4	H	S	3.0	Sol

* Compound solubility tested at concentration of 1 mM in extracellular buffer with 0.5% DMSO, pH 7.4

† This compound was not soluble in pure DMSO, but was soluble in extracellular buffer alone, pH 7.4.

FIGURE 1: (A) Structures of several molecules that potentiate various $GABA_A$ receptor subtypes. Of the many compounds known to modulate $GABA_A$ receptors, only ketamine (*29*), ethanol (*20*, *30*, *31*) [though ethanol modulation seems somewhat complicated (*33*, *34*)], and two benzamide compounds (*32*) have been reported to selectively potentiate δ-subunit-containing receptor subtypes. The structural similarities of JM-II-43A (methyl 6-methyl-2-oxo-4-phenyl-3,4-dihydro-1*H*-pyrimidine-5-carboxylate), monastrol, and other DHPMs (Figure 1B) to pentobarbital and phenobarbital suggest that DHPMs may also potentiate whole-cell currents from $GABA_A$ receptors. (B) A One-step Biginelli reaction (*37*) was used to synthesize derivatives of JM-II-43A. Each compound was tested for solubility and efficacy to potentiate $\alpha1\beta2\delta$ $GABA_A$ receptor currents at 1 mM compound and 1 mM GABA. The solubility of each DHPM was determined qualitatively in aqueous extracellular buffer containing 0.5% DMSO and is classified as soluble (Sol), partially soluble (PSol), or not soluble (NSol). The degree of current potentiation (fold potentiation) is described as the current induced by 1 mM GABA and 1 mM compound divided by the current induced by 1 mM GABA alone ($I_{1\ mM\ cpd.}/I_{1\ mM\ GABA\ control}$).

outer diameter of 1.5 mm and an inner diameter of 1.12 mm (World Precision Instruments Inc., Sarasota, FL) and heat-polished on a microforge to yield pipettes with open-end resistances of 3.0−5.0 MΩ. Pipettes were filled with an intracellular solution composed of 140 mM CsCl, 10 mM tetraethylammonium chloride, 10 mM EGTA [ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 2 mM $MgCl_2$, and 10 mM HEPES brought to pH 7.4 with 5 N CsOH. All measurements were taken with a constant membrane potential of −60 mV, at ambient temperature (~22 °C). An Axopatch 200B amplifier, a Digidata 1322A digitizer, and Clampex version 9.0 (Molecular Devices, Sunnyvale, CA) were used for recording whole-cell currents, as previously described by Hamill et al. (*39*). Data were filtered with the Axopatch 200B internal Bessel filter at 2 kHz with a digital sampling frequency of 20 kHz.

Rapid Solution Application. Rapid applications of ligands to cells expressing the receptor of interest were performed with the cell-flow technique as previously described by Udgaonkar et al. (*36*). Briefly, a cell in the whole-cell current-recording mode in extracellular buffer was suspended from the recording electrode ~200 μm in front of the porthole of a stainless steel U-tube. The solution was actively pumped with a peristaltic pump into one end of the U-tube at a typical rate of ~1.5 cm/s and drawn from the other end of the U-tube with the peristaltic pump at a greater rate. This causes extracellular buffer in the dish to be drawn through the porthole of the U-tube, preventing the leakage of solutions in the U-tube onto the cell. The flow of the solution out of the porthole is regulated by a solenoid Lee Control Valve (Lee Co., Essex, CT) directed by Clampex. After an application period, the solution from the dish is again drawn into the porthole of the U-tube, with rapid removal of the recently applied solution. Solution applications were spaced a minimum of 2−2.5 min apart, permitting receptors time to recover from desensitized states.

Current Measurement Analysis and Correction. Many neurotransmitter receptors undergo rapid desensitization, which can have a significant effect on the observed peak current amplitudes but can be corrected for as previously described (*36*). The observed peak amplitudes of non-δ-subunit-containing $GABA_A$ receptor currents, in the absence or presence of modulators, were corrected for desensitization by fitting the desensitization phase of each current trace with a three-component exponential function. The time constants obtained from the fit were used (i) to compare how compounds alter the rate of receptor desensitization and (ii) to estimate the true current amplitudes by accounting for receptor desensitization (*36*, *40*) that occurs during the rising phase of the receptor current trace (*41*). Measured currents from $GABA_A$ receptors containing a δ-subunit characteristically display limited (if any) desensitization (*42*) and, therefore, were not corrected. To normalize current amplitudes between cells, all receptor currents from a single cell

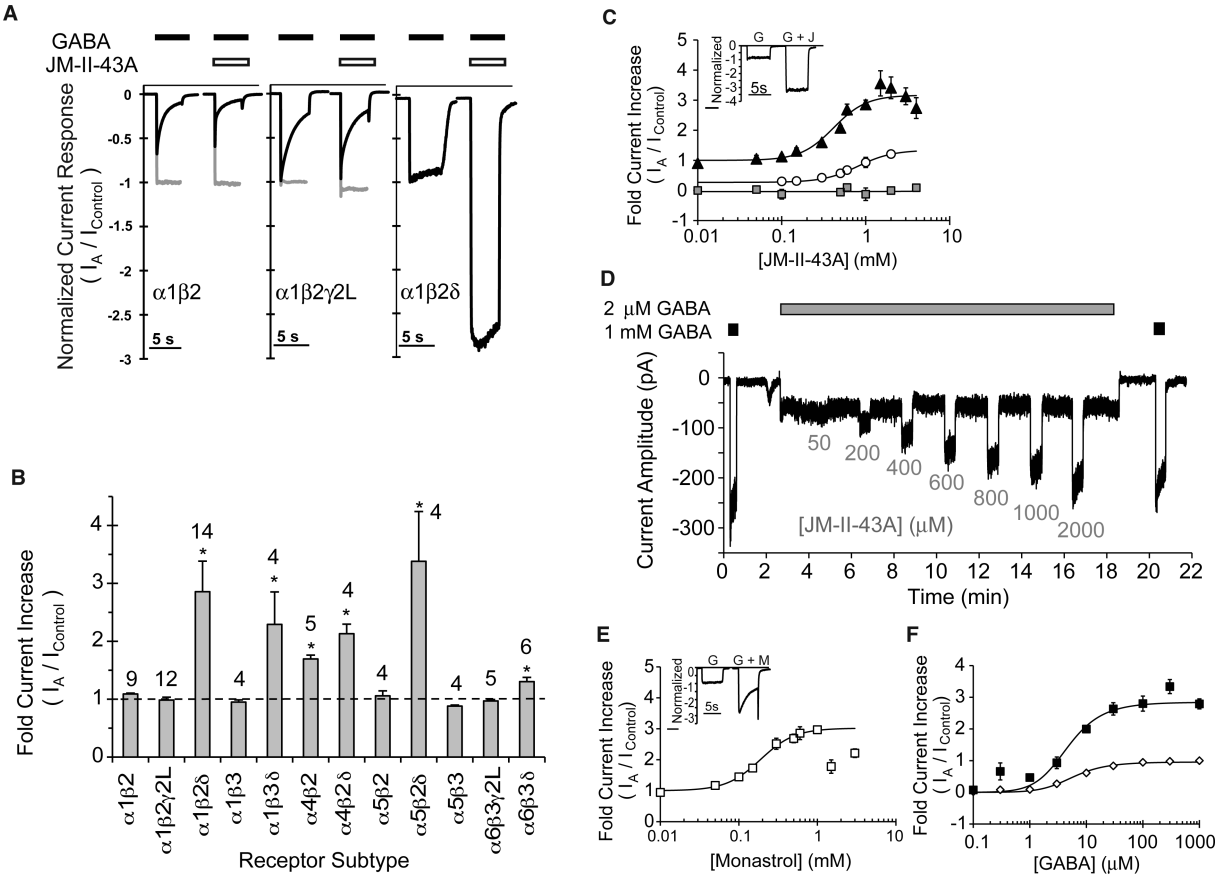

FIGURE 2: (A) Representative traces showing the effects of 1 mM JM-II-43A on currents evoked by 1 mM GABA with three different receptor isoforms, α1β2, α1β2γ2L, and α1β2δ. The current traces for α1β2 and α1β2γ2L receptor subtypes were corrected for receptor desensitization (gray line) (*36*). Current correction for receptor subtype α1β2δ is not needed because limited to no desensitization is observed. (B) GABA (1 mM) with JM-II-43A (1 mM) was applied to various receptor subtypes to test the specificity of JM-II-43A current potentiation. Of the subtypes examined here, only the α4β2 receptor subtype and receptors containing the δ-subunit were potentiated by JM-II-43A. Error bars display the standard error; numbers above each bar represent the number of independent cells measured, and the asterisk denotes a significant difference ($p \leq 0.01$), as tested by a one-way ANOVA statistical test. (C) JM-II-43A dose-dependent curves (from 0.01 to 2 mM) were obtained from α1β2δ receptors for the compound alone (gray squares), in the presence of 1 mM GABA (saturating concentration, ▲) and in the presence of 3 μM GABA (effective concentration for the 25% maximal current response, EC_{25}, ○). Curves were fitted with eq 1 and gave an apparent dissociation constant for JM-II-43A [$*K_{d(JM\text{-}II\text{-}43A)}$] of 410 ± 74 μM at 1 mM GABA and 810 ± 32 μM at 3 μM GABA, with Hill coefficients of 2.1 and 2.2, respectively. The inset shows two representative current traces evoked by 1 mM GABA and by 1 mM GABA with 1 mM JM-II-43A. Current amplitudes are normalized to the peak of the 1 mM GABA current response. (D) Current trace from α1β2δ recombinant receptors in a single cell used to demonstrate the current response upon application of 2 μM GABA and increasing concentrations of JM-II-43A during the 2 μM GABA application. GABA (1 mM) in the absence of JM-II-43A was applied at the beginning and end of the current trace. This trace displays the dose-dependent modulation of the receptor by JM-II-43A, the increase in amplitude of the current by JM-II-43A, and the rapid association and dissociation of the compound with the receptor during a JM-II-43A application period. (E) Monastrol dose-dependent curve obtained with α1β2δ receptors in the presence of 1 mM GABA and fitted with eq 1 to calculate an apparent dissociation constant for monastrol [$*K_{d(monastrol)}$] of 190 ± 28 μM. Potentiation at concentrations of monastrol above 1 mM decreased, likely due to solubility limitations of the compound and/or possible receptor inhibition. The inset shows two representative current traces evoked by 1 mM GABA and by 1 mM GABA with 1 mM monastrol. Current amplitudes are normalized to the peak of the 1 mM GABA current response. (F) GABA dose-dependent curves obtained with α1β2δ receptors in the absence (◇) and presence (■) of 1 mM JM-II-43A were fitted with eq 2 and gave values for the dissociation constant for GABA (K_1) of 9.9 ± 1.1 and 7.0 ± 1.5 μM, respectively. All currents were recorded at ambient temperature (~22 °C), a membrane potential of −60 mV, and pH 7.4. Measurements from a single cell are normalized by dividing the current amplitude evoked in the presence of an experimental compound by a 1 mM GABA control current measurement ($I_A/I_{Control}$). The value of each data point shown in panels C, E, and F is the mean of 4−14 measurements from independent cells and is plotted on a logarithmic scale with error bars representing the standard error.

were divided by the current amplitude evoked by a control measurement of current induced by saturating 1 mM GABA ($I_A/I_{Control}$). Dose-dependent data were plotted with their relative standard errors and fitted with either eq 1 or 2 using nonlinear least-squares regression conducted with Origin version 3.5 data analysis software (OriginLab Corp., Northampton, MA). All reported values of dissociation constants estimated by nonlinear least-squares fitting are reported with relative standard errors.

RESULTS

Most $GABA_A$ receptor subtypes are modulated by a variety of small molecules, including barbiturates such as phenobarbital and benzodiazepines such as diazepam, both of which are anticonvulsants that act by positively modulating (potentiating) currents of $GABA_A$ receptors (*3*). Many different molecules have been reported to potentiate $GABA_A$ receptors (several examples are shown in Figure 1A). With their structural resemblance to phenobarbital and pentobarbital, DHPMs JM-II-43A (methyl 6-methyl-2-oxo-4-phenyl-3,4-dihydro-1*H*-pyrimidine-5-carboxylate) and monastrol were considered likely to potentiate $GABA_A$ receptors (Figure 1A).

JM-II-43A and the other derivatives (except for monastrol, which is commercially available), shown in Figure 1B, were synthesized using a multicomponent Biginelli reaction as previously

described (*37*). The DHPMs synthesized were first tested for their solubility at concentrations of 1−4 mM in aqueous extracellular buffer with 0.5% dimethyl sulfoxide (DMSO); most were soluble at concentrations up to 2 mM. To determine whether the DHPM compounds listed (Figure 1B) potentiate recombinant $GABA_A$ receptors, the cell-flow technique (*36*) was used to rapidly co-apply 1 mM JM-II-43A and 1 mM GABA to various receptor subtypes transiently expressed in HEK293T cells. Representative current traces evoked by 1 mM GABA alone or co-applied with 1 mM JM-II-43A are shown for α1β2, α1β2γ2L, and α1β2δ receptor subtypes (Figure 2A). Currents of specific receptor subtypes, such as α1β2δ, were potentiated, whereas slight increases in the rates of desensitization were observed with currents of α1β2 and α1β2γ2L receptor subtypes (Figure 2A). When co-applied with 1 mM JM-II-43A, 1 mM GABA receptor subtypes α1β2δ, α1β3δ, α4β2, α4β2δ, α5β2δ, and α6β3δ also demonstrated potentiation anywhere from 1.3- to 3.4-fold of the current evoked by 1 mM GABA alone. No changes in corrected peak current amplitudes were observed for currents of receptor subtypes α1β2, α1β2γ2L, α1β3, α4β2, α5β2, α5β3, and α6β3γ2L. However, it was noted that several of these receptor subtypes had small changes in the rates of desensitization. For unknown reasons, limited expression of receptor subtypes α5β3δ, α6β2, and α6β3 was observed when they were transiently transfected in HEK293T cells, preventing the examination of the effect of JM-II-43A on these receptor subtypes. The observed weak expression of the α6β2 receptor subtype agrees with a previous report in which *Xenopus laevis* oocytes were used as an expression system (*43*). However, α6β3 receptors have been successfully expressed in *X. laevis* oocytes (*31*), and it is not known why expression of these receptors is limited in HEK293T cells. The potentiation mechanism of $GABA_A$ receptors by JM-II-43A was further investigated with the α1β2δ receptor subtype because of the high degree of potentiation and satisfactory expression level of the subtype in HEK293T cells. With the α1β2δ subtype, the mean current measured in the presence of 1 mM JM-II-43A and 1 mM GABA was 2.9-fold higher than in the presence of 1 mM GABA alone (Figure 2A−C). The maximum (3.5-fold) potentiation of current occurred with 1.5 mM JM-II-43A. In the presence of 3 μM GABA, currents observed for the α1β2δ receptor subtype are only 25% of the maximal current response (EC_{25}), indicating that the receptors are primarily in a closed-channel state. However, upon co-application of 3 μM GABA and 1 mM JM-II-43A, receptor currents are potentiated up to ~4-fold compared to those evoked by 3 μM GABA alone (Figure 2C). To better describe how JM-II-43A affects the receptors, dose-dependent data (Figure 2C) were fitted with eq 1 to obtain values for the apparent dissociation constant of JM-II-43A [$^*K_{d(JM\text{-}II\text{-}43A)}$].

JM-II-43A may potentiate currents of α1β2δ receptors by preferentially binding to the open-channel form of the receptor, thus shifting the receptor channel-opening equilibrium toward the open-channel state. This mechanism would be similar to that described for the potentiation of the α1β2γ2L(K289M) receptor by phenobarbital (Figure 3) (*3*, *17*). It would require that JM-II-43A bind with higher affinity to the open-channel state than to the closed-channel state of the receptor. Therefore, the dissociation constant of JM-II-43A was determined in the presence of 1 mM and 3 μM GABA to assess the compound's binding affinity for the α1β2δ receptor in the open- and closed-channel states, respectively (Figure 2C). Various concentrations of JM-II-43A were co-applied with either 1 mM or 3 μM GABA, and eq 1 (*44*) was used to evaluate the JM-II-43A dissociation constant, K_d.

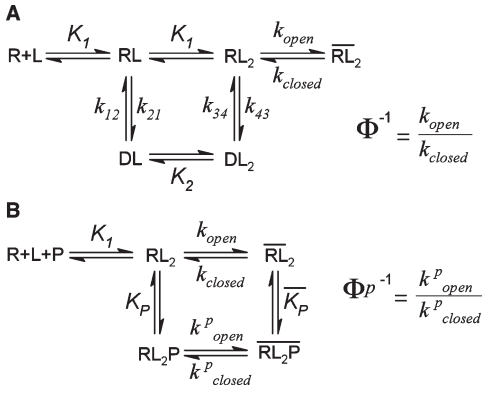

FIGURE 3: (A) Mechanism previously proposed for $\alpha_1\beta_2\gamma_{2\,L}$ $GABA_A$ receptors (*15*), which was originally proposed for the nicotinic acetylcholine receptor by Katz and Thesleff (*50*), with the addition of the desensitized states (DL and DL_2) and the channel-opening equilibrium constant (Φ^{-1}) proposed later (*51*). The mechanism describes the receptor (R) binding two ligand molecules (L) with a dissociation constant K_1. At this stage (RL_2) the receptor is in the closed-channel state, which can change conformation to the open-channel state to allow ion flux across the membrane ($\overline{RL_2}$) and to the subsequent desensitized state (DL_2). DL_2 is a transiently inactive, closed-channel state of the receptor. The equilibrium between the closed- and open-channel states is described by the channel-opening equilibrium constant (Φ^{-1}). (B) This mechanism describes the binding of a potentiating compound (P) to $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors as previously reported (*17*). The mechanism here was simplified by omission of the desensitization states of the receptor and combination of the two steps of ligand binding. The potentiating compound can bind to either the closed- or open-channel state with the relative binding affinities K_P and $\overline{K_P}$, to yield the respective closed-channel (RL_2P) and open-channel ($\overline{RL_2P}$) states of the receptor. The channel-opening and -closing rate constants in the presence of the potentiator are depicted as k^P_{open} and k^P_{closed} and define the channel-opening equilibrium constant (Φ_p^{-1}) in the presence of a potentiating compound.

$$\frac{I_P}{I_o} = I_o + R_{max}[1+(K_d/[Cpd])^h]^{-1} \quad (1)$$

Equation 1, which has been used previously to assess interactions of barbiturates with $GABA_A$ receptors (*17*, *44*), relates the ratio of measured current amplitudes (I_P/I_o) induced by a constant GABA concentration in the presence (I_P) or absence (I_o) of the compound of interest ([Cpd]), JM-II-43A. R_{max} is the maximum I_P/I_o ratio observed when the potentiating compound is co-applied with GABA. The empirical parameter h is equivalent to the Hill coefficient. Because 1 mM JM-II-43A does not saturate α1β2δ receptors at either 1 mM or 3 μM GABA and is not easily soluble at higher concentrations, fitting the dose-dependent data in Figure 2C afforded only the apparent value of its dissociation constant [$^*K_{d(JM\text{-}II\text{-}43A)}$] (the $^*K_d$ is approximately equal to the EC_{50}). For the α1β2δ receptor, the values of $^*K_{d(JM\text{-}II\text{-}43A)}$ were estimated to be 410 ± 74 and 810 ± 32 μM in the presence of 1 mM and 3 μM GABA, respectively (measured constants for various receptors and conditions listed in Table 1). Hill coefficients of 2.1 and 2.2 were obtained for these two fittings, values similar to the values of 2.2 (*45*), 1.5 (*44*), and 1.5−1.9 (*17*) previously observed for phenobarbital modulation of $GABA_A$ receptors.

The apparent dissociation constants of these compounds are relatively large, so it was of interest to investigate if these compounds rapidly or slowly associate with the receptors. To address this question, we designed an experiment similar to that described

Table 1: Apparent Dissociation Constants Obtained for the $GABA_A$ Receptor $\alpha1\beta2\delta$ Subtype and the $\alpha1\beta2\delta$(E177A) Variant

receptor subunit composition	$K_{1(GABA)}$ (μM)	$K_{1(GABA)}$ with 1 mM JM-II-43A (μM)	$K_{d(JM\text{-}II\text{-}43A)}$ with 1 mM GABA (μM)	$K_{d(JM\text{-}II\text{-}43A)}$ with 3 μM GABA (μM)	$K_{d(monastrol)}$ with 1 mM GABA (μM)
$\alpha1\beta2\delta$	9.9 ± 1.1	7.0 ± 1.5	410 ± 74	810 ± 32	190 ± 28
$\alpha1\beta2\delta$(E177A)	5.7 ± 0.26	8.6 ± 2.0	620 ± 120	1140 ± 390	–

Table 2: Parameters Obtained from Fitting eq 3 to the Desensitization Phase of $\alpha1\beta2$ and $\alpha1\beta2\gamma2$L $GABA_A$ Receptor Currents in the Presence and Absence of JM-II-43A[a]

receptor subtype	no. of cells	conditions	mean corrected peak current (pA)	A_1 (pA)	τ_1 (ms)	A_2 (pA)	τ_2 (ms)	A_3 (pA)	τ_3 (ms)	C (pA)
$\alpha1\beta2$	9	1 mM GABA	-4680 ± 930	-1480 ± 410	650 ± 150	-1370 ± 270	1270 ± 210	-1150 ± 210	1590 ± 220	760 ± 200
		1 mM GABA with 1 mM JM-II-43A	-5050 ± 990	-2290 ± 450	68 ± 15	-1170 ± 330	600 ± 180	-1190 ± 240	1900 ± 360	590 ± 170
$\alpha1\beta2\gamma2$L	12	1 mM GABA	-6210 ± 630	-1490 ± 200	970 ± 270	-1880 ± 310	2090 ± 200	-1710 ± 210	2130 ± 150	1150 ± 160
		1 mM GABA with 1 mM JM-II-43A	-6250 ± 930	-2440 ± 780	460 ± 200	-1500 ± 210	1510 ± 320	-1660 ± 180	2090 ± 240	830 ± 110

[a]Paired current traces from a single cell were obtained by application of 1 mM GABA with and without 1 mM JM-II-43A. The desensitization phase of each current trace was fitted with a three-component exponential equation (eq 3) and using nonlinear least-squares regression. The mean values for the parameters are given. A_x and τ_x represent the current amplitude and time constant associated with exponential component x, respectively. C is the current amplitude at the end of the fit region.

by Wallner et al. (*31*), in which whole-cell currents were recorded upon application of the modulating compound at various concentrations while the receptors are continually in the presence of 2 μM GABA. A perfusion system was used to exchange the buffer bathing the cell with buffer containing 2 μM GABA (EC_{15}), followed by increasing concentrations of JM-II-43A co-applied with 2 μM GABA using a U-tube. Not only do these data (Figure 2D) clearly display the dose-dependent effect of JM-II-43A on the receptor, but the rapid change in the current amplitude upon application demonstrates that JM-II-43A is associating and dissociating with the receptor on a relatively fast time scale. In the current trace, the 10−90% response times for the 1 and 2 mM JM-II-43A applications were 155 and 123 ms, respectively.

Monastrol and other DHPMs also potentiated $\alpha1\beta2\delta$ receptors, as shown in Figure 1B. Because monastrol had one of the largest potentiating efficacies at 1 mM in the presence of 1 mM GABA, a full dose-dependent response for this compound was measured (Figure 2E). Fitting of the dose-dependent curve for monastrol yielded a value for the apparent dissociation constant [$^*K_{d(monastrol)}$] of 190 ± 28 μM, indicating that $\alpha1\beta2\delta$ receptors have a higher binding affinity for monastrol than they do for JM-II-43A [$^*K_{d(JM\text{-}II\text{-}43A)} = 410 \pm 74$ μM]. Potentiation of the $\alpha1\beta2\delta$ receptor currents by monastrol declined above 1 mM, which may be due to solubility limitations of monastrol and/or inhibitory activity at higher concentrations. It is interesting to note that while JM-II-43A had no observed effect on receptor desensitization, monastrol dramatically increases the desensitization rate of $\alpha1\beta2\delta$ receptors and causes a large rebound current at the end of the application of ligand (inset, Figure 2E).

No agonist activity on $\alpha1\beta2\delta$ receptors in the absence of GABA was observed for any of the DHPMs tested. However, it is possible that JM-II-43A and the other DHPMs tested potentiate $\alpha1\beta2\delta$ receptor currents by changing the affinity of the receptor for GABA or by acting in cooperation with GABA as an agonist or partial agonist. To test these possibilities, we examined the dose dependence of GABA on receptor currents in the presence and absence of JM-II-43A (Figure 2F). If the mechanism for $\alpha1\beta2\delta$ receptors is similar to that proposed for the $\alpha1\beta2\gamma2$L receptor (Figure 3A), it is possible to estimate the GABA dissociation constant [$K_{1(GABA)}$] by fitting the data with eq 2 (*36*).

$$I_A = I_M R_M L^2 [L^2 + \Phi(L + K_1)^2]^{-1} \quad (2)$$

This equation describes the expected current amplitude (I_A) evoked by a known agonist concentration (L). I_M represents the current from 1 mol of receptors in the open-channel state and R_M the number of moles of receptors on the cell surface. Experimentally, we used the current amplitude at a saturating concentration of the activating ligand (GABA) as the $I_M R_M$ value and the observed current amplitude as I_A. Φ is the channel-closing equilibrium constant (the reciprocal of the channel-opening equilibrium constant), defined in Figure 3A. Fitting the GABA dose-dependent response data from $\alpha1\beta2\delta$ receptors with eq 2 gave values for $K_{1(GABA)}$ of 9.9 ± 1.1 and 7.0 ± 1.5 μM in the absence and presence of 1 mM JM-II-43A, respectively. These values agree well with the previously reported dose-dependent curve of the $\alpha1\beta2\delta$ receptor subtype (*8*). Compounds were also tested, up to concentrations of 4 mM, in the absence of GABA, and no induced currents were observed. Data for JM-II-43A in the absence of GABA are shown in Figure 2C.

$GABA_A$ receptors containing the δ-subunit typically exhibit limited desensitization, and $\alpha1\beta2\delta$ receptor desensitization was not altered by JM-II-43A. However, JM-II-43A did alter the desensitization phase of the current recorded from $\alpha1\beta2$ (Figure 2A), $\alpha1\beta2\gamma2$L (Figure 2A), $\alpha1\beta3$, $\alpha4\beta2$, $\alpha5\beta2$, $\alpha5\beta3$, and $\alpha6\beta3\gamma2$L receptor subtypes. The rate at which ligand-gated ion channels, such as $GABA_A$ receptors $\alpha1\beta2$ and $\alpha1\beta2\gamma2$L, pass into a desensitized state and how modulating compounds change the rate of desensitization can be described by time constants (τ_x). The values of the time constants were obtained from fitting the desensitization phase of each current trace with an exponential function (*46*), shown below:

$$I = A_1(e^{t/\tau_1}) + A_2(e^{t/\tau_2}) + A_3(e^{t/\tau_3}) + C \quad (3)$$

Equation 3 describes the desensitization of current traces, where t is time, A_x represents the relative amplitude of component x, and

C is the current remaining at the end of the GABA application period. In previous studies, ligand-gated ion-channel currents are typically fitted with a two-component exponential function to describe the fast and slow desensitization rates observed (*40*, *46*), but in the presence of DHPMs, a third component was needed for appropriate fitting. Therefore, all traces analyzed for desensitization were fitted with a three-component exponential function so that comparisons could be made between the presence and absence of modulating compounds. The values obtained for τ_x were also used to determine desensitization-corrected amplitudes of peak currents, as previously described (*36*). JM-II-43A changed the desensitization rate of both α1β2 and α1β2γ2L receptors but did not affect the desensitization-corrected current amplitudes (Figure 2A and Table 2). In Table 2, the resulting time constants and relative amplitudes are listed for α1β2 receptors (9 independent cells) and α1β2γ2L receptors (12 independent cells) when 1 mM GABA or 1 mM JM-II-43A with 1 mM GABA was applied to them.

Since JM-II-43A and monastrol potentiated the α1β2δ subtype, these compounds were also tested on receptors that contained the single amino acid mutation E177A within the δ-subunit. $GABA_A$ receptors α1β2δ(E177A) [as well as α4β2δ(E177A)] are reported to have significantly reduced currents (*12*, *14*) due to a decrease in the channel mean open time of the receptor as measured with single-channel recording. The extent of the decrease in whole-cell currents for these mutations varied. The reported amplitudes of averaged whole-cell currents from receptors containing the δ(E177A) subunit range from ~12% [α1β2δ(E177A)] (*12*) and ~33% [α4β2δ(E177A)] (*14*) to ~66% [α1β2δ(E177A)] (Figure 4A) of that of the maximum wild-type currents measured at the same concentrations of GABA. The degree of current reduction due to the mutations varied when maximum currents recorded from independent cells were measured, because whole-cell currents depend on the number of moles of receptors on the cell surface, R_M (eq 2), and on transfection efficiencies for each cell. As a result, a large standard error was observed in the averaged maximum current amplitudes evoked by 1 mM GABA from 34 cells expressing α1β2δ receptors and 19 cells expressing α1β2δ(E177A) receptors (Figure 4A), but the difference was statistically significant ($p < 0.003$). The dose−response curve of α1β2δ(E177A) receptors for JM-II-43A with 1 mM GABA afforded a value of 0.624 ± 0.116 mM for $^*K_{d(JM\text{-}II\text{-}43A)}$ with a Hill coefficient of 1.7 ± 0.3 (Figure 4B). The value of $^*K_{d(JM\text{-}II\text{-}43A)}$ at low (3 μM) concentrations of GABA changed to 1.14 ± 0.39 mM with a Hill coefficient of 1.5 ± 0.3. Very little change in the affinity of α1β2δ(E177A) receptors for GABA was observed in the absence (K_1 = 5.68 ± 0.26 μM) or presence (K_1 = 8.64 ± 1.98 μM) of 1 mM JM-II-43A (Figure 4C). Both 1 mM JM-II-43A and 1 mM monastrol potentiated currents from receptors containing the mutant δ(E177A) subunit, by 3.2- and 2.0-fold, respectively (Figure 4D). These values were directly compared to the approximate ~3-fold potentiation of wild-type α1β2δ $GABA_A$ receptors by 1 mM JM-II-43A or monastrol in the presence of 1 mM GABA, as well as to potentiation by 1 mM phenobarbital and 10 μM tracazolate (Figure 4D).

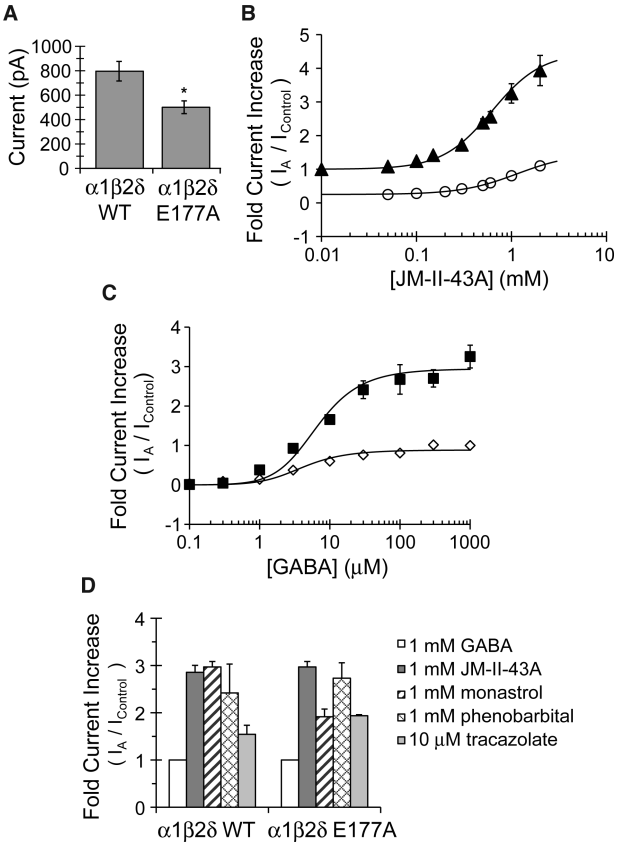

FIGURE 4: Mutated α1β2δ(E177A) $GABA_A$ receptors, which give lower average current responses to GABA vs those of the wild-type receptor (*12*, *14*), were potentiated by JM-II-43A. (A) Mean current amplitudes evoked by 1 mM GABA from 34 independent cells for wild-type α1β2δ and 19 independent cells for mutant α1β2δ(E177A) receptors demonstrate a 47% reduction in current amplitude and are shown with relative standard errors. The difference is statistically significant ($p < 0.003$) using a two-tailed t test assuming unequal variance and an α of 0.05. The somewhat large standard errors, despite 34 and 19 independent measurements, are likely due to large variations in the number of receptors expressed on the surface of each cell. (B) Fitting eq 1 to the dose-dependent response data from α1β2δ(E177A) receptors in the presence of 0.01−2 mM JM-II-43A and 1 mM GABA (▲) gave a value for the apparent dissociation constant for JM-II-43A [$^*K_{d(JM\text{-}II\text{-}43A)(E177A)}$] of 0.62 ± 12 mM, with a Hill coefficient of 1.7 ± 0.3. In the presence of 3 μM GABA (EC_{25}) (○), the value for $^*K_{d(JM\text{-}II\text{-}43A)(E177A)}$ was 1.1 ± 0.39 mM, with a Hill coefficient of 1.5 ± 0.3. (C) Fitting the GABA dose-dependent data with eq 2 gave values for the dissociation constant (K_1) for GABA from α1β2δ(E177A) receptors of 5.68 ± 0.26 μM (◇) and 8.64 ± 1.98 μM (■) in the absence and presence of JM-II-43A, respectively. Independent measurements from three to six cells were taken for every concentration shown in panels B and C and plotted on a logarithmic scale. (D) Comparison of the potentiation due to saturating concentrations of JM-II-43A, monastrol, phenobarbital, or tracazolate co-applied with 1 mM GABA shows that DHPMs positively modulate both α1β2δ and α1β2δ(E177A) $GABA_A$ receptors to a similar or greater extent than do phenobarbital and tracazolate. The means and relative standard errors shown are calculated from 12 independent cells for JM-II-43A and 3−9 independent cells for monastrol, phenobarbital, and tracazolate, normalized to the current response evoked by 1 mM GABA. All current measurements were taken at ambient temperature (~22 °C), a membrane potential of −60 mV, and pH 7.4.

DISCUSSION

Many neurological pathologies, including schizophrenia, depression, anxiety, insomnia, and epilepsy, are linked to $GABA_A$ receptor dysregulation of signal transmission in the mammalian CNS (*25*). Understanding how $GABA_A$ receptors work in the CNS, what molecules modulate these receptors, and how various compounds affect their functions is critical information to aid in the treatment of diseases, such as epilepsy, which affects ~50 million people worldwide (*47*). Compounds that target subsets or specific $GABA_A$ receptor isoforms allow for more detailed and

precise studies and potentially more specific treatment of $GABA_A$ receptor-related disorders. However, the repertoire of known compounds that selectively potentiate currents from $GABA_A$ receptor isoforms containing the δ-subunit was limited to ketamine (*29*), two benzamide molecules (*32*), and ethanol (*30*, *31*). Here we have established that a family of dihydropyrimidinones also has this selective property. Ketamine, a well-characterized allosteric inhibitor of NMDA receptors, was recently reported (*29*) to selectively potentiate currents of recombinant α6β2δ and α6β3δ receptors that were expressed in *X. laevis* oocytes. The degree of potentiation reported for ketamine was greatest when it was tested with GABA concentrations that evoke 1% of maximal current (EC_1) and declined with increasing GABA concentrations. The two benzamide molecules described (*32*) potentiated currents evoked by low (EC_{20}) GABA concentrations when applied to recombinant α4β3δ receptors expressed in cells from an L(tk^-) cell line. The effects of the benzamides when co-applied with higher concentrations of GABA were not reported. Potentiation of δ-subunit-containing receptors by ethanol has been reported at both low and high agonist concentrations (*30*, *31*). However, this observed response seems to be a complicated relationship and necessitates further investigation (*33*, *34*).

The structural similarity of DHPMs to the barbiturates pentobarbital and phenobarbital led us to hypothesize that these molecules may similarly potentiate $GABA_A$ receptor currents, but the DHPMs presented in our study have several novel characteristics. These compounds contrast to other compounds such as ketamine and benzamides, which were reported to specifically potentiate one or two receptor subtypes selectively, whereas DHPMs are somewhat selective for potentiation of a subgroup of receptors containing the δ-subunit, with α4β2 receptors being the exception. In addition, the potentiation of $GABA_A$ receptor currents by DHPMs occurs over the entire effective range of GABA concentrations, without the DHPMs alone showing any agonist activity at concentrations up to 4 mM (Figure 2C). It is possible that these DHPMs may act as agonists at concentrations higher than those tested; however, their solubility prevented examination of this possibility. This finding also contrasts with barbiturates, such as pentobarbital and phenobarbital, which modulate most $GABA_A$ receptor subtypes and display partial agonist activity at high concentrations (*44*, *48*). The observation that DHPMs do not change the affinity of the α1β2δ receptor for GABA suggests that DHPMs interact with $GABA_A$ receptors at an allosteric binding site; however, it is not known if the binding site is novel or the same one to which barbiturates bind. The binding site for DHPMs is evidently not specific to the presence of the δ-subunit as α4β2 receptors were potentiated by DHPMs and these compounds increase desensitization rates of receptor subtypes lacking the δ-subunit. These results, and observations previously reported (*49*) for the potentiation of both α4β3 and α4β3δ receptor subtypes by etomidate, propofol, and the neurosteroid THDOC, indicate that the δ-subunit is not essential for binding and potentiation but likely assists in facilitating potentiation of several receptor subtypes.

The general mechanism for $GABA_A$ receptors presented here (Figure 3) was originally proposed by Katz and Thesleff for the nicotinic acetylcholine receptor (*50*), with the later addition of the desensitized states (DL and DL_2) and channel-opening equilibrium constant (Φ^{-1}) (*51*). This mechanism indicates that observed currents will be affected by any changes in the channel-opening equilibrium caused by receptor mutations or ligands that stabilize either the open-channel or closed-channel receptor state. While the measurement of several kinetic constants, such as channel-opening (k_{open}) and channel-closing (k_{closed}) rate constants, for the specific α1β2δ receptor mechanism must await the development of appropriate reagents, results reported here support the hypothesis that potentiation of α1β2δ receptors and other receptor subtypes by JM-II-43A may be due to the compound preferentially binding to and stabilizing the open-channel state. JM-II-43A has a higher affinity for the wild-type receptor [$^*K_{d(JM\text{-}II\text{-}43A)} = 410 \pm 74\ \mu M$] at a saturating concentration (1 mM) of GABA (receptor predominantly in the open-channel state) than at a low (3 μM) concentration of GABA (receptor predominantly in the closed-channel state) [$^*K_{d(JM\text{-}II\text{-}43A)} = 810 \pm 32\ \mu M$]. It is also possible that DHPMs increase the desensitization rates of α1β2 and α1β2γ2L receptor isoforms because of preferential binding to and stabilization of the desensitized receptor state.

Previous chemical kinetic investigations of the α1β2γ2L-(K289M) receptor genetically linked to epilepsy (*16*) used the mechanism proposed in Figure 3A to explain how the mutation decreases the channel-opening equilibrium constant (Φ^{-1}) 5-fold by reducing the channel-opening rate constant (k_{open}) (*15*). Moreover, the anticonvulsant phenobarbital was found to bind with higher affinity to the open-channel state of the mutated α1β2γ2L(K289M) receptor than to the closed-channel state, shifting the channel-opening equilibrium of the receptor toward the open-channel state (*17*), reaffirming the receptor mechanism. As with the α1β2γ2L(K289M) isoform, $GABA_A$ receptors containing the mutated δ(E177A) subunit together with subunits α1 and β2 have reduced current amplitudes in whole-cell current recordings (Figure 4A) (*12*, *14*). The decrease in whole-cell currents of receptors containing the δ(E177A) subunit is due to a decrease in the mean open time (τ_{open}) of the receptor (the average period of time that the receptor occupies the state able to conduct current before closing), as measured in single-channel current recordings (*14*). The approximate 3-fold increase in the current of α1β2δ(E177A) receptors when in the presence of 1 mM JM-II-43A is consistent with the compound causing a shift in the channel-opening equilibrium toward the open-channel receptor state. Consistent with this hypothesis, the dissociation constant for JM-II-43A for the mutant receptor in the presence of 1 mM GABA (receptor predominantly in the open-channel state) was shifted to a lower value [$K_{d(JM\text{-}II\text{-}43A)(\delta E177A)} = 624.2 \pm 115.7\ \mu M$] compared to that of the wild type [$K_{d(JM\text{-}II\text{-}43A)(\delta WT)} = 410 \pm 74\ \mu M$]. This observation is expected because the mechanism of the $GABA_A$ receptor indicates that the mutated receptor biases the equilibrium toward the closed-channel state, decreasing the number of receptors in the open-channel state at any one time and thus lowering the affinity of the receptor for JM-II-43A. While these observations do not establish that the E177A mutation or application of JM-II-43A alters the channel-opening equilibrium constant of the receptor, they do support this hypothesis. Once appropriate reagents are developed, it will be of interest to determine if changes in the k_{open} or k_{closed} rate constants confirm this hypothesis.

In addition to suggesting that DHPMs potentiate $GABA_A$ receptors by shifting the channel-opening equilibrium to increase receptor currents, our data also support the increasing amount of literature describing GABA as a partial agonist for δ-subunit-containing $GABA_A$ receptors (*8*, *20*, *31*, *49*, *52*). DHPMs seem to increase the efficacy of GABA to open $GABA_A$ receptors while not changing the sensitivity of the receptor to GABA. As previously suggested (*52*), the modulation of GABA efficacy on δ-subunit-containing $GABA_A$ receptors may be an important attribute of tonic currents used to regulate neuronal excitation.

If this is taken one step further, selective modulation of $GABA_A$ receptor subtypes involved with tonic inhibition may yield additional levels of regulation and control. It is interesting to point out that of the receptor subtypes potentiated by DHPMs ($\alpha1\beta2\delta$, $\alpha1\beta3\delta$, $\alpha4\beta2$, $\alpha4\beta2\delta$, $\alpha5\beta2\delta$, and $\alpha6\beta3\delta$), the subunit α4- and α6-containing receptors are modulated to a lesser extent than other subtypes, even though subunits α4 and α6 are currently thought to be the predominant partnering subunits of the δ-subunit in the CNS (*20*). These results, in addition to the colocalization of various GABA subunits with the δ-subunit in immunocytochemical distributions in the rat brain (*53*), support the idea that less abundant minor receptor subtypes may still be functionally relevant. In fact, the $\alpha1\beta2\delta$ receptor subtype, while not a predominant subtype in much of the CNS, has been reported as a significant subtype in specific hippocampal interneurons (*20*) and was described as a silent receptor until it is in the presence of modulators such as the neurosteroid THDOC (*20*). The increased efficacy of GABA on minor receptor subtypes in the presence of DHPMs and other potentiating compounds suggests that less abundant endogenous receptor subtypes in the nervous system may be selectively modulated to play a significant role in regulating the tonic conductance of neurons.

The potentiation efficacy of JM-II-43A and monastrol on $\alpha1\beta2\delta$ receptor currents is comparable to that of phenobarbital and tracazolate (Figure 4D). Phenobarbital, which was reported to potentiate $\alpha1\beta2\gamma2L$ receptors ~1.5-fold (*17*), has a higher efficacy on $\alpha1\beta2\delta$ receptors, increasing maximum currents by 2.5-fold. Although tracazolate was reported to potentiate $\alpha1\beta2\delta$ currents by ~23-fold when it was co-applied with 100 μM GABA to *X. laevis* oocytes (*8*), it increased by only ~1.5-fold the whole-cell currents evoked by 1 mM GABA from $\alpha1\beta2\delta$ receptors expressed in HEK293T cells reported here. At saturating GABA concentrations, 10 μM tracazolate was reported to potentiate $\alpha2\beta3\delta$ receptors expressed in HEK293 cells by approximately 2.7-fold (*54*) and $\alpha1\beta1\delta$ receptors expressed in *X. laevis* oocytes by 2-fold (*19*), which both more closely resemble the results reported here for $\alpha1\beta2\delta$ receptors. These reported differences in the degree of potentiation by tracazolate support previous suggestions that the expression system may affect the degree of potentiation observed for $GABA_A$ receptor currents (*49*). However, the direct comparison of potentiation efficacy of JM-II-43A, monastrol, tracazolate, and phenobarbital with saturating GABA concentrations in HEK293T cells (Figure 4D) demonstrates that these molecules all increase the efficacy of GABA on $\alpha1\beta2\delta$ receptors to a similar extent.

JM-II-43A seems to increase only $\alpha1\beta2\delta$ receptor currents without changing the other properties of the observed current. However, the effects of monastrol on $\alpha1\beta2\delta$ receptors are clearly more complex as the application of 1 mM monastrol causes not only an increase in the overall current amplitude but also the appearance of both a desensitizing phase in the current response and a large rebound current at the end of the application period (inset, Figure 2E). It is not clear how this compound may be causing these features other than to suggest that the compound stabilizes both a conducting state and a nonconducting state.

Monastrol was originally identified as a kinesin inhibitor (*55*), and because of this, the activity of monastrol on $GABA_A$ receptors may raise questions about the specificity of DHPMs and their future as potential therapeutics. Monastrol itself is a very specific inhibitor of the Eg5 kinesin, with a subsequent report showing that the single change of its sulfur atom to an oxygen atom results in the loss of kinesin inhibition (*56*). Various DHPMs have biological activity in several other contexts as well, including modulation and inhibition of calcium channels (*57*). DHPMs that modulate calcium channels were discovered in the process of making derivatives and compounds structurally related to nifedipine and nifedipine-like dihydropyridines (*58*, *59*), compounds that are therapeutically used as vasodilators, antianginals, and antihypertensives. In this report, JM-II-43A was primarily examined for modulation of specific GABA receptor subtypes. However, we have also tested this DHPM on recombinant GluN1N2A NMDA receptors and recombinant $\alpha4\beta2$ nicotinic acetylcholine receptors (nAChRs) separately and transiently expressed in HEK293T cells. It was also tested on endogenous muscle-type nACh receptors of the BC_3H1 cell line. When 1 mM JM-II-43A was co-applied with 10 μM glycine and 100 μM glutamate, there was no observed effect of the compound on the NMDA receptor currents. In contrast, both $\alpha4\beta2$ and muscle-type nAChRs were inhibited ~90% when 1 mM JM-II-43A was co-applied with 100 μM carbamoylcholine. These results were not entirely unexpected as many barbiturates are known antagonists of nAChRs (*60*, *61*), including pentobarbital [one of the barbiturates after which this class of DHPMs was modeled (Figure 1A)], which has a reported EC_{50} of 32 μM using the endogenous receptors of BC_3H1 cells (*61*). With all this in mind, monastrol and the other DHPMs presented in this report may not be directly relevant for therapeutic use. However, they do display $GABA_A$ receptor subtype specificity that will make them good tools for the study of $GABA_A$ receptors. Furthermore, modifications of these compounds may yield molecules that retain the ability to modulate specific $GABA_A$ receptor subtypes while making the molecules more selective for $GABA_A$ receptors than the DHPMs examined here. Such molecules would undoubtedly be desirable for therapeutic purposes.

Chemical libraries of DHPMs can be readily synthesized through a multicomponent Biginelli reaction (*37*) and can afford access to a wide range of molecules having promising biological applications (*57*, *62*). The accessibility of DHPM derivatives and the results presented here showing selectivity for potentiation of $\alpha1\beta2\delta$ $GABA_A$ receptors suggest that DHPM derivatives should be useful as biological tools and may be helpful for the future development of therapeutic agents for targeting $GABA_A$ receptor isoforms containing the δ-subunit.

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