Dipicrylamine Modulates GABA\(_{\rho1}\) Receptors through Interactions with Residues in the TM4 and Cys-Loop Domains

Agenor Limon, Argel Estrada-Mondragón, Jorge M. Reyes Ruiz, and Ricardo Miledi

Neurobiology and Behavior, University of California, Irvine, California (A.L., J.M.R.R., R.M.); Psychiatry and Human Behavior, University of California, Irvine, California (A.L.); and The Queensland Brain Institute, St. Lucia, Queensland, Australia (A.E.-M.)

Received January 16, 2016; accepted February 9, 2016

ABSTRACT

Dipicrylamine (DPA) is a commonly used acceptor agent in Förster resonance energy transfer experiments that allows the study of high-frequency neuronal activity in the optical monitoring of voltage in living cells. However, DPA potently antagonizes GABA\(_{\rho1}\) receptors that contain \(\alpha1\) and \(\beta2\) subunits by a mechanism which is not clearly understood. In this work, we aimed to determine whether DPA modulation is a general phenomenon of Cys-loop ligand-gated ion channels (LGICs), and whether this modulation depends on particular amino acid residues. For this, we studied the effects of DPA on human homomeric GABA\(_{\rho1}\), \(\alpha7\) nicotinic, and 5-HT\(_{3A}\) serotonin receptors expressed in Xenopus oocytes. Our results indicate that DPA is an allosteric modulator of GABA\(_{\rho1}\) receptors with an IC\(_{50}\) of 1.6 \(\mu\)M, an enhancer of \(\alpha7\) nicotinic receptors at relatively high concentrations of DPA, and has little, if any, effect on 5-HT\(_{3A}\) receptors. DPA antagonism of GABA\(_{\rho1}\) was strongly enhanced by preincubation, was slightly voltage-dependent, and its washout was accelerated by bovine serum albumin. These results indicate that DPA modulation is not a general phenomenon of LGICs, and structural differences between receptors may account for disparities in DPA effects. In silico modeling of DPA docking to GABA\(_{\rho1}\), \(\alpha7\) nicotinic, and 5-HT\(_{3A}\) receptors suggests that a hydrophobic pocket within the Cys-loop and the TM4 segment in GABA\(_{\rho1}\), located at the extracellular/membrane interface, facilitates the interaction with DPA that leads to inhibition of the receptor. Functional examinations of mutant receptors support the involvement of the TM4 segment in the allosteric modulation of DABA\(_{\rho1}\) by DPA.

Introduction

Dipicrylamine (DPA) is a hydrophobic anion that intercalates into cellular membranes (Wang and Bruner, 1978) and has been broadly used as a probe to study the physical and biologic properties of cellular membranes (Kleijn et al., 1983; Oberhauser and Fernandez, 1995; Chanda et al., 2005). Notably, DPA allows the study of high-frequency neuronal activity in the optical monitoring of voltage in living cells (Bradley et al., 2009); however, the compound’s unexpected effects on GABA\(_{\alpha}\) receptors (GABA\(_{\alpha}\)Rs) and \(N\)-methyl-D-aspartate receptors have imposed limits on its broad physiologic applications (Chisari et al., 2011; Linsenbardt et al., 2013). Thus, a better understanding of the mechanisms involved in DPA’s effects would be helpful for effective modifications of the DPA molecule to reduce or eliminate its pharmacological activity while preserving its key optical characteristics. Thus far, the mechanism and specificity of the DPA inhibition of GABA\(_{\alpha}\) Rs remain elusive (Chisari et al., 2011). Two nonmutually exclusive scenarios may explain DPA’s effects. In one scenario, DPA may trigger unspecific local perturbations in the surrounding protein/lipid interface that are responsible for conformational changes in the receptor and altered gating. In the other scenario, the antagonism is mediated by site-specific interactions between DPA and transmembrane sites of the receptor (Mennerick et al., 2008; Chisari et al., 2011). To explore the potential mechanism for DPA’s effects, we investigated whether DPA also affects human GABA receptors that are composed of \(\rho1\) subunits (GABA\(_{\rho1}\) receptors). The homomeric nature of this receptor is advantageous for structural modeling studies compared with heteromeric GABA\(_{\alpha}\) Rs composed of \(\alpha\), \(\beta\), and \(\gamma\) subunits. After demonstrating that DPA also negatively modulates GABA\(_{\rho1}\) receptors, we compared in silico structural models and the DPA inhibition of homomeric GABA\(_{\rho1}\), \(\alpha7\) nicotinic acetylcholine (ACh) receptors, and 3 A serotonin receptors (5-HT\(_{3A}\)) receptors to identify structural motifs with the potential to interact with DPA. Finally, we performed a mutagenesis analysis of the GABA\(_{\rho1}\) subunit to determine the congruence between the structural model and DPA effects. Our results demonstrate that DPA is an allosteric modulator of GABA\(_{\alpha}\) Rs and exhibits pharmacological effects on \(\alpha7\) nicotinic ACh receptors but little, if any, effects on 5-HT\(_{3A}\) receptors. The
aromatic residue W475, which is located in the upper part of transmembrane domain 4 (TM4) of GABA\(_A\)-\(1\), appears to participate in the gating of the receptor and in the DPA-mediated inhibition of GABA\(_A\)-\(1\) receptors.

Materials and Methods

Molecular Biology. For the experiments using GABA\(_A\)Rs, we used mRNA that was isolated from rat brain cortices using the FastTrack 2.0 kit (Invitrogen, Carlsbad, CA). For the expression of homomeric channels, we used human \(\alpha_7\) nicotinic receptor cDNA (donated by Eleonora Palma, Università di Roma La Sapienza, Rome, Italy), human 5-HT\(_{3A}\) cDNA (purchased from Missouri S&T cDNA Resource Center, Bloomsburg, PA), and GABA\(_A\)-\(1\), which was cloned from a human retina cDNA library (Martinez-Torres et al., 1998), and introduced the genes into pcDNA3 (Invitrogen). These plasmids were transformed into the Escherichia coli DH5\(a\) strain for storage and amplification. Linearized plasmids were used as templates for cRNA synthesis using the mMessage mMachine kit (Ambion, Austin, TX). Stage V–VI Xenopus oocytes were injected with 50 nl of mRNA or cRNA (concentration of 1 mg/ml) and then maintained in Barth’s solution [88 mM NaCl, 0.35 mM Ca\(_{2+}\), 0.41 mM CaCl\(_2\), 1 mM KC\(_l\), 0.82 mM MgSO\(_4\), 2.4 mM NaHCO\(_3\), 10 mM HEPES (pH 7.4)] with 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma-Aldrich, St. Louis, MO) at 16–17°C until the moment of recording.

Electrophysiological Assay. The oocytes were harvested and prepared as previously described (Miledi et al., 2006), with slight modifications as noted. In brief, Xenopus laevis frogs were anesthetized in tricaine methane sulfonate (MS-222, 0.17%) and euthanized by decapitation, in adherence to protocols approved by the University of California Institutional Animal Care and Use Committee. The ovarian lobes were removed, cut into small pieces, and placed in Ca\(^2+\)-free Barth’s solution with 2 mg/ml collagenase type I (Sigma-Aldrich) for 2 hours in constant rotation. After the enzymatic treatment, isolated stage V–VI oocytes were selected and maintained at 17°C for the remainder of the experiment. Healthy-looking oocytes were injected approximately 24 hours after enzymatic dissociation. Three to four days after injection, the oocytes were impaled with two microelectrodes filled with 3 M KC\(_l\) and voltage clamped at –80 mV using a two-electrode voltage clamp amplifier (Miledi, 1982). The oocytes were continuously perfused with gravity-driven frog Ringer’s solution [115 mM NaCl, 2 mM KCl, 1.8 mM CaCl\(_2\), 5 mM HEPES (pH 7.4)] at room temperature (19–21°C). Data acquisition was performed using WinWCP version 3.9.4 (John Dempster, Glasgow, UK), as previously reported (Limon et al., 2010; Ochoa-de la Paz et al., 2012). DPA was purchased from Bioviet (Hartward, CA). The rest of the substances used were obtained from Sigma-Aldrich.

Data Analysis. The antagonist effect of DPA on GABA currents was determined by measuring the percent inhibition produced by different concentrations of DPA. The concentration of DPA causing a 50% decrease in GABA currents (IC\(_{50}\)) was estimated by fitting the following logistic equation to the experimental data: 

\[ I = I_{\text{max}} + \frac{I_{\text{max}} - I_{\text{min}}}{1 + (\text{EC}_{50}/[A])^n} \]

where \(I\) is the concentration of DPA (in molar concentration), \(I_{\text{max}}\) is the amplitude of the agonist response (in nanoamperes), and \(I_{\text{min}}\) is the Hill coefficient. The time constants of activation and deactivation were measured by fitting the Hill equation in the form 

\[ I = I_{\text{max}} \left( \frac{I_{\text{max}} - I_{\text{min}}}{I_{\text{max}} - I_{\text{min}} + [A]^n} \right) \]

where \(I\) is the current amplitude, \(I_{\text{max}}\) is the maximum current amplitude at the concentration of the agonist [A], \(I_{\text{min}}\) is the agonist concentration that induces 50% of the maximal response, and \(n\) is the Hill coefficient. The peak currents were measured by integrating the area under the decay current using Clampfit version 10.0 software (Molecular Devices, Sunnyvale, CA). The experimental data are shown as the mean ± S.E.M. Statistical differences were determined by Student’s t test when comparing a pair of responses and by Dunnett’s t test when making multiple comparisons with respect to the GABA\(_A\)-\(1\) wild-type (WT) receptor (JMP version 10; SAS Institute, Cary, NC). We considered two groups significantly different when \(P < 0.05\).

Homology Modeling. Three homology models of the GABA\(_A\)-\(1\) \(\alpha_7\) nicotinic, and 5-HT\(_{3A}\) subunits were built. The first model was based on the recently published structure of the \(\beta_3\) homomeric human GABA\(_A\)-\(1\) receptor (Protein Data Bank code 4COF) (Miller and Aricescu, 2014), and the last two models used the structure of the nicotinic acetylcholine receptor from the Torpedo marmorata electric organ (Protein Data Bank code 4AQ9) (Unwin and Fujiyoshi, 2012). We used MODELER version 9.12 (Sali and Blundell, 1993) and built 20 models of each subunit by applying the respective alignments of the UniProt code sequences; P24046 (GABA\(_A\)-\(1\)), P36544 (\(\alpha_7\) nicotinic), and P46098 (5-HT3\(_A\)) (Supplemental Figs. 1 and 2). Then, in each case, we chose the best model based on the TM score and root-mean-square deviation of atomic position.

Each subunit was then projected 5-fold onto its respective template using Molsoft ICM-Pro version 3.5 (Trotrov and Abagyan, 1997), and the few clashes observed at the interfaces were removed by local energy minimization. The entire pentamers were refined by energy minimization using the internal coordinate space, following by optimizing the geometry of the built structures with a fast Dreiding-like force field using Accelrys Discovery Studio version 2.5 (San Diego, CA) (Chen et al., 2006). The human GABA\(_A\)-\(1\) subunit is 52, 56, and 61% homologous to the GABA\(_A\) subunits \(\alpha_1\) (Uniprot P14867), \(\gamma_2L\) (Uniprot P18507), and \(\beta_3\) (Uniprot P28472), respectively. Because the structure of the homomeric \(\beta_3\) GABA\(_A\) receptor was recently solved (Miller and Aricescu, 2014), and GABA\(_A\)-\(1\) and \(\beta_3\) form homomeric receptors and have high homology, we think that using \(\beta_3\) as a template can give a better estimation about the physiologic and molecular aspects of the receptor interacting with dipiprylamine.

Molecular Docking. We used Pocket Finder in ICM, as previously reported (Dey and Chen, 2011). In brief, three (GABA\(_A\)-\(1\)), four (\(\alpha_7\) nicotinic), or eight (5-HT\(_{3A}\)) sites per subunit were identified by the automatic detection of small-molecule binding sites on the homomeric receptors (Supplemental Fig. 3); then, one copy of the three-dimensional structure of DPA (2,4,6-trinitro-N-(2,4,6-trinitrophenyl)aniline) was loaded from ChemSpider into the ICM project (CSID: 8258, http://www.chemspider.com/Chemical-Structure.8258.html). The estimated octanol/water partition coefficient of DPA (log Kow = 3.35) indicates that the compound would be poorly soluble in water; thus, we only considered sites that were located at the extracellular/membrane interface (II in GABA\(_A\)-\(1\), \(\alpha_7\) nicotinic, and II and III in 5-HT\(_{3A}\)) as putative sites for allosteric modulation. One or two molecules were accommodated in each pocket by applying a second local energy minimization. The same procedure was done to dock the DPA molecule to the heteromeric model of the GABA\(_A\) receptor. The images were made using ICM and PyMOL version 1.5.0.4 (Schrödinger, San Diego, CA).

Results

DPA’s Effects on GABA\(_A\)Rs. To determine whether the DPA antagonism originally described for the \(\alpha_1\beta_2\) and \(\alpha_1\beta_2\gamma_2\) GABA\(_A\)Rs extends to other endogenous GABA\(_A\)Rs, we evaluated DPA effects on oocytes that were previously injected with mRNA isolated from rat brain cortex. These mRNA-injected oocytes express a large variety of GABA\(_A\)Rs that are representative of those expressed in the rat brain (Demuro et al., 1999). To ensure maximum activation of all subtypes of mRNA-expressed GABA\(_A\)Rs, we tested DPA with saturating concentrations of GABA. In agreement with Chisari et al. (2011), the antagonist effect of DPA on GABA currents in a concentration-dependent manner (Fig. 1, A and C). This result indicates that the DPA antagonism described for \(\alpha_1\beta_2\) and \(\alpha_1\beta_2\gamma_2\) GABA\(_A\)Rs is qualitatively similar in GABA\(_A\)Rs that were expressed by endogenous rat brain mRNA. Notably, the recovery from DPA...
inhibition was not complete, even after 30 minutes; therefore, to avoid cumulative effects on the concentration-response experiments, each oocyte was tested with DPA only once. The IC$_{50}$ of the aqueous DPA concentration was 62 ± 11 nM (n = 3–5 oocytes per concentration), which is a value similar to that reported for the DPA inhibition of GABAB$_{1}$Rs with a $\alpha_{1}Beta_{2}Y_{2}$ stoichiometry (65 nM) (Chisari et al., 2011).

**DPA Antagonism of Homomeric GABA$_{1}$ Receptors.** GABA$_{1}$ receptors exhibit a high affinity for GABA, demonstrate slow desensitization, and have a pharmacological profile so distinctive that for approximately two decades they were classified as GABA$_{C}$ (Martinez-Delgado et al., 2010). Therefore, we investigated whether GABA$_{1}$ receptors were modulated by DPA. Figure 1, B and C shows that the coapplication of DPA and GABA inhibited the maximal response of GABA$_{1}$ receptors, with an IC$_{50}$ of 1.57 ± 0.2 µM (n = 4). The effects of DPA on GABA$_{1}$ kinetics were different from those observed on GABA$_{1}$Rs from rat brain cortices (Fig. 1A) and heterologously expressed $\alpha_{1}Beta_{2}Y_{2}$ receptors (Chisari et al., 2011). The GABA$_{1}$ receptors exhibited a tail current at the end of the GABA and DPA coapplication (Fig. 1B), indicating that inhibition by DPA is partially relieved faster than the deactivation of GABA$_{1}$ channels is completed.

DPA also produced a 5-fold dextral displacement of the concentration-response curve for GABA (Fig. 2), increasing the EC$_{50}$ from 0.91 ± 0.3 µM to 4.59 ± 1.6 µM (n = 3–5 oocytes per point, P = 0.031; Fig. 2, A and B), and a reduction of the Hill coefficient from 2.34 ± 0.3 to 1.07 ± 0.25 (P = 0.001). DPA antagonism was not surmounted by increasing the concentration of GABA, a result that is consistent with the noncompetitive, negative allosteric modulation of the GABA$_{1}$ receptor (Chisari et al., 2011). DPA Antagonism of Homomeric GABA$_{1}$ Receptors. (A) Effect of 1 mM GABA plus 100 nM DPA coapplication on GABA$_{1}$Rs expressed by mRNA-injected oocytes. (B) Concentration-dependent antagonism of GABA$_{1}$ receptors by DPA. (C) Inhibition curves of DPA on GABA$_{1}$Rs (1 mM GABA) and GABA$_{1}$ (100 µM GABA). No DPA preincubation was used for the experiments shown in (A–C). IC$_{50}$ was 1.57 ± 0.2 µM for GABA$_{1}$ (n = 4 oocytes) and 62 ± 11 nM for GABA$_{1}$Rs (n = 3–5 oocytes per concentration).
when the oocytes were tested with DPA only once (91.1 ± 1.7%; Fig. 3, A and B) than when they were tested consecutive times (maximum inhibition of 42.4 ± 7.6%; n = 4; data not shown), suggesting incomplete recovery from DPA’s effects between applications, even after washout periods of more than 20 minutes. Chisari et al. (2011) previously reported that bovine serum albumin (BSA), a molecular scavenger of DPA, accelerated DPA membrane removal and the antagonism offset of GABA ARs (Chisari et al., 2011); therefore, we analyzed whether a similar mechanism was present in DPA antagonism of GABA<sub>1</sub>. In our experiments, BSA also accelerated DPA washout (Fig. 3, C and D), reducing the τ of antagonism offset from 36.2 ± 5.3 seconds in the absence of BSA to 16.8 ± 1.7 seconds in presence of BSA (P = 0.014, n = 4). This result indicates that incomplete recovery of DPA can be explained by incomplete DPA membrane removal.

Voltage Dependence of DPA Inhibition. Partitioned DPA is negatively charged and generates charge movements when translocating within the plasma membrane in response to changes in voltage. Previous studies have indicated that DPA’s charge movements in oocyte membranes can be described by a Boltzmann function with half activation voltage (V<sub>1/2</sub>) for oocytes of −54 and −59 mV for injected and un.injected oocytes, respectively (Chisari et al., 2011). We performed similar experiments in noninjected oocytes and obtained a V<sub>1/2</sub> of −55 ± 2.3 mV (n = 6) (Fig. 4A). Therefore, we investigated whether the effects of DPA on GABA<sub>1</sub> also varied with changes in voltage. For this study, we exploited the slow desensitization of GABA<sub>1</sub>-mediated currents that facilitate the use of voltage ramp protocols to measure the effects of DPA on steady-state currents. A steady-state GABA current was obtained by subtracting the current elicited by a voltage ramp protocol from −90 to +65 mV (80 mV/s) under control conditions from the ramp-elicited current after the response to 1 μM GABA reached an equilibrium (Fig. 4). The sensitivity of any oocyte’s endogenous component to 3 μM DPA was determined by subtracting the ramp-elicited currents before and after 200 seconds of incubation with DPA. The fraction of steady-state GABA current available after 200 seconds of 3 μM DPA preincubation was obtained by subtracting ramp-elicited currents with DPA from those elicited during the coapplication of 3 μM DPA and 1 μM GABA.

The inversion potential of GABA currents (E<sub>GABA</sub>) before and after DPA was −25.4 ± 3.1 mV and −21.8 ± 1.6 mV, respectively, indicating that DPA did not affect the permeability of the channel (n = 5, P = 0.33). We did observe a small but significant voltage dependence for DPA modulation of the GABA<sub>1</sub> receptor. Figure 4C shows that the percentage of inhibition of the GABA current linearly decreased with positive voltages between 0 and +60 mV (r<sup>2</sup> = 0.99); however, at negative voltages down to −60 mV, the percentage of inhibition remained unchanged, regardless of the voltage. We did not extend the voltage ramp protocol beyond +65 mV because more positive voltages are not normally observed in physiologic conditions. Moreover, because the percentage of inhibition trends asymptotically to ±∞ when close to E<sub>GABA</sub>, we excluded from the analysis the voltage range in which the percentage of inhibition could not be reliably calculated. For comparison purposes, we calculated the DPA-mediated inhibition of the steady-state GABA current at −90 and +50 mV. As observed in Fig. 4, DPA reduced the steady-state GABA current to 20.1 ± 5.3% and 35 ± 5.1% of the control at −90 and +50 mV, respectively (n = 5, P < 0.001, paired Student’s t test). Additionally, we also measured the onset rate of DPA antagonism at −90 and +50 mV once the current was already activated. Fig. 4, E–G shows that DPA inhibition of the GABA current was faster at −90 mV than at +50 mV. The best fit for the onset of DPA’s effects was the sum of two exponential functions with the time constants τ<sub>1</sub> = 2.0 ± 0.9 seconds and τ<sub>2</sub> = 9.6 ± 1.6 seconds at −90 mV (n = 5) and τ<sub>1</sub> = 2.3 ± 0.1 seconds...
and $\tau_2 = 16.9 \pm 1.8$ seconds at $+50$ mV ($n = 5$). $\tau_2$ was significantly slower at positive voltages ($P < 0.05$, Student’s $t$ test) (Fig. 4F).

Previous studies, and our own results, indicate that DPA’s charge movements in oocyte membranes can be described by a Boltzmann function with a $V_{1/2}$ of approximately $-54$ mV; therefore, at membrane potentials of $-90$ mV, the concentration of DPA is expected to be higher in the outer leaflet of the plasma membrane. The faster onset of DPA antagonism at negative potentials suggests that inhibition is favored when DPA molecules are near the protein-lipid interface at the upper part of the receptor. In addition, depolarization during ramp protocols should translocate free or weakly bound DPA with a $V_{1/2}$ of approximately $-55$ mV; however, our experiments indicate that a partial removal of steady-state antagonism is observed at values more positive than $0$ mV. This result suggests that depolarizing voltages are not sufficient to counter: 1) the affinity of DPA for the activated receptor and/or 2) the slow kinetics of DPA dissociation (see Discussion).

**DPA Effects on Homomeric Receptors of the Ligand-Gated Ion Channel Family.** The GABA $\rho 1$ and $\alpha 1$ subunits are only 33% homologous, and receptors containing either of these subunits exhibit profound pharmacological differences. Nevertheless, $\rho 1$ and $\alpha 1\beta 2$ GABA receptors are both antagonized by DPA. Based on the effects of DPA on GABA$\alpha 1$, we hypothesized that if DPA also modulated other homomeric members of the Cys-loop ligand-gated ion channel (LGIC) family, then we could perform comparative studies between the functional effects of DPA and the structure of those receptors. Therefore, we evaluated the effects of DPA on homomeric 5-HT$\alpha 1$ and $\alpha 7$ nicotinic receptors.

DPA did not significantly modify the maximal response of 5-HT$\alpha 1$ receptors elicited by a high concentration of 5-HT (10 $\mu$M) (Fig. 5); small changes in the desensitization of 5-HT-elicited currents were not significant upon statistical testing ($P > 0.05$, paired Student’s $t$ test). By contrast, $\alpha 7$ nicotinic receptors were modulated by high concentrations of DPA. Whereas 1 $\mu$M DPA exhibited almost no effect on maximal $\alpha 7$ nicotinic receptor responses elicited by a saturating concentration of ACh (100 $\mu$M) (97 ± 8% of control; $n = 5$), 5 $\mu$M DPA increased the peak of ACh-elicited currents by 91 ± 15% ($n = 12$; Fig. 5). The potentiation of $\alpha 7$ nicotinic receptors by DPA was observed only for the current activation, without effect on desensitization.

**In Silico Modeling of a Binding Domain for DPA in Homomeric Receptors of the LGIC Family.** The differential effects of DPA on GABA$\alpha 1$, $\alpha 7$ nicotinic, and 5-HT$\alpha 1$ homopentameric receptors provided an opportunity to further
examine the structural components of the receptors that might interact with DPA. We created an in silico model of each receptor. Each model displayed a different pattern of potential pockets that can interact with DPA throughout their extracellular and transmembrane domains, according to their particular architecture. Because the partition coefficient of DPA predicts poor solubility in water, we only considered sites that were located at the extracellular/membrane interface as putative sites for allosteric modulation in each case. The 5-HT_{3A} receptor contains two sites that are each able to accommodate one molecule of DPA, whereas the GABA_{B1} and α7 nicotinic receptors display enough space to contain just one molecule at the protein-lipid interface in each subunit (sites II in GABA_{B1}, I in α7 nicotinic, and I and III in 5-HT_{3A}; Supplemental Fig. 3). These sites were located in the upper half of the transmembrane domain near the superior limit of the membrane in all of the models (Fig. 6).

We identified several hydrophobic aromatic residues interacting with DPA in the three models and/or positively charged arginine residues in the GABA_{B1} and α7 nicotinic receptors. These residues include L207, I281, L285, Y289, Y474, and W475 in GABA_{B1}; F157, F159, R227, Y296, F297, and F493 in α7 nicotinic receptors; and F166, F164, F309, and W472 in the 5-HT_{3A} receptors (Fig. 6). The pattern appears to indicate the existence of certain acidic recognition motifs rich in aromatic, hydrophobic, and even positively charged amino acids that provide π-stacking and electrostatic contributions to stabilize the structure of DPA. This conserved pocket formed by amino acids in the Cys-loop, M1, and M4 domains is reminiscent of the general anesthetic cavity that was reported by Nury et al. (2011), notwithstanding that the specific residues that interact in each case induce a very different physiologic response. Previous results demonstrated that a point mutation in the transmembrane domain 2 of the α1 subunit (V256S) that

---

Fig. 5. Differential effects of DPA on Cys-loop LGIC. DPA (5 μM) was preincubated for 180 seconds to allow adequate membrane partition. Homomeric GABA_{B1}, α7 nicotinic ACh (nACh_{α7}), and 5-HT_{3A} receptors were activated by 100 μM GABA, 100 μM ACh, and 10 μM 5-HT, respectively. Notice that 5-HT_{3A} receptors were not affected by high concentrations of DPA.

Fig. 6. Docking of DPA on Cys-loop homomeric receptors. (Left) Structural model of GABA_{B1} interacting with DPA; the hydrophobic interaction with DPA hinders the appropriate communication of the transmembrane domains with the extracellular compartments disturbing the conformational change in the gating. (Center) α7 Nicotinic ACh (nACh_{α7}) receptor; the additional noncovalent contacts between DPA and the Cys-loop domain potentiate the activation of the channel. (Right) 5-HT_{3A}; the loose hydrophobic interactions do not significantly modify the function of the channel.
renders \(\alpha_1\beta_2\) and \(\alpha_1\beta_2\gamma_2\) receptors insensitive to pregnenolone sulfate (PS) (Akk et al., 2001) also removes sensitivity to DPA (Chisari et al., 2011). Akk et al. (2001) originally described the mutation and concluded that this residue is unlikely to be part of the binding site for PS and may influence PS action indirectly. In agreement with that interpretation, our model of GABA\(_{\alpha_1}\) does not predict interactions between the DPA and the residue equivalent to V256 in \(\alpha_1\beta_2\).

**Interactions between DPA and In Silico Heteromeric \(\alpha_1\beta_2\gamma_2\) GABA\(_{\alpha_1}\) Receptor.** To explore possible interactions between V256 and DPA in heteromeric GABA receptors, we used a model of \(\alpha_1\beta_2\gamma_2\) (Estrada-Mondragón and Lynch, 2015) (Supplemental Fig. 4); in this model, V256 in \(\alpha_1\beta_2\gamma_2\) does not interact with DPA, indicating that V256 is most likely involved in the signal transduction mechanisms following binding by DPA to another site. We also explored hydrophobic pockets in the \(\alpha_1\beta_2\gamma_2\) model; interestingly, \(\alpha_1\) and \(\gamma_1\) subunits share the same pattern of interactions with DPA, with basically the same equivalent residues able to accommodate two DPA molecules each; however, the interactions with \(\beta_2\) subunit are somewhat different (Fig. 4). On the other hand, \(\gamma_2\) and \(\beta_2\) are able to accommodate two DPA molecules each; however, the interactions with \(\gamma_2\) are more similar to those described for the 5-HT\(_3\)A receptor, where the impact on function is minimal. Our model predicts that interactions of DPA with the \(\alpha_1\) subunit are strong drivers of noncompetitive antagonism of DPA in heteromeric GABA\(_{\alpha_1}\) receptors. These interactions do not discard that additional nonspecific membrane perturbations also participate in the inhibition of these receptors. Future comparative studies between heteromeric and homomeric GABA\(_{\alpha_1}\) receptors should provide more information regarding the differences in DPA antagonism between these receptors.

**DPA Effects on GABA\(_{\alpha_1}\) Receptors with Structural Modifications on M4.** Because amino acids at the end of the M4 domain appear to be important for DPA binding, and because interactions between M4 and the Cys-loop are necessary for channel activation (Estrada-Mondragón et al., 2010), we studied the effects of the DPA-mediated antagonism of GABA\(_{\alpha_1}\) receptors mutated in the M4 domain.

We tested the effects of DPA on GABA\(_{\alpha_1}\) mutants in which the C termini of the M4 were shortened by one (S479K or GABA\(_{\alpha_1-1}\)-aa), two (F478X or GABA\(_{\alpha_1-2}\)-aa), or three (I477X or GABA\(_{\alpha_1-3}\)-aa) amino acids (Reyes-Ruiz et al., 2010). These mutants exhibited clear changes in their kinetic properties when activated with GABA (Reyes-Ruiz et al., 2010). Whereas the deletion of one amino acid slowed the activation and decay of the current, the deletion of two amino acids greatly accelerated both parameters (Table 1), suggesting a close involvement of the M4 end termini in the gating of the GABA\(_{\alpha_1}\) receptor. To test the effects of DPA, we preincubated the receptors with 1 \(\mu\)M DPA for 180 seconds and then cotreated them with DPA and the EC\(_{50}\) concentration of GABA for each receptor subtype (see Table 1); this procedure allows for the maximal antagonism of DPA at the EC\(_{50}\) while reducing acute nonspecific effects of DPA on the membrane. Using this procedure, we found that the deletion of up to two amino acids did not affect DPA-mediated antagonism (Fig. 7).

The amplitude of the post-DPA current tail was positively correlated with the decay time of the GABA currents (\(r = 0.93\); Supplemental Fig. 5). The largest tail was observed in GABA\(_{\alpha_1-1}\)-aa and was absent in GABA\(_{\alpha_1-2}\)-aa; these were the mutants with the highest and lowest deactivation time constants, respectively (Fig. 7; Table 1). This result indicates that the post-DPA tail arises from speed differences in GABA current decay and relief from DPA antagonism. Further shortening of M4 produced nonfunctional receptors (Reyes-Ruiz et al., 2010).

Next, we examined the role of the residue W475 that our model implicated in the charge stabilization of DPA within the hydrophobic pocket of each GABA\(_{\alpha_1}\) subunit. As previously demonstrated, the substitution of W475 by electrically charged amino acids (W475R and W475D) produced nonfunctional receptors, indicating the fundamental role of W475 in the gating of the receptor (Estrada-Mondragón et al., 2010). The substitution of W475 with hydrophobic amino acids (W475F, W475L, W475G, and W475A) produced functional channels with less sensitivity and lower efficacy in response to GABA and faster kinetics, and all of the mutants except W475L demonstrated less cooperativity (as reported by the Hill coefficient) than the WT receptor (Table 1). Despite the differences in kinetic and affinity properties between mutants and the WT receptor, we did not observe a statistically significant difference in the antagonistic activity of DPA against W475F, W475L, and W475G with respect to the WT

### Table 1

**Properties of GABA\(_{\alpha_1}\) mutants**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>(N)</th>
<th>(EC_{50}) for GABA</th>
<th>Hill</th>
<th>(N)</th>
<th>GABA Current at the (EC_{50})</th>
<th>Tau of Current Activation</th>
<th>Tau of Current Decay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu M) &amp; (nA) &amp; (s) &amp; (s)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>5 0.73 ± 0.14</td>
<td>3.0 ± 0.5</td>
<td>6</td>
<td></td>
<td>3945 ± 206</td>
<td>5.1 ± 2.8</td>
<td>221 ± 0.8</td>
</tr>
<tr>
<td>−1aa</td>
<td>3 0.41 ± 0.05</td>
<td>3.0 ± 0.1</td>
<td>6</td>
<td></td>
<td>2279 ± 479***</td>
<td>11.6 ± 0.8***</td>
<td>28.2 ± 0.9***</td>
</tr>
<tr>
<td>−2aa</td>
<td>3 2.16 ± 0.06**</td>
<td>3.9 ± 0.7</td>
<td>6</td>
<td></td>
<td>5246 ± 109***</td>
<td>2.4 ± 0.1**</td>
<td>6.2 ± 0.1***</td>
</tr>
<tr>
<td>−3aa</td>
<td>3 1.26 ± 0.15</td>
<td>1.4 ± 0.1**</td>
<td>6</td>
<td></td>
<td>604 ± 129***</td>
<td>7.1 ± 0.1*</td>
<td>22.8 ± 0.7</td>
</tr>
<tr>
<td>W475F</td>
<td>12 4.08 ± 0.49***</td>
<td>1.6 ± 0.1**</td>
<td>5</td>
<td></td>
<td>879 ± 91***</td>
<td>3.6 ± 0.2</td>
<td>6.3 ± 0.4***</td>
</tr>
<tr>
<td>W475L</td>
<td>4 1.72 ± 0.24**</td>
<td>2.3 ± 0.02</td>
<td>5</td>
<td></td>
<td>620 ± 65***</td>
<td>4.5 ± 0.5</td>
<td>12.4 ± 0.4***</td>
</tr>
<tr>
<td>W475A</td>
<td>4 2.72 ± 0.35***</td>
<td>1.4 ± 0.2**</td>
<td>6</td>
<td></td>
<td>634 ± 57***</td>
<td>4.6 ± 1.0</td>
<td>11.0 ± 1.1***</td>
</tr>
<tr>
<td>W475G</td>
<td>4 3.30 ± 0.12***</td>
<td>1.4 ± 0.1**</td>
<td>5</td>
<td></td>
<td>54 ± 7***</td>
<td>3.5 ± 0.2</td>
<td>11.7 ± 1.1***</td>
</tr>
<tr>
<td>W465R</td>
<td>3 NR</td>
<td>NR</td>
<td>3</td>
<td></td>
<td>ND</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>W475D</td>
<td>3 NR</td>
<td>NR</td>
<td>3</td>
<td></td>
<td>ND</td>
<td>NR</td>
<td>NR</td>
</tr>
</tbody>
</table>

ND, not determined; NR, no response to GABA.

*\(P < 0.05\); **\(P < 0.005\); ***\(P < 0.0001.\)
receptor; however, W475A was less sensitive to DPA than the WT receptor (Fig. 7). The IC50 for DPA antagonism, measured after 3-minute preincubation with DPA, of W475A's E C50 was significantly higher (843 ± 35 nM, n = 4, P < 0.001, Dunnett's method) than that for W475F (383 ± 38 nM, n = 4) and WT (309 ± 30 nM, n = 4).

Discussion

This study is the first to report that DPA is a negative allosteric modulator of GABA\(_{\alpha}1\) and a potentiator of \(\alpha7\) nicotinic receptors, with no detectable effects on 5-HT\(_{\beta}\)\(_A\) receptors. The differential pharmacological effects of DPA on distinct receptors of the Cys-loop LGIC family are supportive of a mechanistic model in which DPA modulates receptor activity via site-specific interactions located within transmembrane segments.

Interactions between DPA and In Silico Homomeric LGIC Channels. By using the recently published structure of the anionic β3 homomeric human GABA\(_{\alpha}R\) (Miller and Aricescu, 2014), we adjusted the previous GABA\(_{\alpha}1\) model (Estrada-Mondragón et al., 2010), which now exhibits a higher alignment score in homology and identity (Supplemental Figs. 1 and 2). Our new GABA\(_{\alpha}1\) model predicts interactions between the DPA molecule and several residues located in the upper half of the transmembrane domain to form a hydrophobic cavity that encages DPA in a very stable conformation. This hydrophobic cavity is located behind the upper part of the pore. Recent progress in the search for the gating mechanism along the channel indicates that the upper half of the pore that is lined by the M2 domain of each subunit undergoes considerable deformation, being functionally coupled through loop M2–M3 with loop 2 of the extracellular domain during ligand-induced activation (Prevost et al., 2012; Unwin and Fujiyoshi, 2012). Lipids contribute in a fundamental way to achieve this coupling through critical points conferred by few hydrophobic residues and by electrically charged residues in such interphases (Fernandez Nievias et al., 2008; daCosta et al., 2009; Estrada-Mondragón et al., 2010). Our previous studies have demonstrated that at least two amino acids that form the hydrophobic cavity for DPA, W475 in TM4 and L207 in the Cys-loop, participate in planar hydrophobic interactions that are necessary for the activation of the GABA\(_{\alpha}1\) channel (Estrada-Mondragón et al., 2010); therefore, the presence of DPA inside the hydrophobic pocket may hinder physiologic TM4-Cys-loop interactions, which in turn alter the gating of the channel (discussed later).
A similar cavity was identified in α7 nicotinic receptors, wherein an entire hydrophobic aromatic network distributed along the neighboring pre-TM1 and Cys-loop domains appears to interact with DPA. Based on the potentiation effects elicited by DPA, DPA interactions in this cavity may promote energetic coupling stacking by narrowing the distance between the aromatic residues in the TM1 and Cys-loop domains and those in the TM2–TM3 domain because the participation of interresidue coupling in these domains is known to be important for rapid and efficient gating of the nicotinic receptor, as described by Lee et al. (2009).

In the case of the cationic 5-HT₃A receptor, the interface between extracellular and transmembrane domains appears to be quite different from those of anionic LGIC receptors and even significantly different from cationic receptors, such as α7 nicotinic receptors (Bouzat et al., 2008). Certain charged residues that have been identified as key elements for channel activation, such as R241 in the pre-TM1 domain (Hu et al., 2003; Hu and Peoples, 2008) and K76 in loop 2 (Reeves et al., 2005), are located a considerable distance away from the docked position of DPA molecules in our model. In fact, these regions are only stabilized loosely by hydrophobic residues close to the membrane upper leaflet and are substantially distant from any charged residue. The lack of DPA effects away from the membrane upper leaflet and are substantially distant from any charged residue. The lack of DPA effects, however, the slow recovery of DPA effects also suggests that at least one additional substantially slower K off exists for DPA dissociation. Notably, although the mechanism of DPA-mediated antagonism is likely distinct from that of picrotoxin antagonism, the post-DPA tail current of GABA receptors occurs in a highly similar fashion to the postpicrotoxin tail current described for perch GABA receptors (Qian et al., 2005). Future determination of the constant rates for DPA inhibition and equilibrium modeling may help to decipher the sequential states of DPA binding to GABA receptors; however, based on the results using BSA, realistic models should contemplate the kinetics of DPA partitioning-departitioning in the cell membrane.

DPA antagonism reduced GABA efficacy and produced a right-shift displacement of the concentration-response curve; because affinity is influenced by the gating of the receptor (Colquhoun, 1998; Chang and Weiss, 1999) at this stage, we cannot discard the possibility that conformational changes affecting the affinity for GABA participate in DPA-mediated inhibition of GABA responses. However, a single point mutation of W475 generates GABA receptors with very

![Fig. 8](http://molpharm.aspetjournals.org at ASBET Journals on July 9, 2021)
distinct apparent affinities and distinct decay times. The measurement of binding and activity in the same oocyte system indicated that the slow decay of the GABA current in GABA\(\alpha_1\) results from the lock-on of bound GABA while the receptor is in the open state (Chang and Weiss, 1999). If the same mechanism is present in the mutants studied here, a faster decay of the GABA current would reflect a faster transition of GABA\(\alpha_1\) from the active to the closed state. Because previous studies have already demonstrated that a reduction of the channel opening rate may lead to larger EC\(_{50}\) values and lower efficacy (Chang et al., 2000), we hypothesize that mutants with faster decays will display higher GABA EC\(_{50}\) values and lower efficacy. Indeed, Fig. 8 shows a linear correlation between the time constant of GABA current decay and the EC\(_{50}\) for GABA. Moreover, the maximal response to GABA was not affected by several fold in all W475 mutants (Table 1). Because the post-DPA tail and decay time are correlated, we also analyzed the relationship between the post-DPA tail and the EC\(_{50}\) for GABA. Interestingly, the amplitude of the post-DPA tail, in terms of the percentage of the maximal current, fell exponentially with increases in the apparent GABA affinity of the receptor (Fig. 8B). This indicates that alanine substitution at the 475 position is likely to reduce the general hydrophobicity of the DPA molecule and decreasing the antagonism of the DPA molecule and decreasing the antagonism of the DPA molecule.

To determine whether the apparent affinities and efficacies of gamma-aminobutyric acid (GABA) receptor agonists correlated, we also analyzed the relationship between the EC\(_{50}\) for GABA and the EC\(_{50}\) for GABA. Moreover, the post-DPA tail and decay time are correlated, we also analyzed the relationship between the post-DPA tail and the EC\(_{50}\) for GABA. Interestingly, the amplitude of the post-DPA tail, in terms of the percentage of the maximal current, fell exponentially with increases in the apparent GABA affinity of the receptor (Fig. 8B). This indicates that alanine substitution at the 475 position is likely to reduce the general hydrophobicity of the DPA molecule and decreasing the antagonism of the DPA molecule.

**References**


References

Limon A, Reyes-Ruiz JM, Vaswani RG, Chamberlin AR, and Miledi R (2010) The Cys-loop LGIC family and would be equivalent to the hydrophobic pocket near the interphase of the membrane and the extracellular domain that was identified by photolabeling and nuclear magnetic resonance in nicotinic ACh receptors (Chiara et al., 2009; Bondarenko et al., 2013) and by electron paramagnetic resonance spectroscopy and X-ray crystallography in the prokaryotic gllobacter ligand gated ion channels (Nury et al., 2011; Velisetty and Chakrapani, 2012).


Address correspondence to: Agenor Limon, 2226 Gillespie Neuroscience Research Facility, Irvine, CA 92697-1675. E-mail: alimonru@uci.edu