Analgesic $\alpha$-Conotoxin Binding Site on the Human GABA$_B$ Receptor

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Received April 17, 2022; accepted July 18, 2022

ABSTRACT

The analgesic $\alpha$-conotoxins Vc1.1, RgIA, and PeIA attenuate nociceptive transmission via activation of G protein-coupled GABA$_B$ receptors (GABA$_B$Rs) to modulate N-type calcium channels in primary afferent neurons and recombinantly coexpressed human GABA$_B$R and Cav2.2 channels in human embryonic kidney 293T cells. Here, we investigate the effects of analgesic $\alpha$-conotoxins following the mutation of amino acid residues in the Venus flytrap (VFT) domains of the GABA$_B$R subunits predicted through computational peptide docking and molecular dynamics simulations. Our docking calculations predicted that all three of the $\alpha$-conotoxins form close contacts with VFT residues in both B1 and B2 subunits, comprising a novel GABA$_B$R ligand-binding site. The effects of baclofen and $\alpha$-conotoxins on the peak Ba$^{2+}$ current ($I_{Ba}$) amplitude were investigated on wild-type and 15 GABA$_B$R mutants individually coexpressed with human Cav2.2 channels. Mutations at the interface of the VFT domains of both GABA$_B$R subunits attenuated baclofen-sensitive $I_{Ba}$ inhibition by the analgesic $\alpha$-conotoxins. In contrast, mutations located outside the putative peptide-binding site (D380A and R98A) did not. The key GABA$_B$R residues involved in interactions with the $\alpha$-conotoxins are K168 and R207 on the B2 subunit and S130, S153, R162, E200, F227, and E253 on the B1 subunit. The double mutant, S130A + S153A, abolished inhibition by both baclofen and the $\alpha$-conotoxins. Depolarization-activated $I_{Ba}$ mediated by both wild-type and all GABA$_B$R mutants were inhibited by the selective GABA$_B$R antagonist CGP 55845. This study identifies specific residues of GABA$_B$R involved in the binding of the analgesic $\alpha$-conotoxins to the VFT domains of the GABA$_B$R.

SIGNIFICANCE STATEMENT

This study defines the binding site of the analgesic $\alpha$-conotoxins Vc1.1, RgIA, and PeIA on the human GABA$_B$R receptor to activate Gi/o proteins and inhibit Cav2.2 channels. Computational docking and molecular dynamics simulations of GABA$_B$R identified amino acids of the Venus flytrap (VFT) domains with which the $\alpha$-conotoxins interact. GABA$_B$R alanine mutants attenuated baclofen-sensitive Cav2.2 inhibition by the $\alpha$-conotoxins. We identify an allosteric binding site at the interface of the VFT domains of the GABA$_B$R subunits for the analgesic $\alpha$-conotoxins.

Introduction

Marine cone snail venom is a rich source of peptides called conotoxins that have evolved for defense and prey capture (Jin et al., 2019). $\alpha$-Conotoxins are disulfide-bonded peptides that antagonize nicotinic acetylcholine receptors (nAChR) in the central and peripheral nervous systems and many exhibit exquisite selectivity for nAChR subtypes (Azam and McIntosh, 2009; Lebbe et al., 2014; Abraham and Lewis, 2018).

This work was supported by the Australian National Health and Medical Research Council Program [Grant APP1072113] (to D.J.A.) and the Rebecca Cooper Foundation for Medical Research [Grant PC2019306] (to J.R.M.). A.R.W. acknowledges financial support through the Australian Government Research Training Program Scholarship.

No author has an actual or perceived conflict of interest with the contents of this article.

dx.doi.org/10.1124/molpharm.122.000543

This article has supplemental material available at molpharm.aspetjournals.org.

ABBREVIATIONS: Ala, alanine; DRG, dorsal root ganglion; eGFP, enhanced green fluorescent protein; MD, molecular dynamics; GABA$_B$R, G protein-coupled $\gamma$-aminobutyric acid type B receptor; HEK, human embryonic kidney; $I_{Ba}$, Ba$^{2+}$ current; LB1, lobe 1; LB2, lobe 2; mGlU5, metabotropic glutamate receptor 5; nAChR, nicotinic acetylcholine receptor; PCT, proximal carboxyl terminus; PDB, Protein Data Bank; 7TM, seven-helix transmembrane domain; VFT, Venus flytrap; WT, wild-type.
receptor subunits and the selective GABA<sub>B</sub>R antagonist CGP 55845 blocked the modulation by ω-conotoxins (Cuny et al., 2012).

ω-Conotoxin Vc1.1 modulates Cav2.2 via a site distinct from the orthosteric GABA<sub>B</sub>R binding site for agonists, γ-aminobutyric acid and baclofen. The GABA<sub>B</sub>R agonists typically bind to the B1 Venus flytrap (VT) domain, then activates the GABA<sub>B</sub>R, leading to inhibition of Cav2.2 channels via a pathway involving Gβγ subunits (Galvez et al., 1999). The binding of Gβγ to Cav2.2 modifies it from a “willing” to “reluctant” gating state, thus shifting inactivation to more hyperpolarized potentials while slowing activation kinetics (Zamponi and Currie, 2013). Vc1.1 modifies Cav2.2 channel kinetics through a distinct mechanism from the classic GABA<sub>B</sub>R agonists (increased activation rate and hyperpolarized shift in half-maximum inactivation) (Huynh et al., 2015). Cav2.2 channels in HEK293T cells coexpressing hCav2.2 and GABA<sub>B</sub>R constructs were inhibited by Vc1.1 with mutations that abolish GABA/baclofen binding (B1-S270A and B1-S246A) (Huynh et al., 2015). This finding suggested that Vc1.1 could act at an allosteric site on GABA<sub>B</sub>Rs. In DRG neurons, selective GABA<sub>B</sub>R antagonists, CGP 54626 and CGP 55845, blocked Vc1.1 modulation of Cav2.2-mediated currents (Callaghan et al., 2008; Callaghan and Adams, 2010). However, in HEK293 cells expressing GABA<sub>B1</sub>B1/S270A that attenuates CGP 54626 binding (Galvez et al., 1999; Geng et al., 2013), this compound was unable to antagonize Vc1.1 inhibition of Cav2.2 (Huynh et al., 2015). This suggests that CGP 54626 and CGP 55845 either act allosterically to inhibit Vc1.1 binding or bridge the orthosteric pocket and an allosteric site (distinct from S270 and S246 of B1).

Recent cryo-electron microscopy studies of the GABA<sub>B</sub>R have been elucidated for the receptor bound to a variety of different ligands. The observed structures capture alternate conformations from the inactive apo state to the fully active G protein-bound state (see Shaye et al., 2021). The N-terminal extracellular domains of B1 and B2 each consist of two lobes, LB1 and LB2, of the VFT domains, which trap the ligand in its orthosteric binding site situated on B1 at the B1/B2 interface. Previous structural studies of the GABA<sub>B</sub>R suggest both agonists and antagonists are anchored by a set of polar residues and a key aromatic residue (W182) of LB1. Upon agonist binding, the two lobes close while engaging two bulky aromatic LB2 residues sandwiching the ligand (Geng et al., 2012; 2013). Two major receptor conformations, active and inactive, as well as two intermediate states, have been observed in cryogenic electron microscopy structures, providing insights into the activation pathway of the GABA<sub>B</sub>R upon activation by agonists (Shaye et al., 2020).

The present study used computational docking studies and molecular dynamics simulations, with the crystal structures of the GABA<sub>B</sub>R VFT subunits as templates, to identify amino acid residues with which ω-conotoxins Vc1.1, RgIA, and PeIA likely interact. These residues were mutated to investigate their impact on the activation of the GABA<sub>B</sub>R by the different ligands, as determined by inhibition of hCav2.2 channels expressed in HEK293T cells. We identify an allosteric binding site at the interface of the VFT domains of the GABA<sub>B</sub>R subunits for the analgesic ω-conotoxins.

Materials and Methods

Computational Peptide Docking. Conotoxin-receptor complex structures were predicted via molecular docking using AutoDock Vina (Trott and Olson, 2010; Eberhardt et al., 2021). The GABA<sub>B</sub>R extracellular VFT structure was used as the receptor (Protein Data Bank (PDB) ID: 4MQF, resolution 2.22 Å; Supplemental Data PDB file 1P0) (Apo) (Geng et al., 2013). The ω-conotoxin structures used were obtained as follows: Vc1.1 (PDB ID: 2H8S) (Clark et al., 2006), RgIA (PDB ID: 2JUT) (Ellison et al., 2008), PeIA (PDB ID: 5JME) (Daly et al., 2011), and ImI (PDB ID: 1G2G) (Lamthanh et al., 1999). Discovery Studio Visualizer (BIOVIA, Dassault Systemes, Discovery Studio Visualizer; San Diego, CA) was used to modify parent conotoxins to create the analog RgIA4. The GABA<sub>B</sub>R and all conotoxin structures were converted to PDBQT format using PyRx (Dallakyan and Olson, 2015), the graphical frontend for Autodock Vina. GABA<sub>B</sub>R torsion angles were treated as fixed. Conotoxin backbone torsion angles were fixed, while amino acid sidechain torsion angles were set as flexible. The Vina grid box was centered on GABA<sub>B</sub>R and set with x, y, z dimensions of 82.4 Å × 77.6 Å × 84.5 Å, covering the entire accessible surface of the receptor. The exhaustiveness parameter was set to 256 for all dockings. 2D ligand-receptor interaction diagrams were produced using Schroedinger Maestro (Schroedinger Release 2021-4; Maestro, Schroedinger, LLC, New York, NY). The most favorable docking poses selected for each of GABA<sub>B</sub>R-bound ω-conotoxins were docked into the orthosteric ω-conotoxin binding site, which guided subsequent experimental mutagenesis and electrophysiology studies. The stereochemical quality of each model was evaluated using PROCHECK (Laskowski et al., 1993) and VERIFY3D (Bowie et al., 1991; Lüthy et al., 1992), accessed via the SAVES v6.0 server (saves.mbi.ucla.edu) and with ProSa-Web (Sippel, 1993; Wiederstein and Sippl, 2007). Model quality statistics are presented in Supplemental Table 1. Structural files in PDB format for the top docking-predicted models of Vc1.1, RgIA, PeIA, and RgIA4 bound to the GABA<sub>B</sub>R VFT were provided in Supplemental Data PDB files, PDB 2 (Vc1.1), PDB 4 (RgIA), PDB 5 (PeIA), and PDB 2 (RgIA4), respectively. The top eight energetically favored docking-predicted models for ImI bound to the GABA<sub>B</sub>R VFT are provided in Supplemental Data PDB files, PDB 2 (ImI - model 1), PDB 3 (ImI - model 2), PDB 11 (ImI - model 3), PDB 10 (ImI - model 4), PDB 9 (ImI - model 5), PDB 8 (ImI - model 6), PDB 7 (ImI - model 7), and PDB 6 (ImI - model 8).

Molecular Dynamics Simulations. Energetically favored binding poses for the VFT of GABA<sub>B</sub>R bound to Vc1.1, RgIA, or PeIA, predicted using docking as described above, were used for subsequent all-atom fully solvated molecular dynamics (MD) simulations. Each conotoxin-receptor complex was used as the template. The GABA<sub>B</sub>R orthosteric ω-conotoxin binding site from the crystal structure was modeled in the GABA<sub>B</sub>R orthosteric ω-conotoxin binding site with dimensions of 124 Å × 124 Å × 124 Å. Each box was filled with 39612 TIP3P water molecules (Jorgensen et al., 1983). Approximate box of dimensions 124 Å × 124 Å × 124 Å. Each box was filled with 39612 TIP3P water molecules (Jorgensen et al., 1983). Approximately 128 Na<sup>+</sup> and 122 Cl<sup>-</sup> ions were added to neutralize the charge and produce an approximate ionic concentration of 150 mM. Simulations were performed using GROMACS 2019 (Van Der Spoel et al., 2005; Pronk et al., 2013) and the CHARMM36m forcefield (Huang et al., 2017). The integration time step was set to 2 femtoseconds. Van der Waals interactions were switched to zero between 0.8 and 1.2 nm. Electrostatic interactions were evaluated using the fast smooth particle-mesh Ewald method (Essmann et al., 1995) with a Coulombic potential cutoff of 1.2 nm. Covalent bonds involving hydrogen atoms were constrained using the LINear Constraint Solver algorithm (Hess et al., 1997). For the restraint-free simulations, the velocity rescale thermostat of Bussi et al. (2007), with a coupling time constant of 0.1 picoseconds (ps), was used to maintain the temperature of all simulations at 310 K. The pressure was maintained at 1 bar using isotropic coupling with the Parrinello-Rahman barostat algorithm (Parrinello and Rahman, 1981). The systems were first energy-minimized using the steepest descent algorithm for a maximum of 10,000 steps. Each system was equilibrated by running positional-restraint simulations in which all non solvent heavy atoms were positionally restrained, firstly under a constant particle number, velocity, and temperature ensemble for 100 ps, followed by a
constant particle number, pressure, and temperature simulation for another 100 ps. Subsequently, all restraints were removed, and 1000 nanoseconds (ns) equilibrium simulations were performed for each of the three z-conotoxin-GABABR systems, as well as the apo VFT, producing a total of 4 microseconds (µs) worth of trajectories. Molecular structures were visualized using Visual Molecular Dynamics version 1.9.3 (Humphrey et al., 1996).

HEK293T Cell Culture and Transfection. HEK293 cells expressing large SV40 T antigens (HEK293T) were obtained from American Type Cell Culture Collection (ATCC CRL-3216; RRID: CVCL_0063); Virginia, USA. HEK293T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Thermo Fisher Scientific, Mulgrave, VIC, Australia) containing 10% fetal bovine serum (GIBCO, Thermo Fisher Scientific), 1% penicillin and streptomycin (Pen/Strep, Invitrogen, Thermo Fisher Scientific) and 1% GlutaMAX (Invitrogen, Thermo Fisher Scientific). Cells were incubated in 5% CO2 at 37°C in a humidified incubator and passaged at 10% original density to when ~80% confluency was reached.

HEK293T cells were transiently cotransfected with plasmid cDNAs encoding human Cav2.2 channels (subunits z1m-e37b, z2a1, and z3b; each subunit 2 µg; OriGene Technologies, Inc.; Rockville, MD) and either tricistronic human GABAB1, GABAB2 with enhanced green fluorescent protein (eGFP, 2 µg; custom-designed clone of Adams Laboratory) (wild-type GABAaR) or mutants of GABAaR also tagged with eGFP (2 µg; GenScript; Piscataway, New Jersey) using calcium phosphate transfection (Kumar et al., 2019). Cells were plated on 12 mm glass coverslips and incubated with calcium DNA precipitation mix in growth media at 37°C for 16–18 hours. Afterward, upon changing the transfection media to fresh cell culture media, they were transferred to a 30°C incubator with 5% CO2, 48–72 hours post-transfection, cells were used at room temperature (22°C–24°C) to conduct experiments.

Patch Clamp Electrophysiology. Whole-cell voltage clamp recordings were acquired using a MultiClamp 700B amplifier and digitized through Digidata 1440A (Molecular Devices; San Jose, CA). Data were obtained using pClamp 11 (Molecular Devices) software while maintaining series resistance to <10 MΩ, and the cell capacitance compensated by ≥80%. The external bath solution to record GABAaR-coupled Cav2.2-mediated currents contained (mM): 100 NaCl, 10 BaCl2, 1 MgCl2, 5 CaCl2, 30 TEA-Cl, 10 D-Glucose, and 10 HEPES, pH adjusted to 7.35 using TEA-OH and ~320 mOsmol.kg⁻¹. Patch pipettes were filled with internal solution containing (in mM): 120 Kgluconate, 5 NaCl, 2 MgCl2, 5 EGTA, 10 HEPES, 5 MgATP, and 0.2 NaGTP, pH adjusted to 7.2 using KOH, ~295 mOsmol.kg⁻¹ and had resistance of 1–3 MΩ.

Whole-cell peak Ba²⁺ currents (Iₚ₉) were elicited by a test pulse to 0 mV from a holding potential (V₀) of ~90 mV, sampled at 10 kHz, filtered at 1 kHz, and leak current subtracted using a P/4 pulse protocol. Whole-cell Cav2.2-mediated Iₚ₉ is normalized to the baclofen-sensitive component to eliminate expression artifacts associated with transient transfection of HEK293T cells with human GABAa receptor subunits and Cav2.2 channels. α-Conotoxins Vc1.1, RgIA, PeIA, ImI, and RgIα4 were synthesized as described previously (Calaghan et al., 2008; Daly et at., 2011; Romero et al., 2017) and kindly provided by Dr. Richard Clark (The University of Queensland, Brisbane, QLD, Australia). (±)-Baclofen was purchased from Sigma-Aldrich (St Louis, MO, USA) and CGP 55845 hydrochloride was purchased from Tocris Bioscience (Bristol, UK). All solutions including the drugs (peptides, baclofen, and CGP 55845) were perfused using a peristaltic pump at an exchange speed of 1 ml.min⁻¹ in an experimental chamber (~0.5 ml volume) at room temperature.

Immunocytochemistry. HEK293T cells were transfected with Cav2.2 and GABAaR or the mutants of GABAaR tagged with eGFP using calcium phosphate transfection. After transfection, cells were washed twice with 1x PBS, pH 7.4 for 5 minutes and fixed with Zamponi's solution containing 1.6% formaldehyde (Australian Biostain Pty Ltd.; Traralgon, Australia). Immunostaining was performed using the indirect fluorescence method. The primary antibodies and their dilution used for the receptor subunits were anti-GABAaR1 (goat, 1:250; Santa Cruz Biotechnology, Dallas, TX, USA; sc-7338) and anti-GABAaR2 (rabbit, 1:250; Santa Cruz Biotechnology; sc-28792). They were visualized consequently with Alexa Fluor 647-conjugated donkey anti-goat antibody (1:500; life technology, A21447) and Alexa Fluor 555-conjugated goat anti-rabbit (1:500; Abcam, ab150078). Before staining, cells were permeabilized with 0.1% Triton X-100 for 10 minutes and blocked for 1 hour with a PBS-based blocking solution containing 1% bovine serum albumin and 0.1% Triton X-100. For double immunostaining of the receptor subunits, primary antibodies were added simultaneously and incubated at 4°C overnight, rinsed in three changes of PBS (10 minutes each), and then incubated sequentially for Alexa Fluor 647-conjugated donkey anti-goat antibody followed by Alexa Fluor 555-conjugated goat anti-rabbit; each for 1 hour at room temperature. Negative controls were carried out by omitting the primary antibodies in transfected HEK293T cells and by including the primary antibodies in nontransfected HEK293T cells.

Fig. 1. GABAaR-Vc1.1 structure, illustrating residues targeted for mutagenesis studies and comparison with the baclofen binding site. Computational docking predicted binding of Vc1.1 (blue ribbon) at the intersubunit region of the VFT in ribbon representation (B1 = transparent blue, B2 = transparent red). Residues predicted to form close contacts with z-conotoxin Vc1.1, RgIA, and PeIA and whose Ala mutants result in significantly reduced inhibition of baclofen-sensitive Cav2.2-mediated current are shown as orange spheres, whereas mutant residues resulting in significantly reduced inhibition are shown as yellow spheres. Negative control residues whose mutants exert negligible effect on baclofen-sensitive Cav2.2-mediated currents are shown as gray spheres. (B) Baclofen (large spheres), agonist-binding residues (pink sticks) and residues that interact exclusively with z-conotoxins but not with classic agonists and antagonists (gray sticks).
All coverslips were counterstained with 4',6-diamidino-2-phenylindole (1:5000, 10 minutes, room temperature), mounted (Dako North America Inc.; Carpinteria, CA), sealed, and stored at 4°C. Images were obtained using a Leica SP8 confocal microscope (Leica Microsystems Pty Ltd.; Macquarie Park, Australia) using a 40x oil immersion objective and analyzed using Las X software (RRID: SCR_013673, Leica microsystem).
Data Analysis and Statistics. Patch clamp electrophysiology data are reported as mean ± S.E.M. and n is the number of individual experiments. The significance of peptides effects among different mutants of GABABR was compared with the wild-type (WT) GABABR group. Therefore, multiple comparisons were performed by one-way ANOVA followed by Dunnett’s post hoc test. Values of $P < 0.05$ were considered significant. Clampfit 11 (Molecular Devices; San Jose, CA) and GraphPad Prism 9 (San Diego, CA) software were used to analyze the data.

Control currents were obtained by measuring the peak steady-state whole-cell $I_{Ba}$ ($I_{control}$), peak inhibition of peptides Vc1.1, RgIA, and PeIA ($I_{peptide}$) by applying saturating concentrations, and finally applying a saturating concentration of baclofen ($\approx 50 \mu M$; $I_{Bac}$) to isolate the baclofen-sensitive.
currents. To calculate baclofen-sensitive inhibition of GABA<sub>B</sub> R-coupled Cav2.2 currents by each peptide, we used the following formulas:

\[
\text{Baclofen inhibition} = \frac{(I_{\text{control}} - I_{\text{Bac}})}{I_{\text{control}}} (1)
\]

\[
\text{Inhibition by peptides} = \frac{(I_{\text{control}} - I_{\text{peptide}})}{I_{\text{control}}} (2)
\]

\[
\% \text{Inhibition of baclofen-sensitive current by peptide} = \frac{(I_{\text{peptide}}/I_{\text{Bac}}) \times 100} (3)
\]

Notably, the selective GABAB<sub>R</sub> antagonist, CGP 55845, was applied at the end of each experiment to validate GABAB<sub>R</sub> inhibition by peptides and/or baclofen.

### Results

**Molecular Docking, Simulations, and Identification of α-Conotoxin-VFT Interactions.** The initial binding positions of α-conotoxins Vc1.1, RgIA, and PeIA interacting with the VFT of the GABA<sub>B</sub> R were predicted using automated molecular docking calculations. The docking calculations predicted that all three of the conotoxins bind at the interface between subunits B1 and B2, as well as in the vicinity of the hinge point between lobe 1 (LB1) and lobe 2 (LB2) of both subunits. This constitutes a novel intersubunit site for GABA<sub>B</sub>R ligand binding (Fig. 1A). While the predicted α-conotoxin binding residues include those that interact with classic agonists such as baclofen, residues predicted to interact exclusively with α-conotoxins are situated near the intersubunit cleft in both B1 and B2 subunits (Fig. 1B).

The most energetically favored binding poses are shown in Fig. 2A for Vc1.1, Fig. 3A for RgIA, and Fig. 3C for PeIA. All three α-conotoxins form similar patterns of binding interactions with their surrounding GABA<sub>B</sub>R residues. As an illustration, the 2D ligand interaction diagram of Vc1.1 (Fig. 2C) shows all of the GABA<sub>B</sub>R residues predicted to reside within 5Å of any atom of each of the three α-conotoxins. These receptor residues comprise those that interact closely with known agonists such as baclofen and GABA and include the subunit B1 (LB1) residues S130, S131, and S154 (Geng et al., 2013). Similar interactions are identifiable from 2D ligand diagrams for RgIA (Supplemental Fig. 4A) and PeIA (Supplemental Fig. 4B). Additionally, a small number of residues in the B2 subunit are also involved in α-conotoxin binding. In particular, K168, in LB1, is consistently predicted to form hydrogen bonding (Vc1.1 and PeIA) or salt bridge interactions (RgIA) with all of the conotoxins.

All-atoms, fully solvated MD simulations were subsequently performed on the three α-conotoxin-GABA<sub>B</sub>R VFT complexes to further investigate the roles of key residues of subunits B1 and B2 in facilitating receptor interactions with these toxins. The simulation trajectories for Vc1.1, RgIA, and PeIA bound to the VFT indicate that the conotoxins form strong, persistent, and stable interactions with the key B1 subunit binding site residues. The most significant contacts with B1 are shown as blue time series curves in Figs. 2B, 3B, and 3D, which show relatively high numbers of interatomic contacts between each conotoxin and the subunit exhibiting the most persistent interaction during their respective simulation trajectories, namely, the LB1 residues S130 and S131 for Vc1.1 and PeIA, and the LB2 residue E200 for RgIA. Each of the conotoxins also forms regular, but more intermittent, contacts with the key B2 subunit residue, K168, as shown by the red time series curves in these figures.

### Site-Directed Mutagenesis, Electrophysiology, and Immunocytochemistry

Based on the initial docking calculations, residues that are predicted to form direct hydrogen bonding or salt bridge interactions with the conotoxins were selected for experimental characterization via alanine (Ala) mutagenesis and electrophysiology studies. In addition, a subset of other residues within the vicinity of these tight-binding residues were also selected for experimental validation of their roles in conotoxin activity at GABA<sub>B</sub>R. The positions of the residues selected for experimental Ala mutagenesis studies are summarized in Fig. 1 and are color-coded according to the effects of their mutation to Ala. The effects of baclofen and α-conotoxins Vc1.1, RgIA, and PeIA on whole-cell I<sub>Ba</sub> mediated by WT GABA<sub>B</sub>R-coupled Cav2.2 channels expressed in HEK293T cells are shown in Fig. 4 and Supplemental Fig. 5. Bath application of Vc1.1, RgIA, and PeIA (1 μM) inhibited the baclofen-sensitive I<sub>Ba</sub> amplitude of human Cav2.2 channels cotransfected with WT and mutant GABA<sub>B</sub>R subunits (Fig. 5). In contrast, in HEK293T cells overexpressing single-Ala mutants of GABA<sub>B</sub>R B1 and B2 subunits, baclofen (50 μM) inhibition of I<sub>Ba</sub> amplitude was unchanged, whereas inhibition of I<sub>Ba</sub> by the α-conotoxins was significantly attenuated for all mutants, except for E253A in the presence of PeIA (Fig. 5). Furthermore, two residues that were predicted to be remote from the α-conotoxin binding site, R89 and D380, were also mutated to Ala. Patch clamp recording of HEK293T cells expressing these mutants confirmed that these two residues are inconsequential for α-conotoxin activity. Overexpression of the
Fig. 5. Inhibition of WT and mutant GABA<sub>B</sub>R-coupled Cav2.2 channels expressed in HEK293T cells by analgesic α-conotoxins Vc1.1, RgIA, and PeIA. (A) (i) Vc1.1 (1 μM) inhibition of baclofen-sensitive I<sub>Ba</sub> amplitude mediated by human Cav2.2 channel cotransfected with WT GABA<sub>B</sub>R. Bar graph of the effect of 1 μM Vc1.1 on the baclofen-sensitive I<sub>Ba</sub> in HEK293T cells overexpressing S130A, S131A, S153A, S154A, S130A + S153A, R162A, T198A, E200A, E253A, F227A, and D380A mutants of GABA<sub>B</sub>R B1 subunit and R89A, D165A, K168A, or R207A mutants of GABA<sub>B</sub>R B2 subunit. Dashed line indicates inhibition of baclofen-sensitive current by Vc1.1 with WT GABA<sub>B</sub>R. (ii) Superimposed whole-cell I<sub>Ba</sub> mediated by GABA<sub>B</sub>R-coupled Cav2.2 channels in absence (control, black) and presence of 1 μM Vc1.1 (blue), and 100 μM baclofen (gray). Overexpression of the double mutant (S130A + S153A) abolished the inhibition of GABA<sub>B</sub>R-coupled Cav2.2 channels by both baclofen and Vc1.1. (B) (i) RgIA (1 μM) inhibition of baclofen-sensitive I<sub>Ba</sub> amplitude of hCav2.2 channel cotransfected with WT GABA<sub>B</sub>R. Bar graph of the effect of 1 μM RgIA on the baclofen-sensitive I<sub>Ba</sub> in HEK293T cells overexpressing S130A, S131A, S153A, S154A, S130A + S153A, R162A, E200A, E253A, F227A, and D380A of the B1 subunit and R89A, D165A, K168A, or R207A mutants of the B2 subunit. Dashed line indicates inhibition of baclofen-sensitive current by RgIA with WT GABA<sub>B</sub>R. (ii) Superimposed whole-cell I<sub>Ba</sub> mediated by GABA<sub>B</sub>R-coupled Cav2.2 channels in absence (control, black) and presence of 1 μM RgIA (green) and 100 μM baclofen. Similarly, overexpression of the double mutant (S130A + S153A) abolished RgIA and baclofen inhibition of GABA<sub>B</sub>R-coupled Cav2.2
double mutant (S130A + S153A) abolished the inhibition of GABA<sub>R</sub>-coupled Cav2.2 channels by both baclofen and the z-conotoxins. In the presence of the selective GABA<sub>R</sub> antagonist CGP55845 (1 μM), the effects of baclofen and z-conotoxins on WT and all GABA<sub>R</sub> mutants, except the double mutant (S130A + S153A), were completely abolished. A summary of the inhibition of baclofen-sensitive I<sub>Na</sub> (%I<sub>peptide/I<sub>baclofen</sub></i>) mediated by WT and mutant GABA<sub>R</sub>-coupled Cav2.2 channels by z-conotoxins Vc1.1, RgIA, and PeIA is presented in Table 1.

To confirm that the GABA<sub>R</sub>B Ala mutants were expressed in the HEK293T cell membrane, commercially available antibodies were used to explore the expression and colocalization of the GABA<sub>R</sub> subunits. Positive immunodetection and colocalization were observed for both subunits for WT and GABA<sub>R</sub> mutants (Fig. 6; Supplemental Fig. 2), including those that were predicted to form close contact with the analgesic z-conotoxins (S130A, S153A, E253A, and K168A; Fig. 6). We did not observe immunoreactivity when the primary antibodies directed to GABA<sub>R1</sub> and GABA<sub>R2</sub> were omitted, or where the antibodies were used in nontransfected HEK293T cells (Supplemental Fig. 3).

### Negative Control Peptides Iml and RgIA4

Docking calculations were also performed on the negative control peptides, z-conotoxin Iml, and the RgIA analog, RgIA4 (Romero et al., 2017), both of which are inactive at GABA<sub>R</sub> (Callaghan et al., 2008; Romero et al., 2017; Bony et al., 2022). Both peptides are predicted to form far fewer contacts with subunit B1 and exhibit stronger preferences for binding to B2 compared with the active conotoxins Vc1.1, RgIA, and PeIA. Supplemental Fig. 1 shows all of the binding positions predicted for Iml, none of which lie within the intersubunit region predicted (and experimentally confirmed) to be important for conotoxin activity. For RgIA4 (Fig. 7a, bottom), binding is predicted to occur near the intersubunit region, similar to Vc1.1, RgIA (Fig. 7a, top), and PeIA. However, RgIA4 forms few contacts with the subunit B1 agonist site residues S130 and S131, and instead, is skewed toward close contacts with B2. In a series of experiments testing the activity of 1 μM RgIA4 on GABA<sub>R</sub>-coupled Cav2.2 channels expressed in HEK293T cells, the baclofen-sensitive I<sub>Na</sub> amplitude was reduced to <15% compared with RgIA (Fig. 6B). The preferential interaction with the B2 subunit, as opposed to B1, may explain the lack of activity of RgIA4.

### Molecular Dynamics Predictions of z-Conotoxin-Dependent VFT Structural Transitions

To examine the impact of z-conotoxin binding on the VFT structure, the interlobe separation of the B1 subunit was measured using the minimum distance between I286 and E343 as a proxy, while the separation between B1-R239 and B2-E230 was calculated to represent the intersubunit distance at the juxtamembrane region. Interlobe separation plots for the B1 subunits bound to Vc1.1, RgIA, and PeIA are shown in Fig. 8A. The VFT of all of the z-conotoxin-bound complexes remain open, and none are as closed as the baclofen-bound state (dashed black line). The Vc1.1-bound VFT is marginally more open than apo- and antagonist-bound VFT (blue line), while the PeIA-VFT complex is the next most open structure. In contrast, RgIA induces substantially higher separation between the lobes, resulting in a highly open B1 conformation (green line). z-Conotoxins do not substantially influence the B2 lobe, which remains similar to the initial conformation throughout (Supplemental Fig. 6). Intersubunit separation plots for the VFT dimer are shown in Fig. 8B. Vc1.1 has marginally higher intersubunit separation than apo-VFT. Interestingly, it occasionally induces a “closed” interlobe separation that is even lower than that observed experimentally for baclofen-bound VFT (blue line), particularly toward the end of the microsecond-long simulation. RgIA induces a consistently greater separation than Vc1.1. PeIA causes the most marked increase in intersubunit separation (purple line), with complete detachment of LB2 between the B1 and B2 subunits beyond 400 ns.

### Discussion

#### Discovery of Novel Allosteric Sites for z-Conotoxin Ligand Binding

The initial binding positions of z-conotoxins Vc1.1, RgIA, and PeIA with the VFT of the GABA<sub>R</sub> were predicted using molecular docking calculations. In contrast to the known small-molecule agonists of GABA<sub>R</sub>, which bind exclusively to the B1 subunit, all three of the analgesic z-conotoxins examined form close contact with residues in both B1 and B2 subunits. (Fig. 1). These receptor residues include those that interact closely with known channels. (C) (i) PeIA (1 μM) inhibits baclofen-sensitive I<sub>Na</sub> current amplitude of Cav2.2 channel transfected with WT-GABA<sub>R</sub>B in HEK293 cells to 57.5 ± 3.2% (n = 11). Bar graph of the effect of 1 μM PeIA on the baclofen-sensitive I<sub>Na</sub> in HEK293T cells overexpressing S130A, S131A, S135A, S154A, S130A + S153A, R162A, E200A, E253A, F227A, and D380A of the B1 subunit and R89A, D165A, K168A, or R207A mutants of the B2 subunit. Dashed line indicates inhibition of baclofen-sensitive current by PeIA with WT GABA<sub>R</sub>B. (ii) Superimposed whole-cell I<sub>Na</sub> mediated by GABA<sub>R</sub>-coupled Cav 2.2 channels in the absence (control, black) and presence of 1 μM PeIA (purple) and 100 μM baclofen (gray). Overexpression of the double mutant abolished the inhibition of I<sub>Na</sub> by PeIA. Data represent mean ± S.E.M. One-way ANOVA followed by Dunnett's post hoc test. Statistical significance, **** P < 0.0001 versus WT-GABA<sub>R</sub>B.
agonists such as baclofen and GABA and include the subunit B1 (LB1) residues S130, S131, and S154 (Geng et al., 2013). K168, in B2 (LB1), is consistently predicted to form hydrogen bonding (Vc1.1 and PeIA) or salt bridge interactions (RgIA).

The residues selected for experimental Ala mutagenesis studies are summarized in Fig. 1A. S130, S131, and E253 are known residues important for the activity of classic agonists, such as baclofen. Residues situated near the intersubunit interface region in both B1 (such as F227) and B2 (K168) subunits uniformly show significant interactions with all three α-conotoxins. Baclofen inhibition mediated by mutant GABA_{B}R-coupled Cav2.2 channels was unchanged between WT and all GABA_{B}R mutants except for the double mutant S130A + S153A. This suggests that analgesic α-conotoxins target largely different residues, and unlike baclofen, this interaction might also engage a non-G_{i/o} mediated voltage-independent pathway (Berecki et al., 2014).

Docking predictions for the negative controls, ImI and RgIA4, lend further support to our proposed model, as these peptides lack the identified interactions with these residues. Residues located in LB1, such as W65, S130, G151, S153, H170, and E349, are responsible for anchoring ligands in the binding pocket and interact with both agonists and antagonists. Ligand interaction with the LB2 residue Y250 is unique for agonists, and W278 located in the same domain has been found to only interact with high-affinity antagonists in addition to agonists. The binding footprint of α-conotoxins overlaps those of classic ligands (Fig. 1B), including S131 and Y250 within lobes 1 and 2 of subunit B1 (colored red in Fig. 1B), but also involves residues proximal to the B1/B2 interface (yellow in Fig. 1B).

MD simulations were performed on the three α-conotoxin-GABA_{B}R VFT complexes to further highlight the roles of key residues of subunits B1 and B2 in facilitating receptor interactions with these toxins. The most stable contacts are formed with subunit B1 residues within the agonist pocket (such as S130 and S131), while the B2 subunit residue K168 is also closely involved in binding with α-conotoxins, albeit playing an auxiliary role in anchoring conotoxins to the novel intersubunit/interlobe site. Radioligand binding studies using [3H]GABA and site-directed mutagenesis show that the orthosteric binding site of GABA_{B}R is located in the VFT of GABAB1. The binding of ligands to the VFT B2 subunit has not previously been observed, though the seven-helix transmembrane domain (7TM) of GABAB2 hosts an allosteric binding site and is responsible for G-protein coupling (Kniazeff et al., 2016). Our results suggest that the VFT B2 subunit is involved in the activation of GABA_{B}R by α-conotoxins.

**Putative VFT Structural Transitions and Possible Influences on Activation Pathways Induced by α-Conotoxin Binding.** The conformation of the VFT in the extracellular domain is closely associated with the functional state of GABA_{B}R, and crystal structures indicate that both intrasubunit/interlobe conformations are sensitive to ligand binding. Agonists stabilize the B1 subunit of the VFT in a “closed” configuration, in which the two lobes of subunit B1 (LB1 and
LB2) encase the ligand, as in the case of baclofen (PDB ID: 4MS4; Geng et al., 2013). In contrast, the apo- or antagonist-bound B1 subunit of the VFT exhibits an “open” conformation, in which the lobes are separated (e.g., PDB ID: 4MQF; Geng et al., 2013). Furthermore, the agonist-bound GABABR exhibits close contacts between B1 and B2 in the juxtamembrane region of the VFT, while apo- or antagonist-bound receptors induce a higher separation between the subunits. The interlobe separation of the B1 subunit was measured using the minimum distance between I286 and E343 as a proxy, while the separation between B1-R239 and B2-E230 was calculated to represent the intersubunit distance at the juxtamembrane region. Simulations revealed the promotion of VFT conformations not yet observed in experiments, which are distinct from those previously known to be stabilized by small-molecule agonists and antagonists. Binding of α-conotoxins induced higher separation between LB1 and LB2 in the B1 subunit, resulting in an “open” conformation that exceeds that of the known apo- and antagonist-bound VFT structures (Fig. 7A). Similar structures have previously spontaneously emerged in unbiased MD simulations of the isolated B1 VFT subunit, characterized by Evenseth et al. (2020) as the “wide open” state. In the present simulations, this “wide open” conformation is exemplified by the RgIA-VFT complex (green line) where interlobe opening occurred within 100 ns of the present simulations while the apo-VFT B1 lobe retains its initial classic “open” conformation (Supplemental Fig. 6). Although Evenseth et al. (2020) cautioned against overinterpretation of the “wide open” conformation due to the lack of the B2 subunit in their simulations, our present work shows that association with the adjacent B2 subunit does not hinder the emergence of this predicted B1 conformation. There is little influence of α-conotoxin binding on the interlobe separation of B2 (Supplemental Fig. 7). We propose that the “wide open” B1 conformation, being distinct from that induced by baclofen and other agonists, could trigger the alternative G protein activation pathway demonstrated by Huynh et al. (2015).

In addition to intrasubunit B1 changes, the binding of α-conotoxins may also induce increased distance between the B1 and B2 subunits in the juxtamembrane region, as exemplified by the PeIA-VFT complex (Fig. 7B, purple line), in which LB2 of both subunits are fully separated during the simulation. Such a dimeric conformation has not yet been observed experimentally for GABABR. While the focus of the current work is on identifying the binding site of α-conotoxins on the VFT, we also acknowledge that the absence of the 7TM could influence the variability of the intersubunit distance. However, full-length apo-class receptors exhibit a wider range of conformations, indicating that the absence of the 7TM influences the conformational landscape.

**Fig. 7.** Simulation and docking-predicted binding location and functional analysis for RgIA and its analog RgIA4 at GABABR. (A) Docking-predicted poses of the analog RgIA (top) and RgIA4 (bottom) bound at the initial 4MQF structure, with the α-conotoxins shown as large spheres at the intersubunit region of the VFT (blue = B1, red = B2). Residues previously predicted to form close contacts with Vc1.1, RgIA, and PeIA at the B1 subunit and whose Ala mutants result in significantly reduced inhibition of baclofen-sensitive current are shown as blue spheres. RgIA4 is known to be inactive at GABABR, and docking predicts that it preferentially binds toward the B2 side of the interface, while only partly forming contacts with the main B1 agonist/antagonist site, in contrast to RgIA which binds primarily to the B1 site. (B) (i) RgIA4 (1 μM) had no significant effect on baclofen-sensitive I\textsubscript{Ba} amplitude of hCav2.2 channel cotransfected with WT GABABR. I\textsubscript{Ba} amplitude was reduced by <20% (n = 5) by 1 μM RgIA4 compared with RgIA (1 μM). (ii) Superimposed whole-cell high voltage-activated I\textsubscript{Ba} currents mediated by GABABR-coupled Cav 2.2 in the absence (control, black) and presence of 1 μM RgIA4 (light green) and 100 μM baclofen (gray).
metabotropic glutamate receptor 5 (mGlu5) (Koehl et al., 2019) also exhibits a wide separation of >35 Å at the juxtamembrane region, similar to that observed for the “open,” PeIA-bound VFT conformations. Given that mGlu5 is also a class C G protein-coupled receptor, with a similar structural architecture, it is possible that a broad range of configurational “openness” is also energetically accessible to GABA<sub>B</sub>R even in the presence of the 7TM and lipid membrane. Such large intersubunit separation does not occur in our simulation of the apo-VFT (Supplemental Fig. 6). Further work is required to elucidate the full impact of z-conotoxin binding on the complete structure of GABA<sub>B</sub>R, including experimental determination of a complete z-conotoxin--GABA<sub>B</sub>R complex.

Most intriguing of all is the structural impact of Vc1.1 on the VFT. In the Vc1.1-VFT simulation, the binding of the toxin resulted in greater closure between the subunits B1 and B2 at the juxtamembrane region, similar to that observed for the “open,” PeIA-bound VFT conformations. However, the interlobe separation retains an openness that closely resembles that of the classic antagonist-bound state, though less than the “wide open” conformation present in RgIA- and PeIA-bound VFT. The Vc1.1-bound VFT structure appears to be a novel hybrid conformation composed of an “agonist-like” B1 subunit, but an “agonist-like” dimer complex. The existence of this postulated hybrid state and its functional implications remain to be confirmed experimentally. Nevertheless, it suggests one structural mechanism by which binding of Vc1.1 may activate alternative...
G protein pathways distinct from baclofen and other agonists.

Conclusions

This study elucidated the main site of α-conotoxin interaction with the GABA<sub>B</sub>R<sub>2</sub>, showing that the three α-conotoxins studied bind to both B1 and B2 subunits in the VFT, unlike classic small-molecule agonists and antagonists whose interaction footprints are confined to B1. We propose that α-conotoxin binding at the intersubunit cleft of the VFT may accelerate structural transformations across the dimer, including separation of the subunits at the juxtamembrane region and increase in interlobe distance, triggering a looser conformation in the dimeric complex, similar to that observed for mcIlu5 (Koehl et al., 2019). Such broad structural transitions appear to suggest the initial propagation of conformational shifts from the extracellular VFT to the intracellular 7TM. The downstream interactions across the dimer, including separation of the subunits at the juxtamembrane region and increase in interlobe distance, triggering a looser conformation in the dimeric complex, similar to that observed for mcIlu5 (Koehl et al., 2019).

α-Conotoxin Binding Site on the GABA<sub>B</sub> Receptor

References


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