Conformational ensembles of non-peptide ω-conotoxin mimetics and Ca\(^{2+}\) ion binding to human voltage-gated N-type calcium channel Ca\(_{\text{v}}\)2.2

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Abstract

Chronic neuropathic pain is the most complex and challenging clinical problem of a population that sets a major physical and economic burden at the global level. Ca\(^{2+}\)-permeable channels functionally orchestrate the processing of pain signals. Among them, N-type voltage-gated calcium channels (VGCC) hold prominent contribution in the pain signal transduction and serve as prime targets for synaptic transmission block and attenuation of neuropathic pain. Here, we present detailed in silico analysis to comprehend the underlying conformational changes upon Ca\(^{2+}\) ion passage through Ca\(_{\text{v}}\)2.2 to differentially correlate subtle transitions induced via binding of a conopeptide-mimetic alkylphenyl ether-based analogue MVIIA. Interestingly, pronounced conformational changes were witnessed at the proximal carboxyl-terminus of Ca\(_{\text{v}}\)2.2 that attained an upright orientation upon Ca\(^{2+}\) ion permeability. Moreover, remarkable changes were observed in the architecture of channel tunnel. These findings illustrate that inhibitor binding to Ca\(_{\text{v}}\)2.2 may induce more narrowing in the pore size as compared to Ca\(^{2+}\) binding through modulating the hydrophilicity pattern at the selectivity region. A significant reduction in the tunnel volume at the selectivity filter and its enhancement at the activation gate of Ca\(^{2+}\)-bound Ca\(_{\text{v}}\)2.2 suggests that ion binding modulates the outward spaying of pore-lining S6 helices to open the voltage gate. Overall, current study delineates dynamic conformational ensembles in terms of Ca\(^{2+}\) ion and MVIIA-associated structural implications in the Ca\(_{\text{v}}\)2.2 that may help in better therapeutic intervention to chronic and neuropathic pain management.

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1. Introduction

Chronic pain is a serious health issue that affects more than 25% of the world’s population, and is increasing with the population ages [1]. Approximately, 8% of general population is affected by neuropathic pain [2] that is abnormal signaling due to injury or malfunction in the peripheral or central nervous system resulting in exaggerated pain sensations [3–6]. It originates from plastic changes at peripheral, spinal, or supraspinal sites known as peripheral neuropathies (postherpetic neuralgia, diabetic neuropathies, and focal traumatic neuropathies), central neuropathies (ischemic cerebrovascular injury, spinal cord injury and Parkinson’s disease) and mixed neuropathies (diabetic neuropathies, sympathetically maintained pain) [4]. Neuropathic pain has challenged the biomedical research to develop more effective drugs. Many drugs that treat inflammatory pain, such as Nonsteroidal anti-inflammatory drugs (NSAIDs) or opioids are in general much less effective in relieving neuropathic pain and exhibit efficacy only at high doses or when administered by more invasive (e.g., intraspinal) routes. Currently, neuropathic pain is treated mostly by tricyclic antidepressants, specific serotonin and norepinephrine modulators, and sodium and calcium channel modulators [7]. Currently, only 3 Food and Drug Administration (FDA) approved drugs, gabapentin, pregabalin and ziconotide target voltage-gated calcium channels (VGCCs); however, pregabalin and gabapentin have serious side effects and low efficacy issues [8]. The ω-conotoxin MVIIA or Ziconotide (SNX-11; Prialt\(^\text{TM}\)) is a novel non-opioid analogic drug that is a synthetic version from a large class of marine cone snail neuropeptides [9–11]. This drug has been approved for the symptomatic management of severe chronic pain. It is administered intrathecally to attain optimal analgesic efficacy along with less serious side-effects as it has limited ability to cross blood–brain barrier [11]. This has prompted the development of dozens of small-molecule blockers that are effective in the animal models [12–13].
Animal studies demonstrate that due to peripheral tissue inflammation or nerve injury, Ca_{2.2} (N-type Ca^{2+} channel subunit alpha-1B) expression is enhanced in the dorsal horn [14–15]. Ca_{2.2} deficient mice exhibit hypoesthesia to inflammatory and neuropathic pain [16–18]. Hence, this channel is considered as an important target for treating nociceptive and neuropathic pain [19–22]. Ca_{2.2} is a transmembrane VGCC that is involved in various cell signaling responses and membrane potential alterations that induce calcium influx. As a result, intracellular calcium level elevates to micro molar range [23] that stimulates various calcium channel processes, i.e., gene transcription, membrane excitability, neurotransmitter release, neurite outgrowth and activation of calcium-dependent enzymes such as calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) [24]. The prolonged elevation of intracellular calcium levels is cytotoxic [25] as the intrinsic gating processes and cell signaling pathways tightly regulate the channel activity and trafficking to and from membrane [26]. The blockade of synaptic transmission through Ca_{2.2} serves as a prime mechanism for the reduction of pain signals to the central nervous system [27]. Ca_{2.2} is a complex ion channel protein that is comprised of 4 or 5 different subunits encoded by multiple genes. The largest and most important subunit is α1 subunit of 262 kDa molecular weight that is encoded by CACNA1B gene in human [28]. All Ca_{α1} subunits of VGCC possess a common transmembrane topology in all transmembrane domains. Their important functions include pore conduction, voltage-sensing, gating apparatus and channel regulation [29]. Ca_{α1} subunits have four homologous domains (D1-D4), each having six transmembrane segments (S1-S6), S4 helix is voltage-sensing segment, while the loop between S5 and S6 segments in each domain is involved in pore formation [29]. It involves in ion conductance and selectivity. In addition to α1 subunit, VGCCs are composed of other subunits that are β-subunits, δ-α2β-subunits and γ-subunits. Among them, 4 β- and 2 δ-α2β-subunits, while 8 γ-subunits are involved in enhancing α1-subunit cell surface expression and its interaction with diverse intracellular signaling molecules that modulate the gating properties [30–32].

Animal venoms have high efficacy and selectivity against a wide range of biological targets such as membrane proteins i.e., ion channels, receptors and transporters [33–36]. To date, about 500–700 Conus species have been identified [37] containing valuable neuroactive peptides. Multiple attempts have been made to develop conopeptide mimetics and small molecule inhibitors against VGCCs. Among 21 ω-conotoxins peptides identified so far [38–39], the most well characterized ω-conotoxins are Ca_{2.2} blockers: MVIIA, CVID and GVIA [40]. Here we characterized the published conopeptide-mimetic inhibitors that are based on diverse scaffolds i.e., dendritic scaffold [41], benzothiazole derivative [42], antranilamide derivative [43], antranilamide scaffold [44] and antranilamide compounds with diphenylmethylpiperazine moiety [40] to block Ca_{2.2} (Table S1). Our in-silico analysis provides significant information about the binding pattern and its impact on the Ca_{2.2} channel conformation and activity. By exploring the association of Ca_{2.2} and small-molecule conopeptide-mimetic inhibitors, our study may provide invaluable insights into the potential anesthetic and analgesic impact on pain by multi-targeted inhibition.

2. Methodology

2.1. Molecular modeling of Cav2.2

The amino acid sequence of Homo sapiens α1-subunit of Ca_{2.2} was retrieved through UniProtKB (www.uniprot.org) having an accession number: Q00975. Due to lack of crystal structure, Oryctolagus cuniculus voltage-gated calcium channel, Ca_{a1.1} (PDB ID: 5GJW; resolution: 3.6 Å; sequence coverage: 63% (70-1873AA); sequence identity: 50%:) structure [45] was used as a template to model α1-subunit of Homo sapiens Ca_{2.2} through SWISS-MODEL (swissmodel.expasy.org). The modeled structure of Human Ca_{2.2} (contains 1771 residues (Phe76-Pro1846)) exhibits 0.45 global quality estimation score (GMQE) and 44% sequence similarity. The predicted structure was visualized by UCSF Chimera 1.11.2 [46]. The poor rotamers and outliers in predicted model was refined by WinCoot [47] and subsequently validated by multiple evaluation tools. MolProbity server (http://molprobity.manchester.ac.uk/) was used to analyze the Ramachandran score, Ramachandran outliers, poor and favored rotamers, bad angles and bonds. ERRAT server (https://services.mbi.ucla.edu/ERRAT/) was utilized to calculate the overall quality factor of modeled structure.

2.2. Selection of inhibitors

Initially, 30 inhibitors were retrieved against Ca_{2.2} through extensive literature survey (Table S1). These inhibitors were non-peptide mimetics of ω-conotoxins (MVIIA, CVID and GVIA) isolated from cone snail. From these 30 inhibitors, 7 compounds were filtered out based on their pore binding positions (Table S2). In C1, three important amino acids mimetics (R10, L11 and Y13) of ω-conotoxin MVIIA were attached to dendritic scaffold [41]. In C2-4 benzothiazole scaffold [48,58] and in C5 and C6 contained antranilamide scaffold that projected the side chain mimetics of the key residues (K2, Y13 and R17) in ω-conotoxin GVIA [43,48]. C7 shared a similar pattern to that of C6, except bearing an antranilamide scaffold that was modified by replacing phe-noxyaniline substituent with a diphenylmethylpiperazine moiety [40]. 2D structures of these inhibitors were drawn by ChemDraw Pro 12.0 [49] and converted into 3D coordinates that were further energy minimized using Avogadro® [50] tool through Merck Molecular Force Field (MMFF94) and steepest descent algorithm [51].

2.3. Molecular docking analysis

Molecular docking analysis was performed through PatchDock [52] to evaluate the interaction pattern of Ca_{2.2} with Ca^{2+} ion and selected compounds. For Ca_{2.2} and Ca^{2+} ion docking, Ca^{2+} ion selective and permeable residues of Ca_{2.2} were obtained through UniProtKB (www.uniprot.org/uniprot/Q00975), were provided. For all docking runs, modeled Ca_{2.2} and compounds were subjected to rigid docking though small-scale flexibility, implicitly by allowing few steric clashes and intermolecular penetrations. PatchDock identifies geometric patches through segmentation algorithm, surface matching, filtering and scoring [52]. After detailed comparative analysis of the binding sites of inhibitors, suitable candidate solutions were chosen on the basis of Root Mean Square Deviation (RMSD) clustering. Based on these findings, out of 30 docked compounds (Table S1, Fig. S1) only 7 were shortlisted on the basis of their binding abilities at the ion selectivity and permeability region of Ca_{2.2} pore (Table S2). These compounds were further scrutinized by published IC_{50} values [40–44]. The interactions were carefully evaluated through UCSF Chimera 1.11.2 [46]. Graphical visualization of hydrogen bonding, hydrophobic and electrostatic interactions were analyzed by LigPlus [53] and Discovery Studio 4.5 [54].

2.4. Molecular dynamics simulation analysis

C1 was selected for detailed analysis owing to its binding preference at the SF ring of pore region by interacting with three residues of EEEE motif (E314, E663, and E1365) (Table S2). In order to...
evaluate comparative conformational readjustments, apo-Ca\textsubscript{2,2}, Ca\textsuperscript{2+} ion and C1-bound modeled Ca\textsubscript{2,2} (1771 residues; Phe76-Pro1846) complexes were subjected to MD simulation assay through Visual Molecular Dynamics (VMD) [55] and Nanoscale Molecular Dynamics (NAMD) tools [56]. For all three systems, psfgen plugin of VMD was utilized to generate protein structure files (PSF). These structures were solvated by Heimit Grubmuller’s SOLVATE program [57] that fills empty space inside the pores by surrounding it with water. The undesired water molecules were removed. The solvated structures were embedded in the remaining system by deleting unwanted water molecules in the hydrophobic region of Ca\textsubscript{2,2} using VMD.

An automated system of VMD generated lipid bilayer by Membrane Builder by specifying 1-palmitoyl 2-oleoyl phosphatidylcholine (POPC) membrane patch of length 128 Å in X and Y coordinates and CHARMM27 force field [58]. VMD was utilized to align partially solvated Ca\textsubscript{2,2} tetramer with POPC lipid membrane. Ca\textsubscript{2,2} was settled in the membrane with the hydrophobic residues; while, the orientation of protein was adjusted to avoid its overlap with lipid molecules. Subsequently, the overlapping water molecules were removed. VMD Solute plugin was used for the solvation of entire system by placing it in the specified size water box. Ionization of all systems was performed by VMD Autoionize plugin that generates specific ionic concentration of NaCl (0.4 mmol/L) and transmutes water molecules into ions. The membrane patches were initially equilibrated by short simulation runs (0.5 ns) while keeping the system static, except lipid tails to disorder in fluid-like bilayer. The temperature was set to 300 K by employing Langevin dynamics to maintain constant temperature and 1 atm pressure (by Langevin piston method) throughout production simulation. Minimizations were carried out for 1000 steps. NAMD 2.9 was used to perform the equilibrium MD simulations for all systems. Instead of standard CHARMM parameter file, a modified parameter file containing “NBFIX” with correction for carbonyl oxygen-Ca\textsuperscript{2+} ion interaction (distance restrained to 2.85 Å) was used. This correction is essential for the selectivity filter (SF) in Ca\textsubscript{2,2}. SwissParam [59] was utilized for the topology file generation of compounds. The periodic boundary conditions (PBC) and Particle Mesh Ewald (PME) grid size was also set for all systems in the NAMD configuration files. The outputs were analyzed having disordered lipid tails. MD simulations were run with full dynamics that are essential for the setting of unusual atomic positions in the systems, followed by minimization and equilibration. Harmonic constraints were enforced on Ca\textsubscript{2,2}. The minimization and equilibration steps were executed for 0.5 ns by NAMD2 [56]. The full systems were then equilibrated by keeping protein channel unconstrained for 0.5 ns. Fully equilibrated systems were analyzed by computing Root Mean Square Deviation (RMSD) and Root mean square fluctuation (RMSF) plots through VMD. The final production runs were carried out for 100 ns. The resulting outputs were analyzed by VMD tool [55] and UCSF Chimera 1.11.2 [46].

3. Results

3.1. Model analysis of Ca\textsubscript{2,2}

The crystal structure of Oryctolagus cuniculus Ca\textsubscript{a,1.1} [60] (PDB ID: 5GJV; resolution: 3.6 Å; sequence coverage: 63%; sequence identity: 50%) was selected as a template to model 3D structure of Ca\textsubscript{2,2} through SWISS-MODEL (swissmodel.expasy.org). An RMSD value of 0.196 Å was observed upon superimposition of template and target structures. Ramachandran plot designated the presence of 90.23% residues of Ca\textsubscript{2,2}-modeled structure in the favored region, 9.60% residues as favored rotamers, 0.17% residues as poor rotamers and 1.46% residues as Ramachandran outliers. The observed ERRAT quality factor value was 81.46%.

Human Ca\textsubscript{2,2} also known as N-type calcium channel consists of 2,339 residues with a molecular mass of ~262 kDa comprises 4 domains (D\textsubscript{1–D4}); each contains 6 transmembrane (S1–S6) \(\alpha\)-helices (Fig. 1A) [45]. Two helices (S5 and S6) from each domain constitute a pore that is essential for the conduction of Ca\textsuperscript{2+} ions (Fig. 1) [45]. The voltage sensor domain (VSD) consists of 4 helices (S1–S4) in each domain (Movie S1). Any voltage change across the cell membrane is sensed by VSD that surrounds the pore region. In between the S5 and S6 helices, a P-loop is present that acts as a selectivity filter for Ca\textsuperscript{2+} ion permeability [45]. The N- and C-termini of channel are localized in the cytoplasmic environment. In addition to these four domains, there are two additional domains named as EF-hand (helix-loop-helix domain) and IQ (isoleucine-glutamine motif) that are crucial for Ca\textsuperscript{2+} binding. EF-hand is a helical domain that is flanked by a 12-residue loop from both sides [61]. It is involved in binding with Ca\textsuperscript{2+} ions through undergoing multiple conformational changes [61]. IQ motif in IQ domain interacts with Ca\textsuperscript{2+}/calmodulin.

3.2. Ca\textsuperscript{2+} ion coordination

As voltage-gated calcium channels (VGCCs) allow Ca\textsuperscript{2+} ions to pass through the pore region, Ca\textsuperscript{2+} ions were coordinated with the modeled Ca\textsubscript{2,2} structure. Among surrounding residues, Glu314 and Glu1365 residues potentially coordinated with Ca\textsuperscript{2+} ion having bond lengths of 3.12 Å and 3.25 Å, respectively (Fig. 2).

3.3. Molecular docking analysis

Multiple reports suggested that non-peptide analogues of oc-conotoxins may target Ca\textsubscript{2,2} channels [38,41,43,70,83–85]. These non-peptide conotoxin mimetics mimic the scaffolds of oc-conotoxins MVIIA, CVID and GVIA [62]. In total, out of 30 docked compounds (Fig. S1; Table S1), 7 [40–44] were selected on the basis of their binding pattern at the pore region of Ca\textsubscript{2,2} shared by S5 and S6 helices along with P-loop of each domain (Fig. 4, S2). These compounds contain dendritic, benzothiazole or anthranilamide scaffolds, attached with Y, LK and R residues (Fig. 3, Table S2). The IC\textsubscript{50} values of 7 selected compounds against Ca\textsubscript{2,2} are listed in Table S2. Compound C1 exhibits a dendritic scaffold. C2-4 contain benzothiazole, C5-6 have anthranilamide scaffold, whereas C7 holds a modified anthranilamide scaffold with a phenoxylamine group that is replaced into diphenylmethylpiperazine moiety. These compounds may be used for the treatment of chronic and neuropathic pain by blocking Ca\textsubscript{2,2} VGCCs.

Compound C1 interacted with the residues of pore and S6 segments of Ca\textsubscript{2,2}. It exhibited favorable associations with 3 of the selectivity filter (SF) ring residues (Glu314, Glu663 and Glu1365) (Fig. 4I). Compounds C2 and C3 binding was promoted through hydrophobic association with residues of D\textsubscript{i} (pore), D\textsubscript{in} (S5, pore and S6) and D\textsubscript{tv} (pore and S6). Ala1652, Thr1653, Ser1696 residues of Ca\textsubscript{2,2} were involved in hydrogen bonding with both compounds (Fig. 4II, 4III). C4 exhibited association with D\textsubscript{in} (S5, pore and S6) and D\textsubscript{tv} (pore and S6), while a single hydrogen bond was observed with O-atom of Thr1653 residue (Fig. 4IV). Contrary to C2-C4, compounds C5-C6 showed interactions with the residues of D\textsubscript{i} (S5, pore and S6) and D\textsubscript{tv} (S6). Moreover, a single H-bond was observed via Asn697 and Met313 residues, respectively (Fig. 4V, 4VI). Compound C7 exhibited a favorable number of hydrophobic associations with the residues of D\textsubscript{i} (S5, pore and S6), D\textsubscript{in} (S6), D\textsubscript{tv} (pore) and D\textsubscript{tv} (pore). Furthermore, Met347, Asn697, Thr1653 and Thr1363 residues exhibited hydrogen bonding (Fig. 4VII). The schematic binding details are indicated in Fig. S2. These findings illustrate that only compound C1 exhibited...
bonding with three residues (E314, E663, and E1365) of the Ca²⁺ ion SF ring, therefore, compound C1 may be a good choice for MD simulation assay.

3.4. Molecular dynamics simulation analysis

Compound C1 was selected for detailed binding pattern and conformational ensemble study based on its localization at the SF ring and central cavity of pore. In order to explore the overall stability and time-dependent conformational transitions in Ca₂⁺ upon binding to C1 and Ca²⁺ ions, molecular dynamics (MD) simulations were performed by utilizing Visual Molecular Dynamics (VMD) and Nanoscale Molecular Dynamics (NAMD) tools. Systems preparation (apo-Cav2.2, Cav2.2-Ca²⁺ ion and Cav2.2-C1) for MD simulations were carried out by wrapping the Cav2.2 structural model in 1-palmityol 2-oleoyl phosphatidylcholine (POPC) membrane to create a native-like surrounding (Fig. S5). All systems were minimized and equilibrated to analyze the passage of Ca²⁺ ions through embedded ion channel in the lipid membrane.

The overall protein stability and time-dependent interactions were monitored by generating PDB files at different time intervals. Subsequently, underlying conformational and structural changes in the protein channel in association with Ca²⁺ ion and C1 were analyzed. Root mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration (Rg), radial pair distribution function g(r) and number of hydrogen bonds were calculated throughout 100 ns time scale.

To examine the convergence in all three systems, RMSD values computed through Ca-atoms were plotted against time (Fig. 5A). RMSD scores observed over 100 ns for apo-Ca₂⁺ and both complexes displayed an abrupt rise up to 10 nm. All simulated structures acquired a stable state at 40 ns. The apo-Ca₂⁺ and Ca²⁺-
bound Caᵥ₂.₂ attained a stable state within a range of 11–15 nm whereas, C₁-bound Caᵥ₂.₂ demonstrated an increasing trend in the RMSD profile (18–19 nm). RMSF plot revealed certain fluctuating residues at the loop regions of Caᵥ₂.₂ between DII and DIII (Fig. 5B, Table 1). Glu₃₁₄, Glu₆₆₃, Glu₁₃₆₅ and Glu₁₆₅₅ residues of SF ring involved in Ca²⁺ ion selective permeability exhibited lower fluctuations during simulation assays; however, these residues were more stable in Caᵥ₂.₂-Ca²⁺ complex as compared to apo-Caᵥ₂.₂ and Caᵥ₂.₂-C₁ complex. In addition to SF ring, Gly₃₅₃, Ala₇₀₆, Ala₁₄₁₃ and Ala₁₇₀₅ residues (the G/A/A/A ring) at inner gate of channel and interacting residues lying at S₄-S₅ linker were quite stable in both complexes. Major fluctuations were observed in the loop regions, while α-helices and ion selectivity residues remained stable during the course of simulation.

Radius of gyration (Rg) values were calculated for both complexes and apo-Caᵥ₂.₂ to get insight into compactness of protein

**Fig. 2.** Ca²⁺ ion coordination at the selectivity filter (SF) ring of Caᵥ₂.₂. (A) Top view of Caᵥ₂.₂. The four homologous repeats exhibit a clockwise arrangement, when viewed from extracellular side. VSD of each domain is labeled by specific colors. Glu residues responsible for the Ca²⁺ ion selectivity and permeability are shown in red sticks. (B) Interactions are analyzed by LigPlus [53]. (C) The positions of Glu residues are labeled in respective colors. The distances between them are illustrated by dotted lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Fig. 3.** 2D structures of selected mimetics. C₁, MVIIA (dendritic scaffold attached to Y₁₃, L₁₁ and R₁₀ residues); C₂, CVID (benzothiazole scaffold attached to K₂, Y₁₃ and R₁₇ residues); C₃, GVIA (benzothiazole scaffold attached to K₂, Y₁₃ and R₁₇ residues); C₄, GVIA (benzothiazole scaffold attached to K₂, Y₁₃ and R₁₇ residues); C₅, GVIA (anthranilamide scaffold attached to K₂, Y₁₃ and R₁₇ residues); C₆, GVIA (anthranilamide scaffold attached to K₂, Y₁₃ and R₁₇ residues); C₇, GVIA (modified anthranilamide scaffold attached to K₂ and R₁₇ residues) [41,48,38,43,40]. The scaffolds are indicated in grey color.
throughout MD simulation. Rg analysis provides quantitative information about changes in the tertiary structures linked to the compactness of the simulated system. Higher Rg values from the original depict the protein structure expansion. The apo-Ca_{2.2} exhibited a decreasing trend in Rg values from 56.74 Å to 52.5 Å, highlighting the compactness of system at 40 ns onwards. The structural equilibrium for Ca^{2+}-bound Ca_{v2.2} was observed throughout the simulation assay with the Rg values ranging between 55.79 Å and 57.17 Å, indicating the consistent system compactness (Fig. 5C). The highest values of Rg for C1-bound Cav2.2 complex was detected as 58.96 Å during 1 ns of MD simulation. Afterward, Rg values gradually decreased up to 52 Å at 70 ns of time scale and remained stable up to 100 ns. Consequently, Cav2.2-C1 complex exhibited a tight packing as compared to Ca^{2+}-bound Cav2.2 suggesting more firmness in channel upon binding to C1. MD simulation trajectory files for both complexes were analyzed for hydrogen bond interactions, which remained stable during the entire simulation time (Fig. 5D). The highest numbers of hydrogen bonds in Ca^{2+}-bound and C1-bound Cav2.2 were observed at 45.8 ns (403) and 89.4 ns (417), respectively.

Radial pair distribution function g(r) provides the probability of finding particles at a certain distance r. G(r) values were calculated for four residues (Glu314, Glu663, Glu1365 and Glu1655) that form SF ring to explore their distances with Ca^{2+} ion (Fig. 6A,B). The atom OE1 of Glu314 exhibited interactions with Ca^{2+} ion. OE1 atoms of Glu314 and Glu1365 residues more likely preferred to localize (11832.6 and 10041.5 times) in the vicinity of Ca^{2+} ion at a distance of 2.15 Å (Table 2). Similarly, OE1 atoms of Glu663 and Glu1655 residues exhibited highest peaks at distances of 2.25 Å and 4.85 Å, respectively. Interatomic distance calculation among Glu314, Glu663, Glu1365 and Glu1655 residues suggested that Glu1365-Glu1655 residues were closer to Ca^{2+} ion at a distance of 3.75 Å whereas, Glu663-Glu1655 residues exhibited a distance of 8.65 Å.

Fig. 4. Binding analysis of compounds C1-7 and Ca_{2,2}. C1-7 are shown in dark green, dark pink, dark sky blue, chartreuse, orange, dark magenta and firebrick colors in circles I-VII, respectively. The channel interacting residues are shown in grey wires and labeled in black color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Furthermore, g(r) plot results for Ca\(^{2+}\) ion and OE1 atoms of 4 glutamate residues (Glu314, Glu663, Glu1365 and Glu1655) were validated by distance calculation analysis through UCSF Chimera 1.11.2 for the PDB files generated at 10 ns and 99 ns of simulation time. Evidently, distances for Glu314, Glu663 and Glu1365 residues to Ca\(^{2+}\) ion were slightly reduced, while Glu1655 residue moved away (4.86 Å to 6.04 Å) from Ca\(^{2+}\) ion, indicating the movement of Ca\(^{2+}\) ion towards Glu314, Glu663 and Glu1365 residues (Fig. 6C,D).

Table 1
Root Mean Square Fluctuation (RMSF) for apo-Ca\(^{2+}\), Ca\(^{2+}\)-Ca\(^{2+}\) and Ca\(^{2+}\)-C1 complex during 100 ns MD simulation.

| Residues            | Apo-Ca\(^{2+}\) (nm) | Ca\(^{2+}\)-Ca\(^{2+}\) ion (nm) | Ca\(^{2+}\)-C1 (nm) |
|---------------------|-----------------------|----------------------------------|---------------------|
| Selectivity filter (SF) ring |                       |                                  |                     |
| Glu314              | 2.39                  | 1.18                             | 2.24                |
| Glu663              | 2.26                  | 1.35                             | 2.14                |
| Glu1365             | 2.27                  | 1.22                             | 2.06                |
| Glu1655             | 2.26                  | 1.12                             | 2.69                |
| G/A/A/A ring        |                       |                                  |                     |
| Gly353              | 2.42                  | 1.57                             | 1.28                |
| Ala706              | 2.24                  | 1.59                             | 1.22                |
| Ala1413             | 2.06                  | 1.60                             | 1.76                |
| Ala1705             | 2.22                  | 1.48                             | 2.22                |
| S4-S5 linker residues|                       |                                  |                     |
| Pro222              | 2.36                  | 1.84                             | 1.51                |
| Leu223              | 2.41                  | 1.68                             | 1.31                |
| Ser608              | 2.50                  | 1.92                             | 1.83                |
| Ile609              | 2.28                  | 1.76                             | 1.80                |
| Asn1281             | 2.13                  | 1.50                             | 2.07                |
| Val1282             | 2.09                  | 1.50                             | 2.07                |
| Lys1297             | 2.42                  | 1.82                             | 2.44                |
| Ala1598             | 2.46                  | 2.13                             | 2.41                |
3.5. Conformational change analysis

Through comparative MD simulation analysis for Cav2.2-Ca\(^{2+}\) ion and Cav2.2-C1 complexes in comparison to apo-Cav2.2, details of critical residues including gating charge residues of VSDs, ion and inhibitor interacting residues at the pore region, pore lining residues, hydrophobic residues in the inner gate, S4-S5 linker interacting residues to S6 segments of pore domain were observed.

Moreover, the influence of these residues in the opening and closing of Cav2.2 inner gate was explored in both systems, particularly at SF and ion permeable regions of Cav2.2.

For Cav2.2-Ca\(^{2+}\) ion complex, all 4 domains (DI-DIV) were superimposed at 99 ns time scale (Fig. 7A). Though multiple structural variations were observed in all 4 domains (DI-DIV) of Cav2.2, divergence was more prominent at the loop and linker regions that allow the movement of individual segments during voltage sensing and inner gate opening/closing. These structural variations exhibited by DI-DIV domains may lead to asymmetric tetrameric conformation of Cav2.2. In this conformation, S1-S4 VSDs of Cav2.2 bear similar but non-identical structural features. Each Cav2.2 VSD contains four transmembrane \(\alpha\)-helices (S1-S4). Upon superimposition, these transmembrane segments (S1-S4 VSDs) revealed an RMSD value of \(~\)1.3 Å (Fig. 7A). Positively charged residues (Arg and Lys) of S4 segments at every third and fourth position were labeled as R1-R6. The human Cav2.2 S4 segment exhibits R1-R5 on VSDI, R2-R6 on VSDII, R1-R6 on VSDIII and R2-R6 residues on VSDIV (Fig. 7A). In each VSD, S4 segment-specific gating charged residues were aligned at one side (Fig. 7B). R1-R4 residues were positioned above the conserved occluding Phe residue in CTC (charge transfer center), while R5 and R6 residues were lined below (Fig. 7C). Extracellular pointing of 4 out of 6 conserved \(\mathbf{R}\) residues allow forming a depolarized or upstate generation. The upstate
levels may vary among different structures or within the domains of same structure. These levels are determined by gating charges due to residues above the bulky hydrophobic residue (Phe) of CTC \[63\]. The CTC is composed of conserved negative or polar residues including a highly conserved occluding bulky hydrophobic residue localized on S2 segment plus an invariant Asp residue, localized on S3 segment. These residues along with another polar or negatively charged residue (An1 and An2) of S2 segment contribute in the transmembrane movement by interacting with gating charged residues on S4 segment. In VSD tetramer, Glu134/518 residue localized on S2 of VSDI, II, Asp1187 of VSDIII and Asn1507 of VSDIV interact with R2 and R3, while R5 interacts with Glu144/528/1197/1517 of S2 segment. As evident in rabbit Cav1.1 \[60\], in human Cav2.2, Asp residues of VSDII and VSDIV S3 segments were observed in the formation of CTC (Fig. 7).

To observe structural conformations, VSDs were superimposed for both complexes (Ca\(^{2+}\)-ion and C1-bound Cav2.2) revealed two rings of charged residues at the outer vestibule. The inner ring is composed of four negatively charge residues (Glu314I, Glu663II, Glu1365III and Glu1655IV; the EEEE motif) neighbored by Asp664II and Arg1650IV \[64\]. The outer ring is composed of Asp325I, Arg1634IV and Glu1651IV. The hydrophobic ring was composed of Ile319I, Val668II, Val1370III and Ile1660IV residues that parted the outer and inner rings (Fig. 8B-C). Recent studies showed that \(-\)conotoxin GVIA forms salt bridges with the outer ring of Cav2.2; however, the apolar residues of hydrophobic ring sterically hinder the binding of toxin with the outer ring \[64\]. In our study, Ca\(^{2+}\)-bound Cav2.2 exhibited a wide groove at the pore region surrounded by negatively charged residues that may allow Ca\(^{2+}\) ion binding at inner rings. Similarly, compound C1 interaction was mediated by SF ring in the pore. Such conformation may allow the appropriate binding of C1 with the pore residues of Cav2.2.

3.7. Analysis of Cav2.2 pore domain region

VSDs and pore structure are coupled in such a manner that S4 segment of VSD is connected to S5 of the pore domain via S4-S5 linker. Subsequently, a helical structure surrounds the pore domain and runs parallel to the intracellular side of membrane. Each VSD is localized adjacent to the neighboring pore domain and influences the gate of its own domain as well as of neighboring domain. Channel pore region and VSDs are coupled together due to direct interactions of S4-S5 linkers and S6 segments via Gly353 (S6I), Ala706 (S6II), Ala1413 (S6III) and Ala1705 (S6IV). Sequence alignment (Figs. S3, S4) and structural comparison of Cav1.1 and Cav1.2 channels indicate that instead of Gly residue in the S6I segments of Ca\(^{2+}\)-ion-bound Cav2.2 is shown in spring green color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
makes ‘G/A/A/A ring’ [65]. Recent studies potentiate that mutations of G/A/G/A residues impact the movement of VSDs [66]. The G/A/A/A position in the S6 is faced toward the S4-S5 linker to induce a direct interaction between them for tight packing. In apo-Cav2.2, the G/A/A/A ring residues lacked close interactions with loop residues localized between the S4-S5 linker and the S5 segment (Fig. 9B). In Ca2+-bound Cav2.2, Gly353, Ala706 and Ala1413 residues were involved in interaction with the neighboring residues of loop region to facilitate the opening of channel pore at the entry gate for Ca2+ ions (Fig. 9D). In contrast, in Cav2.2-C1 complex, Ala1705 with Lys1597 and Ala1598 residues of the similar loop were involved in channel activity (Fig. 9F). Due to lack of Gly353, Ala706 and Ala1413 involvements in interaction, this loop moved towards S6 segment that prevented its interaction with S4-S5 linker residues and narrowed the tunnel at the inner gate to close the passage for ion flow. Lys218-Leu223 (S4I-S5I) and Val1814-His1844 (IQ domain) regions of apo-Cav2.2 and Cav2.2-Ca2+ ion complex attained loop conformations (Fig. S7), while in Cav2.2-C1 complex; these regions adopted helical conformations (Fig. S8). The helical break of S4III was involved in kinking or bending the segment. S3III was positioned in parallel with the membrane as compared to other VSDs. The position of S4-S5 linker was also varied in all VSDs. In all systems, loop regions exhibited more fluctuations, except SF region of channel pore. Similarly, more conformational readjustments were visible at the EF-hand and IQ domains in all systems. The IQ domain was more inclined to inner gate of channel in Cav2.2-Ca2+ ion complex.

Fig. 8. Outer vestibule of four domains of Cav2.2. (A) Sequence alignment of Cav2.2 outer vestibule region in all four domains. The highlighted region signifies the selectivity filter lining residues. (B) Extracellular and cytosolic views of Cav2.2-Ca2+ ion complex outer vestibule. (C) Extracellular and cytosolic views of Cav2.2-C1 complex outer vestibule. Coloring scheme for residues are: hydrophobic, gray; polar, green; negatively charge, red and positively charge, blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Fig. 9. Conformational transition analysis in apo-Ca_{2.2}, Ca_{2.2} complex with Ca^{2+} ion and C1. (A), (C) and (E) Membrane topology and side views of apo-Ca_{2.2}, Ca_{2.2}-Ca^{2+} ion and Ca_{2.2}-C1 complexes. D_{1}-D_{4} domains are indicated in blue, gold, green and violet red colors, respectively. EF-hand domain is shown in sky blue, IQ domain is in orchid, Ca^{2+} ion in gray and C1 is indicated in dark green color. (B), (D) and (F) Cytosolic view of apo-Ca_{2.2}, Ca_{2.2}-Ca^{2+} ion and Ca_{2.2}-C1 complexes. The dotted lines at the pore region indicate a cooperative unit. Residues of G/A/A/A ring on S6 and their interacting residues on S4-S5 linkers are labeled in black color. The spheres indicate their side chains. (G), (H) and (I) Side views of channel having superimposed 4 domains of apo-Ca_{2.2}, Ca_{2.2}-Ca^{2+} ion and Ca_{2.2}-C1 complexes, respectively embedded in membrane at 67 ns time-scale. The domain segments are labeled in black color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Fig. 10. Architecture and tunnel comparison in apo-Ca2.2, Ca2.2-Ca2+ and Ca2.2-C1 complexes. The VSDs have been omitted for better illustration. (A), (B) and (C) Side views of permeation path of pore domains for apo-Ca2.2, Ca2.2-Ca2+ ion and Ca2.2-C1 structures, respectively. The ion conducting passage, calculated by MoleOnline (https://mole.upol.cz/online/) is illustrated by purple-colored tunnel. DI-IV are indicated in blue, gold, green and violet red colors, respectively. Each segment of domain is labeled in the respective domain color. The Ca2+ ion is indicated in gray and C1 in dark green color. Pore radii along with the pore distances is displayed for each structure. Top red arrows indicate the ion selective and permeable regions localized at the narrowest tunnel (radius close to 0). Furthermore, the closest part of hydrophobic area (position close to 0) formed by Val351, Phe704, Phe1411 and Phe1703 residues is indicated by red arrows (bottom). The blue to yellow colored plot demonstrates the hydrophobic strength of pore forming residues. (D), (E) and (F) Circle displays extracellular view of SF residues of the pore region are indicated by red sticks for apo-Ca2.2, Ca2.2-Ca2+ and Ca2.2-C1 structures, respectively. Cytoplasmic view of the occluding residues of inner gate (Val351, Phe704, Phe1411 and Phe1703) is shown in stick representation. (G), (H) and (I) Tunnel forming residues along with their distances measured by UCSF Chimera 1.11.2 for apo-Ca2.2, Ca2.2-Ca2+ and Ca2.2-C1 structures, respectively. Dotted lines indicate distances between adjacent residues of tunnel. Adjacent to tunnel, only two domains were shown to avoid ambiguity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
repeats demonstrated that P-loop regions of DIII and DIV were longer than the P-loops of DI and DII (Fig. 9). In addition, P-loops of DI and DIII exhibited 1–2 pairs of antiparallel β-strands, respectively (Fig. 9). P-loop of DIII exhibited an extended conformation and more protrusion into the extracellular space. Through the P-loop region, α1 and α2 subunits were associated together. Upon superimposition of all 4 domains at 10, 60, 67 and 99 ns time scales, varying structural preferences were observed for individual VSD segments (Figs. S6, S7 and S8). Pronounced variations were visible in S3 and S4 segments (VSDII) of apo-Ca2.2 and Ca2.2-Ca2+ ion complex, while in Ca2.2-C1 complex, VSDIII exhibited more bends within membrane (Fig. 9G-I, S6, S7 and S8). P1 and P2 helices of S3 and S4 segments were structurally similar in Ca2.2-Ca2+ ion complex than that of Ca2.2-C1 complex and apo-Ca2.2. S5 and S6 segments were conformationally dissimilar in both complexes. In case of Ca2.2-Ca2+ ion complex, S4-S5 linker helices of DIII were more divergent than others.

At the pore region, Glu residues (Glu314, Glu663, Glu1365 and Glu1655) localized at the periphery of ion selectivity region are clustered to regulate the tunnel passage (Fig. 10). The apo-Ca2.2 displayed a wide tunnel radius at SF region as compared to other two complexes (Fig. 10A-C). Underneath the selectivity filter vestibule, a typical hydrophobic cavity passes along with the side portals penetrated by transverse membrane lipids, a feature similar to rabbit Cav1.1 [45] and bacterial Na+ channels [67]. The asymmetric S6 bundle of Ca2.2 is tightly screwed at the inner activation gate. The apo-Ca2.2 and Ca2.2-C1 complex exhibited narrow tunnels in comparison to Ca2.2-Ca2+ ion complex, indicating a closed pore conformation through channel (Fig. 10A-C-G-I). Three aromatic residues (Phe704, Phe1411 and Phe1703), localized at the corresponding positions at S6II-IV along with Val351 of S6I (pore-blocking S6 residues) mediated the pore seal formation at the cytosolic region (Fig. 10D-F). Below the aromatic ring, hydrophobic residues of S6I (Leu and Gly/Ala) and S6II-IV (Val and Ala) facilitated in the channel closure. Furthermore, hydrophobic residues (Val351, Phe704, Phe1411 and Phe1703) lined at the narrowest point along the pore enclosed the entrance to the SF vestibule.

3.8. Structural comparison of human Ca2.2

In order to explore the open or close state of channel, Ca2.2-Ca2+ ion and Ca2.2-C1 complexes were superimposed with apo-Ca2.2 simulated structure, relative to the pore domains. These structures revealed an RMSD value of ~1.24 Å (Fig. 11). The VSDs of apo-Ca2.2 possessed a depolarized or ‘up’ conformation and a closed inner gate, suggesting a potentially inactive state. All 4 homologous repeats/domains exhibit a counterclockwise arrangement when viewed from intracellular side and remain conserved in all eukaryotic calcium and sodium channels [60]. In our analysis, there was a subtle clockwise rotation of VSDs in Ca2+-bound Ca2.2 VSDIII relative to apo-Ca2.2, with the exception of VSDII that moves counterclockwise. Overall, a counterclockwise movement was detected in VSDIII of Ca2+-bound Ca2.2 relative to apo-Ca2.2 (cytosolic view). The observations of inner gate suggested more distances among Ca atoms of S6III for Ca2+-bound Ca2.2 in comparison to apo-Ca2.2 and C1-bound Ca2.2, whose inner gate is closed, while shorter distances were observed for S6I, S6II and S6IV segments. Due to a kink in S6IV segment of C1-bound Ca2.2, apo-Ca2.2 exhibited more distance. Similarly, S6 segments of both apo and C1-bound Ca2.2 were

Fig. 11. Structural comparison of apo-Ca2.2 with Ca2.2, Ca2+ and C1-bound human Ca2.2. The pore domain superimposition demonstrates a clockwise rotation of VSDs in Ca2.2-Ca2+ ion complex (periplasmic view along the channel axis) relative to the apo-Ca2.2, with the exception of VSDIII, that moves counterclockwise. Overall, C1-bound Ca2.2 reveals a subtle counterclockwise rotation.
different than that of Cav2.2–Ca\textsuperscript{2+}–complex Overall, the inner gates of both apo-Ca\textsubscript{2,2} and C1-bound Ca\textsubscript{2,2} shared a similar conformational pattern. An inward movement of S6 segments in Ca\textsubscript{2,2}-C1 complex and a reduced pore size at the activation gate suggests that the gate is potentially closed. Thus the “up” (depolarized) conformation of VSDs and closed pore dimension may signify a potentially inactivated state of Ca\textsubscript{2,2} upon C1 inhibitor binding.

4. Discussion

Chronic pain is considered as a major issue that affects the overall life quality of patient. Approximately, ~1.5 billion people are suffering with this condition globally [68]. Recently, a mouse model expressing N-type Ca\textsubscript{2,2} VGCCs at the plasma membrane of peripheral somatosensory neurons via α2δ-1 accessory subunit revealed the role of these channels in pain modulation [69], suggesting that Ca\textsubscript{2,2} Ca\textsuperscript{2+} channel may prove to be a potential drug target for novel analgesic therapies. Purposefully, the mimicry of venom peptides may hold a considerable promise due to lack of detailed structural information of neuronal ion channel. The non-peptide mimetics of MVIIA and GVIA o-conotoxins isolated from marine cone snail efficiently bind to neuronal Ca\textsuperscript{2+} channels at low concentrations [8,62]. Here in this study, out of 30 known non-peptide analogues of o-conotoxins (Table S1, Fig. S1), 7 peptides (Fig. 3; Table S2) were shortlisted on the basis of their binding abilities at the Ca\textsubscript{2,2} pore region. These blockers bearing dendritic, benzothiazole or anthranilamide scaffolds (Fig. 3) may be used for the effective management of chronic and neuropathic pain by blocking Ca\textsubscript{2,2} VGCCs.

Subsequently, through in silico analysis, we compared conformational ensemble of Ca\textsubscript{2,2} bound to conopeptide-mimetic alkylphenyl ether-based analogue MVIIA (C1) [41] and Ca\textsuperscript{2+} ion to demonstrate potential anesthetic and analgesic impact of inhibitor. C1, initially proposed by Horwell group [70] contains a dendritic scaffold that mimics three key residues (Arg10, Leu11 and Tyr13) of MVIIA conotoxin [41]. In our analysis, the dendritic scaffold was found to be hydrophobically associated with Met313, Phe346 and Met347 residues of Ca\textsubscript{2,2} (52 Å) structure was more compact than Ca\textsuperscript{2+}–bound Ca\textsubscript{2,2} (57 Å) indicating that Ca\textsuperscript{2+}–dependent structural destabilizing effects may lead in the channel opening (Fig. 5C). Possibly, Ca\textsuperscript{2+}–ion-dependent opening of voltage-gated channel may create a functional binding site for synaptic vesicles in the N-type channels as reported in the recent studies [72,73]. Another evidence suggests that channel lacking DII–DIII linker region is dramatically less sensitive to MVIIA and GVIA conotoxins than the full-length construct [74]. The presence of more structural rearrangements in the DII–DIII linker region of C1-bound Ca\textsubscript{2,2} may perhaps facilitate key residues for toxin binding. Our simulations delineate significant conformational changes at the proximal carboxyl-terminus (EF-hand and IQ domain helix) of Ca\textsubscript{2,2} that attains an upright orientation upon Ca\textsuperscript{2+} ion permeability, (Fig. 57; Movie S2). In contrast, movement of C1-bound Ca\textsubscript{2,2} IQ domain helix was totally different (Fig. S8; Movie S3). Possibly, such movements may be linked with the extent of channel opening at the activation gate. At IQ domain, a competitive binding of calmodulin (CaM) and Ca\textsuperscript{2+} binding proteins (CaBPs) influences the Ca\textsuperscript{2+}–dependent facilitation and inactivation of the Voltage-gated channels [75–78], while EF-hand motifs serve as Ca\textsuperscript{2+} ion sensors. Indeed, following the ion selectivity by Glu residues, such oscillations in the IQ helix and EF-hand orientation may provide a docking site for CaM association to induce auto-inhibitory mechanism by Ca\textsuperscript{2+} binding [79].

Evidently, SF ring is localized at the TM pore region with a large external vestibule or central cavity lined by the S6 segments and the intracellular activation gate formed due to intersection of S6 helices that exhibited more variations. Though tunnel lengths were quite comparable (41.2 Å, 40 Å and 40.2 Å, respectively), in contrast to apo-Ca\textsubscript{2,2}, both complexes (Ca\textsubscript{2,2}–Ca\textsuperscript{2+} and Ca\textsubscript{2,2}–C1) displayed remarkable changes in the architecture of channel tunnels (Fig. 10; Movie S4, S5). These findings illustrate that C1 binding to Ca\textsubscript{2,2} induces more narrowing of the pore size at the activation gate as compared to Ca\textsuperscript{2+}–bound Ca\textsubscript{2,2} due to differences in the hydrophilicity pattern at the selectivity region (Fig. 10F and 10G). Generally, the inner lining of tetrameric channel pore remains in the hydrophilic environment to allow the ion passage through the hydrophobic gate [80]. Subsequent occluding residues (Val351, Phe704, Phe1411 and Phe1703) facing the intracellular side contribute in the enhancement of hydrophobicity. Such dynamic structural rearrangements within the selectivity filter are crucial for channel gating [81]. In our analysis, a pronounced reduction in the tunnel volume at the selectivity filter and its enhancement at the activation gate of Ca\textsuperscript{2+}–bound Ca\textsubscript{2,2} suggests that ion binding allows an outward splaying of pore-lining S6 helices to open the voltage gate. Recent evidence supports the movement of S6 helices in both sodium and potassium channels that is initiated at the hinge-point in the middle of the helix [82]. Collectively, given the analgesic efficacy and minimal side effects of o-conotoxins [40], our study reveals MVIIA-associated structural implications and subtle changes in the Ca\textsubscript{2,2} for the development of better therapeutic intervention for chronic and neuropathic pain. Clearly, MD-based conformational analysis of pain blocking o-conotoxins may largely help in the potent inhibition of human presynaptic ion channels for devising promising therapeutic strategy.

CRediT authorship contribution statement

Sameera: Formal analysis, Investigation, Writing - original draft. Fawad Ali Shah: Formal analysis, Visualization. Sajid Rashid: Conceptualization, Project administration, Resources, Supervision, Validation, Writing - review & editing.

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Declarations of interest

None.
Appendix A. Supplementary data

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