Cyclic Peptides as T-Type Calcium Channel Blockers: Characterization and Molecular Mapping of the Binding Site

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ABSTRACT: T-type calcium (Ca_v3) channels play a crucial role in the generation and propagation of action potentials in excitable cells and are considered potential drug targets for the treatment of neurological and cardiovascular diseases. Given the limited pharmacological repertoire for these channels, there is a great need for novel potent and selective Ca_v3 channel inhibitors. In this study, we used *Xenopus* oocytes to heterologously express $\text{Ca}_v3.1$ channels and characterized the interaction with a small cyclic peptide, PnCS1. Using molecular modeling, PnCS1 was docked into the cryo-electron microscopy structure of the human $\text{Ca}_v3.1$ channel and molecular dynamics were performed on the resultant complex. The binding site of the peptide was mapped with the involvement of critical amino acids located in the pore region and fenestrations of the channel. More specifically, we found that PnCS1 reclines in the central cavity of the pore domain of the $\text{Ca}_v3.1$ channel and resides stably between the selectivity filter and the intracellular gate, blocking the conduction pathway of the channel. Using Multiple Attribute Positional Scanning approaches, we developed a series of PnCS1 analogues. These analogues had a reduced level of inhibition, confirming the importance of specific residues and corroborating our modeling. In summary, functional studies of PnCS1 on the $\text{Ca}_v3.1$ channel combined with molecular dynamics results provide the basis for understanding the molecular interactions of PnCS1 with $\text{Ca}_v3.1$ and are fundamental to structure-based drug discovery for treating Ca_v3 channelopathies.

KEYWORDS: T-type calcium channels, toxin-based therapeutics, cyclic peptide, molecular modeling

Voltage-gated calcium (Ca_v) channels are key regulators of the generation and propagation of action potentials in excitable cells and are the subject of intensive research in the neurological and cardiovascular fields. Mutations in genes encoding Ca_v channels have been associated with serious disorders or illnesses. As a result, these channels are considered potential drug targets for the treatment of cardiac disorders, pain syndromes, and epilepsy.¹ There is therefore a great need for novel potent and selective Ca_v channel modulators.

Topologically, the pore-forming α -subunits of Ca_v channels consist of four covalently linked homologous domains (DI–DIV). Each domain is composed of six transmembrane helices (S1–S6). The first four helices (S1–S4) comprise the voltage-sensing domain (VSD), whereas S5 and S6 form the central ion-conducting pore. Depolarization of the membrane results in an outward movement of positive charges in S4 of the VSD. This movement causes a conformational change that leads to the opening or closing of the pore.² At each interface between pore-forming helices of different domains, close to the selectivity filter (SF), a fenestration occurs perpendicular to the channel direction. It connects the central pore to the ion channel's lipid-facing side of the ion channel, embedded in the membrane.³ Fenestrations are common features in voltage-

gated ion channels and were originally proposed as sites for the ingress of hydrophobic drugs into the channel core of voltage-gated sodium (Na_v) channels.^{4,5}

While recombinant Ca_v1 and Ca_v2 channels require auxiliary subunits including $\alpha 2/\delta$ -, β -, and γ -subunits for normal gating behavior,^{6–8} the autonomous function of Ca_v3 core subunits is supported by characterization of recombinantly expressed Ca_v3 channels. These pore-forming α -subunits are encoded by 10 different genes and have been divided into three subfamilies: Ca_v1 ($\text{Ca}_v1.1$ – $\text{Ca}_v1.4$), Ca_v2 ($\text{Ca}_v2.1$ – $\text{Ca}_v2.3$), and Ca_v3 ($\text{Ca}_v3.1$ – $\text{Ca}_v3.3$).

Ca_v1 and Ca_v2 channels are high-voltage-activated (HVA) channels, conducting currents classified as L-, P/Q-, N-, and R-type.⁹ The family of Ca_v3 is classified as low-voltage-activated (LVA) channels, mediating a transient or T-type current.^{10,11} Two main LVA T-type Ca^{2+} channel subtypes are present in

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Figure 1. Electrophysiological characterization of PnCS1 on the $\text{Ca}_v3.1$ channel. (A) Current traces before (black) and after (blue) application of $10\ \mu\text{M}$ PnCS1. In $10\ \text{mM}$ Ba^{2+} , currents were elicited by depolarizing pulses from -90 to $+60$ mV. Holding potential was -90 mV. First 300 ms of the depolarizing pulses are shown. (B) Concentration–response curve indicating the concentration dependence of the PnCS1-induced decrease of the Ba^{2+} peak current. Data are means $\pm$ SEM ($n \geq 3$). (C) Normalized voltage–current relationship in control (black symbols) and peptide ($10\ \mu\text{M}$ PnCS1, blue symbols) conditions. (D) Steady-state activation (open symbols) and inactivation (closed symbols) curves in control (black symbols) and peptide ($10\ \mu\text{M}$ PnCS1, blue symbols) conditions. Data are means $\pm$ SEM ($n = 4$). (E) Normalized $\text{Ca}_v3.1$ channel current during wash-in and wash-out of PnCS1 over time.

the heart, namely, $\text{Ca}_v3.1$ ($\alpha 1\text{G}$) and $\text{Ca}_v3.2$ ($\alpha 1\text{H}$).¹² They are not modulated by the dihydropyridines blocking Ca_v1 channels or by the cone snail and spider toxins blocking Ca_v2 channels.⁶ At present, no widely used therapeutic agents that block T-type calcium currents are known. Mibrefradil is a nondihydropyridine calcium channel blocking agent with a moderate selectivity for T-type over L-type calcium channels,^{13,14} but it was withdrawn from the market in 1998 because of severe drug interactions. Other drugs currently on the market with a potential inhibitory effect on Ca_v3 channels are penfluridol, flunarizine, and ethosuximide.^{15,16} Flunarizine is one of the most often used medicines in the case of migraine prevention. Previous studies reported that flunarizine potently inhibits T-type calcium channels but also native N- and L-type calcium channels.^{17–19} The peptides kurtoxin (found in the venom of the scorpion *Parabuthus transvaalicus*) and ProTx-I and ProTx-II (found in the venom of the tarantula *Thrixopelma pruriens*) are among the few known LVA calcium channel gating-modifier toxins.^{20–22} Compared to Na_v and voltage-gated potassium (K_v) channels, peptide ligands for T-type Ca_v s are poorly investigated. To fully explore the therapeutic potential of targeting Ca_v3 channels, it is necessary to fill this void and characterize novel Ca_v3 channel ligands, preferably with high potency and selectivity.

Given the homology between Ca_v and Na_v channels and the limited pharmacological repertoire for Ca_v3 channels, we investigated the peptide PnCS1 as a novel Ca_v3 channel inhibitor. In recent studies published by our group, we used a chimeric approach to design small cyclic inhibitors for Na_v

channels.^{23,24} It started out with two well-known Na_v channel blockers, namely, the *Conus kinschitai* toxin μ -KIIIA, a cone snail peptide consisting of 16 amino acids and 3 disulfide bonds, and the *Phoneutria nigriventer* toxin1 (PnTx1), a spider toxin consisting of 78 amino acids and 7 disulfide bonds. Based on their sequence and secondary structural homology, a common pharmacophore was found and used for the design. The resulting designed hybrid peptide, Pn, consisted of 13 amino acids and 2 disulfide bridges. Based on Pn, three series of mutant peptides were generated. The first series of Pn mutants (PnM1–PnM4) and the second series (PnM5–PnM9) include sequence variations compared with Pn. PnCS1–PnCS4 peptides (series three) were cyclic variations of PnM9 from the second series. One of the obtained cyclic peptides (PnCS1) showed an effect on different Na_v channels, with a submicromolar inhibitory potency for the $\text{Na}_v1.2$ channel.²³ Based on the promising results of PnCS1, a fourth series of small cyclic peptides was generated using a Multiple Attribute Positional Scanning (MAPS) approach. Using this approach, every noncysteine residue was replaced with alanine, lysine, or glutamic acid. Unfortunately, none of these peptides displayed improved activity or selectivity for the Na_v channels investigated compared to PnCS1.²⁴

In the present study, we investigated the *ex vivo* activity of PnCS1 at Ca_v3 channels using two-electrode voltage clamp electrophysiology on oocytes expressing Ca_v3 subunits and found that this cyclic peptide was active on the three members of the Ca_v3 family. The nature of the interactions of PnCS1 with T-type calcium channels was further examined using

Figure 2. Molecular dynamics simulation of the PnCS1- $\text{Ca}_v3.1$ complex. Upper left shows the cryo-EM structure of the apoprotein $\text{Ca}_v3.1$ channel, with the domains named and color-coded for future reference. Extra-transmembrane loops were omitted from the figure to reduce visual noise. The bottom figures are rotated a quarter clockwise turn relative to the top figures. After simulation, it appears (emphasized by the blue arrows) that the selectivity filter is fully disrupted by the binding of the peptide, which causes a large conformational change and eventually the reversible destabilization and inactivation of the $\text{Ca}_v3.1$ channel. Domain I in chartreuse green (lighter green hue), domain II in dark gray (dark gray hue), domain III in lime green (darker green hue), domain IV in gray (lighter gray hue); the ligand is shown in magenta in order to contrast the protein channel. Bindings of the selectivity filter are detailed in Figure S6.

molecular modeling. PnCS1 was docked into the cryo-electron microscopy (EM) structure of the human $\text{Ca}_v3.1$ channel,¹⁰ and molecular dynamics (MD) showed corresponding results to the *ex vivo* experiments, thereby allowing for an in-depth analysis of the formed interactions between PnCS1 and the $\text{Ca}_v3.1$ channel and revealing a unique binding site for peptide toxins. Future experiments need to confirm these results.

RESULTS

Electrophysiological Characterization of PnCS1 on $\text{Ca}_v3.1$. Two-electrode voltage clamp electrophysiology on oocytes expressing the $\text{Ca}_v3.1$ channel was used to evaluate the activity of PnCS1. Current traces of $\text{Ca}_v3.1$ before (control) and after application of 10 μM PnCS1 are shown in Figure 1A. The concentration-dependent inhibitory effect of PnCS1 was determined by measuring the current inhibition in the presence of increasing PnCS1 concentrations. The calculated IC_{50} value yielded $13.1 \pm 3.3 \mu\text{M}$ with a Hill coefficient of 1.4 ± 0.2 . Upon application of 100 μM peptide, a maximum percentage inhibition of $90.2 \pm 6.5\%$ was observed (Figure 1B).

Further electrophysiological characterization was performed with 10 μM PnCS1. Steady-state activation and inactivation curves showed no significant shift in the voltage dependency of

$\text{Ca}_v3.1$ channels upon peptide binding (Figure 1C, D). For the midpoint of activation, $V_{1/2}$ values yielded $-36.2 \pm 2.8 \text{ mV}$ in control conditions and $-39.0 \pm 3.4 \text{ mV}$ in the presence of 10 μM PnCS1. For the inactivation curves, the $V_{1/2}$ shifted from $-59.3 \pm 2.8 \text{ mV}$ to $-54.2 \pm 3.3 \text{ mV}$ in control and peptide situations, respectively. A wash-out study with 10 μM PnCS1 showed that binding of PnCS1 to $\text{Ca}_v3.1$ was reversible (Figure 1E). The state dependence of inhibition was investigated by using the following protocol; In control, a series of depolarizing pulses were applied to an oocyte expressing $\text{Ca}_v3.1$ channels, followed by addition of 10 μM PnCS1 and no pulsing for 2 min. Subsequently, a similar series of pulses were applied. After the 2 min incubation, an immediate inhibition was observed, indicating that the open state is not required for peptide interaction with the channel (data not shown).

Parent peptides, PnTx1 and $\mu\text{-KIIIA}$, and hybrid peptide, Pn, were then evaluated for their inhibition against the three $\text{Ca}_v3.1$ - $\text{Ca}_v3.3$ channel isoforms. Application of PnTx1 at a concentration of 1 μM showed no effect, whereas $\mu\text{-KIIIA}$ (1 μM) and Pn (10 μM) displayed a small level of inhibition (Figure S1).

PnCS1 was evaluated for inhibition and potency across subtypes $\text{Ca}_v3.2$ and $\text{Ca}_v3.3$, and the peptide was found to be

Figure 3. Upper left shows the PnCS1- $\text{Ca}_v3.1$ complex. Fenestrations II–III and III–IV are indicated. Same color coding as in Figure 2. Protein channel residues in black, and toxin residues in blue. The insets show PnCS1 surrounded by hydrophobic residues on the S6 helices (bottom left, upper right) and the specific coordination of PnCS1 by polar residues (bottom right) in the central pore. The hydrogen bonds are indicated by black dashed lines. The backbone carbonyl oxygen (O) and amide (N) are labeled.

equipotent across all three isoforms of the Ca_v3 family (Table S1).

Computational Studies. PnCS1 in Complex with $\text{Ca}_v3.1$. Obtaining a Model of the Complex. A cryo-EM structure of human $\text{Ca}_v3.1$, with a resolution of 3.3 Å, was recently reported¹⁰ and was used to elucidate the mechanism by which PnCS1 interacts with the channel. Initially, a molecular model for PnCS1 was generated prior to docking. After simulating the cyclic peptide, an ensemble of 15 PDB structures was extracted from the MD trajectory, from 10 to 100 ns with an interval of 6 ns. As a reference, toxin μ -KIIIA (PDB ID: 2LXG) was used, as PnCS1 is derived from the original Pn peptide's cyclic variations, which is closely related to μ -KIIIA conotoxin.²⁰ All selected models adopted a structure highly similar to the reference μ -KIIIA, as reflected in the root-mean-square deviation (RMSD) of the ensemble (1.2 ± 0.4 Å across all residues).²³ One of the models in the set, #14, was randomly selected for docking experiments and compared with the experimental structure of μ -KIIIA. As previously described for Pn,²³ the short helix in the μ -KIIIA toxin is also missing in PnCS1. The absence of a helix can be attributed to the reduced size of PnCS1 that is formed by 10 amino acid residues instead of 16 in μ -KIIIA, compromising the accommodation of a helical structure. Despite this, the RMSD was still low and showed strong resemblances across pairs of four residues of the selected model of PnCS1 structures in the NMR ensemble of μ -KIIIA (0.5 ± 0.1 Å).

Results on protein–protein docking by the ClusPro server showed dense clustering of calculated structures for PnCS1 in complex with native $\text{Ca}_v3.1$, with 161 iterations in its most favorable cluster (score = -1078.9 kcal/mol, lowest energy = -1179.7 kcal/mol). In 4 of the 12 clusters of the obtained PnCS1- $\text{Ca}_v3.1$ channel complex (1, 3, 5 and 7), the cyclic peptide is located in the central pore and extending into the

fenestrations between pore-forming segments of domains II–III and III–IV (Figure S2). Other clusters were docked on the outside of the channel but showed unfavorable results. Since the first cluster had the most favorable clustering results, based on the number of dockings per cluster first and energy results second, a model of this cluster was selected for further MD simulation. The R6E mutant of PnCS1 showed less dense clustering, with 131 iterations in its most energetically favorable cluster (score = -1051.2 kcal/mol, lowest energy = -1114.7 kcal/mol). Of the 21 clusters, only clusters 4, 6, 18, 19, and 21 were visibly located on relatively the same location but with very different conformations. We opted to use the first cluster of the PnCS1[R6E] mutant docking due to its largest cluster size and subjected it to a MD simulation.

MD Simulation. Upon binding of PnCS1, conformational changes occurred in the $\text{Ca}_v3.1$ channel. In the initial model of the docked complex, the S5–S6 helices from domain III were inching away from the same helices of domain IV (Figure 2, see blue arrows). This effect became more pronounced during the 400 ns MD simulation, opening up the fenestration between domains III and IV to accommodate residues Trp6–Asn8–Arg9 of the cyclic peptide and breaking the symmetry of the $\text{Ca}_v3.1$ channel in the complex. Allosteric effects caused by PnCS1 increased the distance between the VSD of domain II and the VSD of domain III (Figure 2, see red dashed lines), while the distance between VSD I and IV steadily decreased.

The average displacement of each residue in the $\text{Ca}_v3.1$ channel over the MD simulation highlighted interacting residues of the $\text{Ca}_v3.1$ channel with the PnCS1 peptide (Figure S5). The largest spike corresponded to a loop converging toward a stable conformation during the MD trajectory. Low root-mean-square fluctuation (RMSF) values, by residue, were observed for residues directly involved in interactions with the ligand (indicated by circles in Figure S5).

Figure 4. Saddle-shaped conformation of the PnCS1[R6E] mutant in the central pore of $\text{Ca}_v3.1$. Same color coding as in Figure 2. PnCS1[R6E] resides in the central pore of the protein while leaving the protein itself rather undisturbed.

This illustrates the stability of their interaction with the peptide, which was especially notable in domain IV, with mostly stable hydrophobic interactions. The RMSD of the PnCS1 peptide in complex with the $\text{Ca}_v3.1$ channel was extremely stable along the simulation's trajectory, despite the intrinsic flexibility of the peptide itself owing to the high degrees of freedom of its residues. Due to extensive intra- and intermolecular interactions in the binding site, it showed remarkable stability in the docking site (Figure 3, S4 and S5).

Considering the SF, a substantial disruption was observed when the $\text{Ca}_v3.1$ channel was complexed with PnCS1. The characteristic hydrogen-bonding network within the SF was disconnected between domains III and IV (dark green in Figure S6) and, to a lesser extent, between domains IV and I (light gray in Figure S6). This is also in line with what is seen in Figure 2, where the breakage of these bonds leads to a virtual spacing of the domains, especially the domain III opening.

According to the molecular model of PnCS1 used in the molecular dynamics simulations with the $\text{Ca}_v3.1$ channel, the guanidine moiety of PnCS1-Arg6 interacted not only with the terminal carboxyl of PnCS1-Cys10 but was also involved in a stable intermolecular interaction with the $\text{Ca}_v3.1$ -Glu923. The terminal amine of PnCS1-Cys1 was within hydrogen-bonding distance to the backbone carbonyl of $\text{Ca}_v3.1$ -Thr921. The same nitrogen of PnCS1-Cys1 interacted with the carboxyl moiety of the $\text{Ca}_v3.1$ -Glu923 residue belonging to the SF. In addition, the carbonyl of PnCS1-Arg3 interacted with the nitrogen in the amide moiety residue of $\text{Ca}_v3.1$ -Gln922. An extra intramolecular hydrogen bond of the backbone carbonyl in PnCS1-Trp7 and the nitrogen of PnCS1-Cys10 stabilized the cyclic peptide conformation in the binding site. Additionally, the positively charged guanidine moiety of PnCS1-Arg2 was accommodated in the II–III fenestration, making phenyl–cation stacking interactions with $\text{Ca}_v3.1$ -Phe917 and hydrophobic interactions with $\text{Ca}_v3.1$ -Phe956.

Hydrophobic interactions were observed for the side chains of Trp4 and Trp7 in PnCS1. The latter residue, PnCS1-Trp7, was located in the III–IV fenestration in close proximity to Val1505, Met1508, Phe1817 and Val1820 of the $\text{Ca}_v3.1$ channel. The PnCS1-Trp4 residue in the central pore was in

close proximity to Leu353, Phe384, Ile387, Asn388, and Leu1819 and the intracellular gate. It seemed to exert steric hindrance on Asn388, which caused a π -helix to be formed in S6 of domain I. The same happened in S6 of domain II, likely due to PnCS1-Arg2 interacting with $\text{Ca}_v3.1$ -Phe956, which caused a shift of residues by a quarter turn and made $\text{Ca}_v3.1$ -Val960 part of the intracellular gate instead of Leu959.

R6E Mutant of PnCS1. Triggered by the observed interactions of PnCS1-Arg6 with the $\text{Ca}_v3.1$ channel in the modeled complex, a R6E mutant of PnCS1 was produced to further delineate the details of these putative interactions. Analysis of the 150 ns MD trajectory of PnCS1[R6E] revealed that the mutant peptide remained very stable in the docking site. However, in contrast to parent peptide PnCS1, PnCS1-[R6E] did not show observable hydrogen bonding with residues in the $\text{Ca}_v3.1$ channel. Second, several intramolecular hydrogen bonds contorted the mutant peptide heavily into a hyperbolic saddle shape. PnCS1[R6E]-Trp4 resided in the III–IV fenestration. PnCS1[R6E]-Trp7 had PnCS1[R6E]-Arg2 and PnCS1[R6E]-Arg3 on either side of the indole moiety, making a cation–aromatic–cation stacking interaction and adding to the self-stabilizing property of the mutant R6E. They were pointed toward $\text{Ca}_v3.1$ -Phe956, showing interaction with the II–III fenestration (Figure 4).

In contrast to the complex of the $\text{Ca}_v3.1$ channel with PnCS1, the mutant PnCS1[R6E] exerted minimal conformational changes onto the protein channel, causing the protein channel to remain relatively similar to its apo-structure. A small disruption in the hydrogen bonding network occurred at the SF but not of the same magnitude as with the parent PnCS1 peptide (Figures S7–S9). Unlike the significant movement of the $\text{Ca}_v3.1$ domains in complex with PnCS1, the $\text{Ca}_v3.1$ domains in the PnCS1[R6E] complex remained relatively undisturbed.

Electrophysiological Characterization of PnCS1 Mutants. Following the identification of Cys1, Arg3, Trp4, Arg6, and Trp7 as important residues in the PnCS1- $\text{Ca}_v3.1$ channel complex (Figure 3), we were interested in further characterizing the involvement of these individual residues in the binding to the $\text{Ca}_v3.1$ channel and thus, by using a MAPS approach, structure–activity relationship studies were per-

formed. In this approach, residues are systematically replaced with amino acids possessing various chemical properties. To cover a broad range of chemical space, Arg3, Trp4, Arg6 and Trp7 were replaced with alanine, lysine, or glutamic acid. In addition, we created a backbone cyclized version of the parent peptide PnCS1, PnCS3, where the two cyclizing Cys-residues, Cys1 and Cys10, were replaced with an Ala and a Gly residue, respectively. This resulted in 12 cyclic peptides (Table 1) which were evaluated for activity using two-electrode voltage clamp electrophysiology on oocytes expressing $Ca_v3.1$ and compared to the activity of the parent peptide PnCS1.

Table 1. MAPS Mutants Based on PnCS1^a

peptide	sequence
PnCS1	CRRWARWNRC*
PnCS3	<u>ARR</u> WARWNRC
PnCS1[R3A]	CR <u>A</u> WARWNRC*
PnCS1[R3E]	CR <u>E</u> WARWNRC*
PnCS1[R3K]	CR <u>K</u> WARWNRC*
PnCS1[W4A]	CRR <u>A</u> ARWNRC*
PnCS1[W4E]	CRR <u>E</u> ARWNRC*
PnCS1[W4K]	CRR <u>K</u> ARWNRC*
PnCS1[R6A]	CRRWA <u>A</u> WNRC*
PnCS1[R6E]	CRRWA <u>E</u> WNRC*
PnCS1[R6K]	CRRWA <u>K</u> WNRC*
PnCS1[W7A]	CRRWAR <u>A</u> NRC*
PnCS1[W7E]	CRRWAR <u>E</u> NRC*
PnCS1[W7K]	CRRWAR <u>K</u> NRC*

^aMutations are underlined in bold. *Amidated C-terminal.

At a concentration of 10 μ M, the Glu analogues showed a complete loss of inhibition. A reduced level of inhibition was displayed by the Ala and Lys mutants, with PnCS1 analogues [R3A], [R6A], [W7A], [R3K], and [W7K] showing a reduction in inhibition of more than 50% (Figure 5). In addition, the importance of the N-terminal amino group and the C-terminal amide of PnCS1 was investigated in PnCS3 by replacing the N- and C-terminal Cys residues and subsequently cyclizing the peptide via the backbone. PnCS3 showed a substantial reduction in potency, with a maximal inhibition of $24.7 \pm 3.6\%$ at 100 μ M (Figure 5). Although some peptide mutants showed inhibition at 10 μ M (Figure 5), no IC_{50} could be determined due to low potency of these peptides.

DISCUSSION

Peptide toxins that interact with ion channels are invaluable tools to study channel function and provide the basis to explore the physiological roles of ion channel subtype diseases including pain, cardiovascular, stroke, epilepsy and others. The initial goal of this study was to characterize new peptide-based Ca_v3 channel blockers. We focused on the $Ca_v3.1$ channel, which is of particular interest because of its possible role as a pacemaker current in the heart. The highest level of T-type calcium currents in the human body is found near birth and decreases gradually. As a result of pathological conditions, the currents may reappear.⁶ To date, very few selective T-type current inhibitors have been described.

While screening for ligands that bind to the $Ca_v3.1$ channel, we identified PnCS1, a small cyclic peptide that was initially developed as a small cyclic Na_v inhibitor.^{23,24} Our electrophysiological studies suggested that PnCS1 acts like a pore blocker obstructing the ion flow by binding in the pore region

Figure 5. Electrophysiological characterization of PnCS1 analogues (10 μ M) on $Ca_v3.1$. (A) Representative whole-cell current traces in control (black) and peptide (blue) conditions are shown. The dotted line indicates the zero-current level. In 10 mM Ba^{2+} , currents were elicited by depolarizing pulses to -25 mV from a holding potential of -90 mV. (B) Quantification of the percentage inhibition of $Ca_v3.1$ after application of the PnCS1 analogs. Data are means $\pm$ SEM ($n \geq 3$). Statistical significance was determined using one-way ANOVA with Dunnett's post-test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ compared to PnCS1.

of the $Ca_v3.1$ channel rather than acting as a voltage-sensor modifier, since no significant shift in the half activation potentials can be observed. The effect of PnCS1 on $Ca_v3.1$ channels is reversible as PnCS1 can be washed out completely. Molecular dynamic simulation studies further confirmed PnCS1's role as a blocker and not as a gating modulator. Since the few previously reported venom-derived peptides known to interact with $Ca_v3.1$ channels including kurtosin, ProTx-I, and ProTx-II inhibit T-type calcium channels by modifying channel gating,^{25,26} PnCS1 is the first peptide known to date to act as a pore blocker on T-type calcium

channels. Additionally, PnCS1 resides stably between the selectivity filter and the intracellular gate of the $\text{Ca}_v3.1$ channel, while in general peptide toxins targeting Ca_v2 channels are known to block the channel pore by binding within the outer vestibule of the pore.^{27–29} Thus, displaying the uniqueness of the PnCS1 peptide.

Molecular modeling approaches were used to locate the binding site of PnCS1 that extends from the central pore of the $\text{Ca}_v3.1$ channel into two so-called fenestrations of the ion channel. Extensive interactions between the cyclic peptide and its binding pocket stabilize the complex. So far, only one structure is available for the $\text{Ca}_v3.1$ channel bound with a channel blocker.¹⁰ As described for this complex with Z944, PnCS1 strongly interacts with the S5–S6 helices of domain II through hydrogen bonding with amino acids in the fenestration between pore-forming segments of domains II and III, known to be a druggable target site.^{30,31} Both PnCS1 and Z944 are involved in hydrogen bonding with the $\text{Ca}_v3.1$ -Thr921 in the pore of the channel. Additional hydrogen bonds occur with $\text{Ca}_v3.1$ -Gln922 and eventually with the SF-Glu923. PnCS1-Arg6 appears to play a crucial role in the structural stability of the peptide during the interaction by stabilizing PnCS1's flexible backbone while simultaneously binding to a SF residue. On the other hand, PnCS1-Arg2 formed stabilizing cation– π interactions with Phe917 and Phe956 in the II–III fenestration of the $\text{Ca}_v3.1$ channel. The same $\text{Ca}_v3.1$ -residues interact with Z944 to stabilize the hydrophobic pocket. The binding in the II–III fenestration conjures a first π -helix in S6 of domain II in both the PnCS1- $\text{Ca}_v3.1$ and Z944- $\text{Ca}_v3.1$ complexes. PnCS1-Trp4 interacts with a part of the intracellular gate, similar to the isobutyl moiety of Z944, and forces a second π -helix in S6 of domain I.¹⁰ Confirming the importance of the observed interactions of PnCS1-Arg6 with the $\text{Ca}_v3.1$ channel in the modeled complex, PnCS1[R6E] shows no important or discernible interaction with $\text{Ca}_v3.1$. The stability of the trajectory can be attributed to the many intramolecular hydrogen bonds, thereby locking itself in place. Further experimental studies, where the peptide is tested on the $\text{Ca}_v3.1$ channel with mutations in the inner vestibule, are required to verify this binding site.

The binding mode of PnCS1 not only is consistent with the binding mode of Z944 but also seems to combine this with the allosteric effect of antagonists described for $\text{Ca}_v1.2$. $\text{Ca}_v1.2$ channel blockers are widely prescribed in the therapy of cardiovascular-related diseases and can be subdivided into three chemical classes of drugs that bind to three separate binding sites.³ While phenylalkylamines and benzothiazepines are thought to physically block the pore,^{3,32} the 1,4-dihydropyridines interact with the external, lipid-facing side of the III–IV fenestration in the ion channel. Their binding breaks the 4-fold symmetry in the channel, affecting the selectivity filter allosterically and hereby inducing a high-affinity Ca^{2+} binding that blocks the pore.³ Comparable to the insertion of dihydropyridines to the III–IV fenestration, PnCS1-Trp7 stabilized the peptide through hydrophobic interactions in the same III–IV fenestration, which embeds Asn8 and Arg9 of the cyclic peptide. To accommodate these residues, allosteric changes occur in the structure of the $\text{Ca}_v3.1$ channel, breaking its symmetry. This loss of symmetry is related to an increased Ca^{2+} affinity in the pore for dihydropyridines, accounting for the pore-blocking effect.³ In the complex with PnCS1, we observed a loss of the

characteristic hydrogen-bonding network at the SF in the MD trajectory.

In the cryo-EM structure of Z944 in complex with $\text{Ca}_v3.1$,¹⁰ the III–IV fenestration is not occupied by the small ligand Z944. Instead, it is plugged by a phosphatidylcholine diglyceride with the head pointing to the center of the cavity near Z944. Zhao et al. mention that the presence of this fatty acid residing in the fenestration requires future investigation. The residence of larger, interacting residues in this fenestration is in line with what we see with PnCS1. The hydrophobic interactions made in at least one of the fenestrations of the channel protein, contribute also to the stabilization the ligand.¹⁰

How exactly the peptide can get into the inner pore remains unclear. Further experimental, computational, and structural studies are definitely required to elucidate this mechanism. The importance of stabilizing interactions in fenestrations of voltage-gated ion channels was already demonstrated by elucidating the binding of cannabidiol (CBD) at Na_v channels. CBD binds at the interface of a fenestration and the central hydrophobic cavity of a mutated Na_v channel, stabilizing the inactivated state of the channel protein while physically blocking the ion-influx pathway.³⁰ The binding site for PnCS1 is, similar to the CBD-binding site in Na_v channels, located at the pore end of the fenestrations. This may facilitate the ingress of hydrophobic molecules into the channel interior and may possibly provide a pathway for PnCS1 to enter the channel and inhibit the calcium currents. These fenestrations are shown to have similar conformations in both the resting and open states of the Na_v channel.³¹ This could explain our observation that the inhibition of $\text{Ca}_v3.1$ channels by PnCS1 is not state-dependent.

Molecular dynamic studies demonstrated the importance of Cys1, Arg3, Trp4, Arg6, and Trp7 of PnCS1 in binding to $\text{Ca}_v3.1$. We therefore developed a series of mutants in which we replaced these specific amino acids one by one. The introduction of a negative charge resulted in a complete loss of inhibition for PnCS1[R3E], [W4E], [R6E], and [W7E] when evaluated for binding to $\text{Ca}_v3.1$. Replacing these amino acids with neutral or positive residues led to a strong reduction in inhibition for PnCS1[R3A], [W4A], [R6A], [W7A], [R3K], [W4K], [R6K], and [W7K] compared to PnCS1. Furthermore, we investigated the importance of the N- and the C-terminal of the peptide by replacing Cys1 and Cys10 with an Ala and Gly residue, respectively, and backbone cyclized the peptide (PnCS3). This altered cyclization method resulted in a significant loss of PnCS3 inhibition at $\text{Ca}_v3.1$. The observed reduction in activity corroborates our modeling results, suggesting that residue Cys1, Arg3, Trp4, Arg6, and Trp7 as well as the N- and C-terminal play a significant role in the binding of PnCS1 and the $\text{Ca}_v3.1$ channel. In conclusion, functional studies characterizing the activity of PnCS1 on $\text{Ca}_v3.1$ channels, combined with molecular dynamics results, have provided the means for understanding the molecular interactions of PnCS1 with the $\text{Ca}_v3.1$ target and lays out a framework for structure-guided drug discovery for the treatment of a variety of Ca_v3 channelopathies.

METHODS

Peptide Synthesis. Pn was purchased from GenicBio Ltd. (Shanghai, China). PnCS1, PnCS3, and all PnCS1 mutants were chemically synthesized using standard Fmoc solid-phase synthesis protocols on a Symphony Automated Peptide

Synthesizer (Protein Technologies, Tucson, AZ, USA) and oxidized or backbone cyclized as previously described by Peigneur and co-workers.^{23,24}

***Xenopus laevis* Surgery.** Stage V–VI oocytes were isolated by partial ovariectomy from *Xenopus laevis* frogs (African clawed frog) as described previously.³³ Mature female frogs were purchased from CRB Xénopes (Rennes, France) and housed in the Aquatic Facility (KU Leuven) in compliance with the regulations of the European Union (EU) concerning the welfare of laboratory animals as declared in Directive 2010/63/EU. The use of the frogs was in accordance with license number LA1210239 of the Laboratory of Toxicology & Pharmacology, University of Leuven. All animal care and experimental procedures agreed with the guidelines of the “European Convention for the protection of vertebrate animals used for experimental and other scientific purposes” (Strasbourg, 18.III.1986). After anesthetizing the frogs by a 15 min submersion in 0.1% tricaine methanesulfonate (pH 7.0), the oocytes were collected. The isolated oocytes were then washed with a 1.5 mg/mL collagenase solution to remove the follicle layer.

Expression of Ca_v3 Channels in *Xenopus laevis* Oocytes. Rat Ca_v3.1, human Ca_v3.2, and rat Ca_v3.3 were expressed in *Xenopus laevis* oocytes by linearizing the plasmids and subsequent *in vitro* transcription using a commercial T7 mMESSAGE mMACHINE transcription kit (Ambion, Carlsbad, CA, USA). Defolliculated *Xenopus* oocytes were injected with 50 nL of cRNA at a concentration of 1 ng/nL by using a microinjector (Drummond Scientific Company, Broomall, PA, USA). The oocytes were incubated in a solution containing (in mM): NaCl, 96; KCl, 2; CaCl₂, 1.8; MgCl₂, 2; and HEPES, 5 (pH 7.5). This solution was supplemented with 50 mg/L gentamicin sulfate and 90 mg theophylline.

Electrophysiological Recordings. Two-electrode voltage clamp recordings were performed at room temperature (18–22 °C) by using a GeneClamp 500 amplifier (Molecular Devices, San Jose, CA, USA) controlled by a pClamp data acquisition system (Axon Instruments, Union City, CA, USA). Whole-cell currents from oocytes were recorded 5–10 days after mRNA injection. The bath solution composition was the following (in mM): BaCl₂, 10; NaOH, 90; KOH, 1; EDTA, 0.1; and HEPES, 5 (pH 7.5). Voltage and current electrodes were filled with 3 M KCl. Resistances of both electrodes were kept between 0.8 and 1.5 MΩ. The elicited Ca_v3.1, Ca_v3.2, and Ca_v3.3 currents were filtered at 2 kHz and sampled at 4 kHz using a four-pole low-pass Bessel filter. Leak subtraction was performed by using a -P/4 protocol.

For the electrophysiological analysis of peptides, a number of protocols were applied from a holding potential of −90 mV. Currents for Ca_v3.1 and Ca_v3.2, carried by Ba²⁺, were evoked by 300 ms depolarizing pulses to −25 mV or V_{max} (the voltage corresponding to maximal Ba²⁺ current in control conditions). Currents for Ca_v3.3, carried by Ba²⁺, were evoked by 1 s depolarizing pulses to −25 mV or V_{max} . The current–voltage relationships were determined by 600 ms step depolarizations between −90 and +60 mV, using 10 mV increments. The values of I_{pBa} were normalized to the maximal Ba²⁺ current amplitude and plotted as a function of voltage. To investigate the effect of the peptide toxins on the activation and steady-state inactivation, a standard two-step protocol was used. In this protocol, 600 ms conditioning 10 mV step prepulses ranging from −90 to +60 mV were followed by a 400 ms test pulse to −25 mV. Data were normalized to the maximal Ba²⁺

current amplitude, plotted against the prepulse potential, and fitted by using the Boltzmann function: $I_{Ca}/I_{max} = \{(1 - C)/(1 + \exp[(V - V_h)/k])\} + C$, where I_{max} is the maximal I_{Ba} , V_h is the voltage corresponding to half-maximal inactivation, V is the test voltage, k is the slope factor, and C is a constant representing a noninactivating persistent fraction (close to 0 in control). The concentration–response relationship was determined by fitting the data with the Hill equation: $y = 100/\{1 + [IC_{50}/(\text{toxin})]^h\}$, where y is the amplitude of the toxin-induced effect, IC_{50} is the toxin concentration at half-maximal efficacy, toxin is the toxin concentration, and h is the Hill coefficient.

Data Analysis. All electrophysiological data are presented as means ± SEM of at least three independent experiments ($n \geq 3$) unless otherwise indicated. All data were analyzed using pClamp Clampfit 10.4 (Molecular Devices, Downingtown, PA, USA) and OriginPro 9 (Originlab, Northampton, MA, USA) or GraphPad Prism 8 software (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was determined using one-way ANOVA with Dunnett's post-test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ comparing to PnCS1.

Computational Studies. The protein structures of the Ca_v3.1 channel (PDB ID: 6KZO and 6KZP) were procured from the RCSB protein databank.³⁴ Structurally, the difference between the two structures lies in the S6 π -helix of domain II in 6KZP. To determine which structure to use for the computational studies, a short simulation of 100 ns with PnCS1 and the Ca_v3.1 channel (PDB ID: 6KZO) was run and confirmed the same conformational change in the S6 helix of domain II, and therefore 6KZP, was used as an induced fit starting model. The small ligand Z944 and a phosphatidylcholine diglyceride in the III–IV fenestration were manually removed from the Ca_v3.1 channel (PDB ID: 6KZP). The amino acid sequence of the PnCS1 peptide is available in the article by Peigneur et al.²³ So far, no experimental structure of this cyclic peptide was available. Therefore, a starting structure was generated through the tLEaP module and the cyclic structure was manually torsioned to obtain an initial model for PnCS1. Missing hydrogens were added to the apo-structure of the Ca_v3.1 channel and the peptide, and in both models cystine bridges were defined. Short extracellular loops that were missing in the apo-structure were generated through AMBER's³⁵ tLEaP module; missing intracellular loops were considered too large to model accurately.

Particle mesh Ewald molecular dynamics (PMEMD)³⁶ was employed on the initial model of PnCS1, using the ff14SB protein and TIP3P water force field. First, a minimization was carried out for 20 000 cycles, starting with the steepest descent method and ended the last 5000 cycles with the conjugate gradient method. Hereafter, the SHAKE algorithm was activated with step size set to 0.002.³⁷ Heating was done in two parts: first heating to 100 K, then to 300 K, both for 50 000 steps and using the Langevin thermostat.³⁸ Density and equilibration were run with the use of the Berendsen barostat.³⁹ The production run was carried out for 400 ns with the simulation being restarted every nanosecond through Bash-scripted methods to reduce the possibility of being stuck in a local minimum.

An ensemble of 15 snapshots of the PnCS1 peptide was extracted from the PnCS1–Ca_v3.1 simulation, with an evenly spaced interval. From the extracted models, structure 14 was chosen (see Results for more information) and used to create

the PnCS1[R6E] model by virtual mutation of PnCS1-Arg6 to a glutamate residue.

The ClusPro docking server was used to perform protein–protein docking to obtain a model on the complexes of PnCS1 and its PnCS1[R6E] mutant with the Ca_v3.1 channel.⁴⁰ The docking results are categorized into *balanced*, *electrostatic-favored*, *hydrophobic-favored*, and *van der Waals + electrostatics*. The balanced results were selected to favor neither type of interaction. The structure for each best-ranked complex in the best cluster, being the cluster with the most favorable iterations, was subjected to a MD simulation mainly similar to the protocol used for PnCS1 mentioned above. Most simulation parameters remained the same as previously stated, except for the extended minimization and production run. The former lasted for 22 500 cycles and 7500 cycles with the conjugate gradient method, and the latter was extended to 200 ns. Analysis of the trajectory, difference in the distance between atoms, RMSF, and RMSD by the C α atoms were calculated with the Cpptraj module.⁴¹ Calculation of the Gibbs free binding energy (ΔG) was done via the Molecular Mechanics Poisson–Boltzmann Surface Area (MMPBSA) program.⁴² The MMGBSA module was used, because of its time-efficient calculations, with 2000 frames of the stable trajectory taken into account (interval set to 1). The graphs of this section were generated through Python's pandas and matplotlib libraries, and the figures were generated through UCSF Chimera⁴³ and Inkscape. The apoprotein simulation was included to provide insight into the stability of the selectivity filter and as a basal control of the simulation parameters.

The authors considered the added value of the membrane to be minimal since it is unlikely that the ligand, with its polar amino acids, would reside at the apolar interface between the membrane and the channel. Binding at this outer surface of the channel would also not fit with the available biological data, which indicates that the ligand is a pore blocker. The docking experiment also showed predominant binding predictions in the central pore.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsptsci.1c00079>.

IC₅₀ values obtained for PnCS1 on Ca_v3 channel isoforms; Gibbs free binding energy calculations; quantification of the percentage inhibition of Ca_v3.1, Ca_v3.2 and Ca_v3.3 after application of PnTx1, μ -KIIIA and Pn; top results of each cluster's binding pose of the PnCS1-Ca_v3.1 complex; simplified depiction of the location of the PnCS1 peptide in the central pore of the Ca_v3.1 channel; RMSD PnCS1-Ca_v3.1 complex; RMSF PnCS1-Ca_v3.1 complex; interactions of PnCS1 and the Ca_v3.1 channel; RMSD PnCS1[R6E]-Ca_v3.1 complex; RMSF PnCS1[R6E]-Ca_v3.1 complex; interactions of PnCS1[R6E] and the Ca_v3.1 channel; RMSD Ca_v3.1-apo; RMSF Ca_v3.1-apo; interactions Ca_v3.1-apo (PDF)

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Author Contributions

[†]A.-S.D. and J.R. contributed equally to this work. A.-S.D., J.R., S.P., and J.T. designed the study; A.-S.D. conducted all electrophysiology experiments; S.P., C.I.S., and J.T. designed the peptides; J.R. performed all computational studies; O.C. performed peptide synthesis; A.-S.D. and J.R. wrote the manuscript; and M.V., C.I.S., D.J.C., E.L., S.P., and J.T. supervised the writing and editing of the manuscript.

Notes

The authors declare no competing financial interest.

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