

A single S6_{III} mutation uncouples the dual gating effects of Pc1a on Na_v1.7

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Abstract

Voltage-gated sodium (Na_v) channels couple membrane depolarization to pore opening and fast inactivation. The bullet-ant toxin δ -paraponeritoxin-Pc1a (Pc1a) is an unusual membrane-spanning Na_v agonist that facilitates activation while suppressing fast inactivation. It remains unclear if and how these two effects are coupled. We previously identified a Pc1a-binding site between voltage-sensing domain in repeat II (VSD_{II}) and pore domain III (PD_{III}) in Na_v1.6 and found that mutation of an S6_{III} residue could restore fast inactivation without abolishing the toxin-induced shift of activation. Here we use human Na_v1.7 as a structurally tractable model to define this mechanism. A cryo-EM structure of Pc1a-bound Na_v1.7 complex reveals a membrane-spanning binding pose nearly identical to that observed in Na_v1.6. In the sensitized Na_v1.7-M3 mutant (N1420K/Y1429D/S1430N), veratridine (VTD) dose-dependently suppresses Pc1a-induced persistent current. Ensuing mutational analysis pinpointed residues that bidirectionally tune Pc1a-induced persistent current. We focused on L1449A in S6_{III}, which nearly abolishes Pc1a-induced persistent current while preserving the hyperpolarizing shift of activation. In the absence of toxin, L1449A shifts activation toward more depolarized voltages and both fast and slow inactivation toward more hyperpolarized voltages, indicating a redistribution of channel gating toward inactivation-prone states. Together, these results separate the activation- and inactivation-modifying actions of Pc1a and identify Leu1449_{S6III,29} as a conserved determinant coupling Pc1a binding to fast inactivation.

Introduction

Voltage-gated sodium (Na_v) channels initiate and propagate electrical signals in excitable cells (1-4). To ensure repetitive firing, Na_v channels possess the unique properties of instantaneous activation upon membrane depolarization and ensuing fast inactivation, the latter involving displacement of the Ile-Phe-Met (IFM) motif to its receptor site next to the intracellular gate, a mechanism defined as the “wedge model” (5-7). Activation and fast inactivation are therefore tightly coupled, yet the structural determinants that coordinate these processes remain incompletely understood.

Gating-modifier toxins (GMTs) provide powerful tools to probe Na_v channels function and mechanism. Most characterized peptide GMTs act on the extracellular side of the voltage-sensing domains (VSDs) or the pore domain (PD) (8-10). By contrast, δ -paraponeritoxin-Pc1a (Pc1a), a 25-residue toxin from the bullet ant *Paraponera clavata*, forms a single transmembrane helix that wedge between VSD_{II} and PD_{III} as revealed by its complex structure with $\text{Na}_v1.6$ (11-15). Pc1a facilitates Na_v activation, slows activation kinetics and strongly suppresses fast inactivation, thereby producing a persistent sodium current. Our previous structural observations suggested that Pc1a might act as a molecular constraint linking VSD_{II} movement to pore rearrangement (15).

One observation from the $\text{Na}_v1.6$ study was particularly informative and prompted us to further dissect the underlying mechanism. Substitution of Leu1456 S6_{III} ,²⁹ based on a generic residue numbering system (16), with alanine substantially restored fast inactivation in the presence of Pc1a, whereas the toxin retained its ability to shift activation toward hyperpolarized voltages. This result suggested that Pc1a-facilitated activation and suppression of fast inactivation can be functionally separated, and that an S6_{III} residue downstream of the toxin-binding site contributes to the anti-inactivation effect. We reckoned that human $\text{Na}_v1.7$ might provide a good scaffold for structural dissection of such functional separation, as its open-state structure has been captured in the presence of veratridine (VTD).

Two conformations of VTD-bound $\text{Na}_v1.7$ have been determined: an inactivated state

with VTD bound at site I, which overlaps the IFM receptor site, and an open state with VTD bound in the central cavity at site C (7). Comparison of these structures with Na_v1.7 in other conformational states highlights substantial rearrangements of the intracellular S6 helices during gating and provide a structural framework for testing how perturbations within the pore influence the functional consequences of Pc1a treatment. Notably, alanine substitution of Leu1449_{S6III,29} in Na_v1.7, corresponding to Leu1456_{S6III,29} in Na_v1.6, nearly abolished VTD-induced persistent and tail currents while enhancing VTD-mediated inhibition.

Here we combine cryo-EM, whole-cell electrophysiology, and sequence-guided mutagenesis of both the channel and the toxin to dissect the action of Pc1a on Na_v1.7. We first define molecular determinants of Pc1a subtype sensitivity despite a conserved binding pose. We then show that VTD functionally opposes Pc1a-induced persistent current, and use this observation to interrogate a set of pore residues. Mutagenesis characterizations reveal both Pc1a-enhancing and Pc1a-suppressing substitutions, supporting the idea that this region forms an allosteric gating network rather than a simple toxin-contact surface. We next focus on Na_v1.7-L1449A, which produces a pronounced loss of Pc1a-induced persistent current, thereby separating Pc1a's effects on activation and fast inactivation. Characterization of Na_v1.7-L1449A's gating properties, together with structural determination of its Pc1a-bound complex, reveals how a single residue in S6_{III} leverages the ability of Pc1a to suppress fast inactivation.

Results

Pc1a induces persistent current and facilitates activation of Na_v1.7

Pc1a was characterized to have subtype preference for Na_v1.6. To characterize its effect on Na_v1.7, we conducted systematic whole-cell voltage-clamp recording of HEK293T cells that transiently expressed human Na_v1.7. Pc1a produced a concentration-dependent persistent current, quantified as the current remaining at 40-45 ms relative to the peak current ($I_{40-45\text{ms}}/I_{\text{max}}$), and shifted the voltage dependence of activation toward more negative potentials

(Fig. 1A-1D). Concentration–response analysis yielded EC₅₀ values of $0.42 \pm 0.08 \mu\text{M}$ and $0.41 \pm 0.06 \mu\text{M}$ for currents elicited by a step to 0 mV and -20 mV, respectively (Fig. 1B). Across 10 nM to 10 μM Pc1a, activation was progressively shifted toward more negative voltages (Fig. 1C), while the current-voltage relationship changed in parallel with the emergence of persistent current (Fig. 1D). We therefore used two separable functional readouts throughout the study: persistent-current fraction ($I_{40-45\text{ms}}/I_{\text{max}}$) to report suppression of fast inactivation and the activation-voltage shift ($\Delta V_{1/2}$) to report modulation of activation.

We determined a cryo-EM structure of the Na_v1.7-Pc1a complex at an overall resolution of 3.4 Å (Fig. 1E). As expected, Pc1a is resolved as a membrane-spanning helix at the interface between VSD_{II} and PD_{III}, adopting a binding pose nearly identical to that observed in the Na_v1.6-Pc1a complex (Fig. 1E). Pc1a binding is accompanied by a pronounced rearrangement of VSD_{II}, characterized by an inward tilt of the S3-S4 hairpin and outward displacement of the intracellular portions of S2_{II} and S0_{II} (Fig. 1F).

Molecular determinants for subtype sensitivity to Pc1a

Pc1a acts more potently on Na_v1.6 than on Na_v1.7. Sequence alignment and our Na_v1.6-Pc1a structure previously identified three subtype-divergent residues near the extracellular toxin interface as determinants of this difference (15). To test the reciprocal prediction in Na_v1.7, we introduced the reciprocal mutations N1420K/Y1429D/S1430N to potentially enhance Na_v1.7's sensitivity to Pc1a, and name the resulted mutant to Na_v1.7-M3 (Fig. 2A).

Indeed, Na_v1.7-M3 showed substantially increased functional sensitivity to Pc1a compared with WT Na_v1.7 (Fig. 2A and 2B). Persistent-current concentration-response analysis yielded EC₅₀ values of $0.050 \pm 0.005 \mu\text{M}$ and $0.042 \pm 0.004 \mu\text{M}$, compared to $0.42 \pm 0.08 \mu\text{M}$ and $0.41 \pm 0.06 \mu\text{M}$, for WT, at 0 mV and -20 mV, respectively. Thus, the reciprocal substitutions increased the potency of Pc1a by approximately 8-10-fold. Na_v1.7-M3 also reached a larger maximal persistent-current fraction than WT in the concentration-response measurements. Together with the reduced Pc1a sensitivity produced by the reciprocal

substitutions in Na_v1.6, these data identify Asn1420_{P2III,42}, Tyr1429_{P2III,51} and Ser1430_{P2III,52} (16) as important determinants of subtype-dependent Pc1a recognition and functional sensitivity.

The membrane-spanning portion of Pc1a contains a cluster of hydrophobic residues on the interface with the channel within the bilayer. We generated two toxin variants, Pc1a-4A (I6A/L7A/L10A/L11A) and Pc1a-AG (L2A/I6G), to test the contribution of this surface (Fig. 2C and 2D). As expected, both Pc1a variants produced little persistent current even at concentrations up to 100 μM. Nevertheless, both Pc1a-4A and Pc1a-AG produced concentration-dependent hyperpolarizing shifts in Na_v1.7 activation, but with substantially reduced efficacy compared with WT Pc1a (Fig. 2D). Thus, perturbation of the hydrophobic toxin surface severely impairs suppression of fast inactivation while also weakening, but not abolishing, facilitation of activation. This observation provides an initial indication that Pc1a control of activation and fast inactivation can be uncoupled.

VTD and site-I-associated residues modulate Pc1a-induced persistent current

The Pc1a-binding site is spatially separated from the canonical IFM-associated inactivation region, raising the question of how a toxin bound at the VSD_{II}-PD_{III} interface suppresses fast inactivation. We used veratridine (VTD) as a functional perturbation, as VTD can bind to site I and cause the movement of S6_{III}, which is critical in the process of inactivation (7) (Fig. 3A, left). To maximize the dynamic range of the Pc1a response, we performed this experiment on the sensitized M3 background. After establishing a robust persistent current with 1 μM Pc1a, we applied 0.1, 1, 3, 10, 30 or 100 μM VTD and quantified $I_{40-45\text{ms}}/I_{\text{max}}$ (Fig. 3A, right). Low concentrations of VTD (0.1-1 μM) produced little detectable change, whereas higher concentrations progressively reduced the persistent current induced by Pc1a, with significant inhibition evident from 3 μM VTD in the current dataset (Fig. 3A, right). Because VTD can occupy more than one site and stabilize different Na_v1.7 conformations, these data do not demonstrate direct steric competition between VTD and Pc1a. Instead, they show that the

functional state promoted by Pc1a can be opposed by VTD and provided the rationale for testing residues associated with the VTD site-I/fast-inactivation region.

We next introduced alanine substitutions at ten residues associated with the VTD site-I/fast-inactivation region: Gly1316_{S4III,53}, Ile1321_{S5III,16}, Leu1449_{S6III,29}, Phe1452_{S6III,32}, Ile1456_{S6III,36}, Phe1460_{S6III,40}, Gln1463_{S6III,43}, Lys1465_{S6III,45}, Ile1472_{S6III,52} and Ile1756_{S6IV,33} (16). We quantified $I_{40-45\text{ms}}/I_{\text{max}}$ at matched Pc1a concentrations (1 μM , 10 μM and, where tested, 100 μM) (Fig. 3B and 3C). The mutations produced a broad spectrum of Pc1a responses rather than a uniform loss of toxin activity.

G1316A, I1321A, F1460A, Q1463A, K1465A and I1472A increased Pc1a-induced persistent current relative to WT. F1452A and I1456A showed only modest differences from WT and were therefore considered broadly WT-like in the context of the screen. By contrast, L1449A and I1756A showed the strongest reductions in persistent current, and the L1449A/I1756A double mutant likewise exhibited only a small persistent-current fraction (Fig. 3B). Thus, substitutions within this region can enhance, largely preserve or strongly reduce the ability of Pc1a to suppress fast inactivation.

The bidirectional nature of these effects is notable. If these residues simply constituted a Pc1a-binding epitope, alanine substitutions would be expected predominantly to weaken toxin action. Instead, mapping the mutations onto the structure revealed a spatial organization of their functional effects (Fig. 3C). Mutations that enhanced Pc1a-induced persistent current clustered mainly toward the intracellular portion of S6_{III} and its surrounding region, whereas Phe1452_{S6III,32} and Ile1456_{S6III,36}, which showed relatively modest effects, occupied an intermediate region of S6_{III}. In contrast, L1449A, which strongly reduced persistent current, was positioned further toward the extracellular side of S6_{III}, along with another strongly affected residue, Ile1756_{S6IV,33}. This spatial stratification suggests that these residues form a functionally organized gating network rather than a simple toxin-contact surface. The intracellular S6_{III} cluster may participate in conformational rearrangements associated with fast inactivation, whereas the relatively insensitive residues may constitute a more

structurally stable segment or pivot within this network. Conversely, the strong effects of L1449A and I1756A suggest that these positions are important for coupling Pc1a binding to suppression of fast inactivation. Given its pronounced phenotype and the previous identification of the equivalent Nav1.6 residue in Pc1a-dependent fast inactivation, we selected L1449A for detailed mechanistic analysis.

Despite exhibiting only a small persistent-current fraction even at high Pc1a concentrations (Fig. 3B), L1449A remained responsive to Pc1a at the level of activation. Increasing concentrations of Pc1a progressively shifted the activation curve of L1449A toward more hyperpolarized voltages (Fig. 3D), with a magnitude comparable to that observed in WT Nav1.7. Thus, L1449A does not cause a general loss of Pc1a responsiveness, but instead preferentially disrupts the ability of Pc1a to suppress fast inactivation while preserving its modulation of activation. These results demonstrate that the hyperpolarizing shift in activation is not sufficient to prevent fast inactivation and establish Leu1449_{S6III,29} as a key determinant that functionally separates the two major gating effects of Pc1a.

L1449A reshapes Nav1.7 gating while preserving Pc1a binding

Next, we set out to determine the cryo-EM structure of Nav1.7-L1449A treated with 20 μ M Pc1a. In the 3D EM reconstruction at an overall resolution of 2.2 Å (Fig. 4A), Pc1a remained well resolved at the VSD_{II}-PD_{III} interface and adopted an overall binding pose closely resembling that in the WT Nav1.7-Pc1a complex. Despite the preserved Pc1a binding pose, structural comparison revealed conformational differences in the pore. Viewed from the intracellular side, the activation gate of L1449A appeared more constricted than that of the WT Pc1a-bound channel (Fig. 4A, middle). Consistently, the side-view comparison showed an inward displacement of S6_{III} toward the pore axis in L1449A, accompanied by local rearrangement of the S4-S5_{II} region (Fig. 4A, right). These structural differences suggest that L1449A alters the conformation of the intracellular pore despite preserving the overall binding pose of Pc1a.

We next examined whether these structural differences were accompanied by altered gating properties. In the absence of toxin, L1449A shifted voltage-dependent activation toward more depolarized potentials compared with WT Na_v1.7 (Fig. 4B, left), indicating that channel opening becomes less favorable. In contrast, both steady-state fast inactivation and slow inactivation were shifted toward more hyperpolarized potentials (Fig. 4B, middle and right). Thus, L1449A exerts opposing effects on activation and inactivation, disfavoring channel opening while promoting entry into inactivated states at more negative voltages. The functional bias toward inactivation is therefore consistent with the more constricted intracellular gate and inward displacement of S6_{III} observed in the L1449A-Pc1a structure. Together, these results indicate that Leu1449_{S6III,29} is an important determinant of the gating equilibrium of Na_v1.7 and that its substitution biases the channel toward inactivation-prone conformations.

Structural comparison with the open-state Na_v1.7 structure further provides a framework for understanding how this altered conformational preference opposes the effect of Pc1a (Fig. 4C). Pc1a is positioned between VSD_{II} and PD_{III}, where it engages S4-5_{II} and S6_{III}. In WT Na_v1.7, Pc1a acts as a structural constraint at this interface, restricting the rearrangement of S6_{III} associated with fast inactivation and thereby promoting persistent current. L1449A shifts S6_{III} further toward the pore axis and favors a more constricted intracellular gate, consistent with an increased intrinsic tendency toward the inactivated configuration. This increased drive toward inactivation counteracts the restraint imposed by Pc1a, allowing S6_{III} to undergo the inactivation-associated rearrangement despite continued toxin binding.

Together, these results provide a structural and functional explanation for the uncoupling observed with L1449A. Pc1a remains bound to the mutant channel and retains its ability to facilitate voltage-dependent activation, but its restraint of S6_{III} is no longer sufficient to prevent fast inactivation. Leu1449_{S6III,29} therefore acts as a critical S6_{III} determinant that controls how Pc1a binding is translated into suppression of fast inactivation. The similar phenotype previously observed for the equivalent Na_v1.6-L1456A substitution further

suggests that this coupling mechanism is conserved across Na_v subtypes.

Discussion

This study uses Pc1a to dissect two coupled but separable components of Na_v gating: facilitation of voltage-dependent activation and suppression of fast inactivation. Direct evidence for this functional separation comes from Na_v1.7-L1449A. Although Pc1a-induced persistent current is nearly abolished in this mutant, the toxin retains its ability to shift activation toward hyperpolarized voltages, indicating that these two gating effects arise from distinct, although structurally coupled, consequences of toxin binding.

The structural and functional properties of Na_v1.7-L1449A provide a mechanistic explanation for this uncoupling. Pc1a retains an overall WT-like binding configuration in the Na_v1.7-L1449A-Pc1a structure. Instead, L1449A shifts intrinsic gating toward inactivation-prone states and is accompanied by displacement of S6_{III} toward the pore axis and a more constricted intracellular gate. These observations are consistent with recent models in which fast inactivation involves allosteric coupling between IFM engagement and rearrangement of the intracellular S6 bundle (17). Our results extend this framework by identifying Leu1449_{S6III,29} as an S6_{III} determinant that modulates whether a Pc1a-bound channel can proceed into fast inactivation.

The bidirectional effects of mutations along S6_{III} further suggest that this helix does not contribute uniformly to fast-inactivation control. Mutations toward the intracellular portion predominantly enhanced Pc1a-induced persistent current, whereas substitutions in the intermediate region had relatively modest effects and L1449A, positioned further outward, strongly reduced persistent current (**Figure 3B and 3C**). This spatial organization suggests that different regions of S6_{III} may make distinct contributions to the conformational transitions underlying fast inactivation. Previous Na_v1.7 studies similarly revealed substantial structural plasticity of intracellular S6_{III} during modulation of inactivation, supporting the idea that S6_{III} functions as a spatially differentiated coupling element rather than a rigid gate

(18). Although the intrinsic gating properties of the remaining mutants require further characterization, their opposing effects highlight a broader S6_{III} gating network that tunes the Pcl_a-stabilized state.

The equivalent Nav1.6-L1456A substitution produces a similar separation between activation modulation and suppression of fast inactivation, suggesting that this S6_{III} locus represents a conserved determinant of Pcl_a-dependent gating (15). Together, our present and previous studies establish that facilitation of activation and suppression of fast inactivation can be mechanistically uncoupled and identify S6_{III} as a critical element through which modulation at the VSD-pore interface is translated into fast-inactivation control. Pcl_a therefore provides a molecular probe for dissecting activation-inactivation coupling in Nav channels.

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Author Contributions

X.J., N.Y. and T.W conceived the project. X.J., N.Y. and T.W designed experiments; T.W performed all experiments related to cryo-EM studies, including protein expression, purification and 3D reconstructions; Q.G. and X.J. carried out experiments related to electrophysiology; J.Z, H.H synthesized the peptide; T.W, Q.G., X.F., Z.L, N.Y. and X.J. analyzed data; T.W., N.Y. and X.J. wrote the manuscript with input from all authors. All authors approved the final manuscript.

Competing Interest

The authors declare no competing interests.

Methods

Transient co-expression of human Na_v channels with β 1

The human Na_v channels and β 1 subunit were co-expressed as described previously (19, 20). The optimized coding DNAs for human Na_v1.7 (Uniprot: Q15858) was cloned into the pCAG vector with twin Strep-tag and FLAG tag in tandem at the amino terminus, and β 1 (Uniprot: Q07699) was cloned into the pCAG vector without affinity tag (21, 22). Na_v1.7 variants carrying individual or combined mutations were generated using QuickChange site-directed mutagenesis and confirmed by PCR sequencing. HEK293F cells (Invitrogen) were cultured in SMM 293T medium (Sino Biological Inc.) under 5% CO₂ in a Multitron-Pro shaker (Infors, 130 r.p.m.) at 37 °C, and transfected with plasmids when the cell density reached 2×10^6 cells per ml. For 1 liter cell culture, approximately 2 mg plasmids (1.5 mg for Na_v channel plus 0.5 mg for β 1) were pre-incubated with 4 mg 25-kDa linear polyethylenimines (PEIs) (Polysciences) in 20 mL fresh medium for 15-30 min before adding into cell culture. Transfected cells were cultured for 48 h before harvesting.

Protein purification of Na_v channels

The protein purification protocol for Na_v channels with β 1 is identical to the one we previously described (19, 20). 1 L transfected cells for Na_v1.7-WT and 4 L for Na_v1.7-L1449A were harvested by centrifugation at 800 g and resuspended in the lysis buffer containing 25 mM Tris-HCl (pH 7.5) and 150 mM NaCl. The suspension was supplemented with 1% (w/v) n-dodecyl- β -D-maltopyranoside (DDM, Anatrace), 0.1% (w/v) cholesteryl hemisuccinate Tris salt (CHS, Anatrace), protease inhibitor cocktail containing 2 mM

phenylmethylsulfonyl fluoride (PMSF), aprotinin (3.9 $\mu\text{g/ml}$), pepstatin (2.1 $\mu\text{g/ml}$), and leupeptin (15 $\mu\text{g/ml}$) and 10 μM Pc1a. After incubation at 4 °C for 3 h, the cell lysate was ultra-centrifuged at 20,000 g for 45 min, and the supernatant was applied to anti-Flag M2 affinity gel (Sigma) by gravity at 4 °C. The resin was rinsed four times with the W buffer that contains 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.02% glycol-diosgenin (GDN, Anatrace), the protease inhibitor cocktail and 10 μM Pc1a. Proteins were eluted with W buffer plus 200 $\mu\text{g/ml}$ FLAG peptide (Sigma). The eluent was then applied to Strep-Tactin Sepharose (IBA) and the purification protocol was similar to the previous steps except elution buffer, which was W buffer plus 2.5 mM D-Desthiobiotin (IBA). The eluent was then concentrated using a 100-kDa cut-off Centricon (Millipore) and further purified by Superose-6 column (GE Healthcare) in W buffer. Another 20 μM Pc1a was added to the purified protein one hour before cryo grids preparation.

Cryo-EM data acquisition

Aliquots of 4 μl freshly purified Na_v channel complexes were placed on glow-discharged home-made GO grids (Quantifoil Au 300 mesh, R1.2/1.3), which were provided by Prof. Hong-Wei Wang from Tsinghua University (23). Grids were blotted for 2.5 s and plunge frozen in liquid ethane cooled by liquid nitrogen with Vitrobot Mark IV (Thermo Fisher).

For $\text{Na}_v1.7\text{-}\beta1\text{-Pc1a}$, electron micrographs were acquired on a Titan Krios electron microscope (Thermo Fisher) operating at 300 kV and equipped with Gatan K2 Summit detector and GIF Quantum energy filter. Movie stacks were automatically collected, using

AutoEMation (24) with a slit width of 20 eV on the energy filter and a preset defocus range from -1.5 μm to -1.0 μm in super-resolution mode at a nominal magnification of $\times 64,000$, yielding a calibrated pixel size of 1.0742 Å. Each stack was exposed for 2.56 s with 0.08 s per frame, resulting in 32 frames per stack, accumulating a total electron dose of approximately $50\text{ e}^- \text{Å}^{-2}$. Meanwhile, dose weighting was performed (25). The defocus values were estimated with Gctf (26).

For Nav1.7-L1449A- β 1-Pc1a, electron micrographs were acquired on a 300 kV Titan Krios G4 cryogenic electron microscope (Thermo Fisher Scientific), equipped with a Gatan K3 direct electron detector and a GIF Quantum energy filter. Micrographs were captured using Falcon IVi (Thermo Fisher Scientific) in EC mode at a nominal magnification of $\times 130,000$, corresponding to a calibrated pixel size of 0.933 Å. Data were acquired at a preset defocus range of -1.5 μm to -1.0 μm . Each EER-format movie stack was exposed within a 0.79- μm spot size and accumulated a total electron dose of approximately $50\text{ e}^-/\text{Å}^2$ in EPU (Thermo Fisher Scientific).

Image processing

For Nav1.7- β 1-Pc1a, A total of 4,564 movie stacks were automatically collected. 1,908,933 particles were automatically picked in Cryosparc (27). After 2D classification, a total of 381,777 good particles were selected. After several rounds of heterogeneous refinement using the 3D reconstruction of Nav1.7- β 1- β 2 (EMDB code: EMD-32368) as a reference and 3D

classification, a total of 266,073 particles were selected and applied to non-uniform refinement, resulting in final reconstructions at overall resolutions of 3.4 Å.

For Nav1.7-L1449A-β1-Pc1a, A total of 9,135 movie stacks were automatically collected. Following motion correction and CTF estimation performed using cryoSPARC. A total of 2,652,320 particles were automatically picked. After 2D classification, 637,846 good particles were selected and applied to heterogeneous refinement. 422,153 good particles were applied to 3D classification and finally a total of 282,202 particles were subject to non-uniform refinement, yielding 3D map with overall resolutions of 2.2 Å. Resolution was estimated with the gold-standard Fourier shell correlation 0.143 criterion (28) with high resolution noise substitution (29).

Model building and structure refinement

Model building was performed based on the map acquired in the data processing. The starting model of Nav1.7 (PDB code: 7W9K) was fitted into the EM map by Chimera (30). Each residue was manually checked in COOT (31). The chemical properties of amino acids were considered during model building.

Structure refinement was performed using phenix.real_space_refine application in PHENIX (32) real space with secondary structure and geometry restraints. Over-fitting of the overall model was monitored by refining the model in one of the two independent maps from the gold-standard refinement approach and testing the refined model against the other map (33).

Whole cell electrophysiology

Whole-cell patch-clamp recordings were performed with modifications to previously established procedures (34-36). HEK293T cells were transiently co-transfected with plasmids encoding human Na_v1.7 and eGFP using Lipofectamine 2000 (Invitrogen). Sodium currents were recorded under the same instrumentation and solution conditions reported previously.

Baseline gating properties were characterized using standard voltage-clamp protocols. For activation, cells were held at -120 mV for 200 ms and subsequently depolarized from -90 to +80 mV in 5-mV increments for 100 ms. Steady-state fast inactivation was measured from a holding potential of -90 mV using 200-ms conditioning pulses ranging from -150 to 0 mV in 10-mV increments, followed by a 50-ms test pulse to 0 mV. For steady-state slow inactivation, cells were held at -90 mV and subjected to 9-s conditioning pulses from -130 to +20 mV in 10-mV increments. A 40-ms recovery pulse at -120 mV was then applied before a 50-ms test pulse to 0 mV. Activation and inactivation relationships were derived from the resulting current measurements and fitted as described below.

Pc1a was dissolved in dimethyl sulfoxide (DMSO; Sigma) to prepare a stock solution, which was stored at -80 °C. Working solutions were freshly diluted to the indicated concentrations, with the final DMSO concentration maintained below 0.1%. Following establishment of the whole-cell configuration, recordings were allowed to stabilize for approximately 5 min before Pc1a application. Increasing concentrations of Pc1a were then perfused until the response at

each concentration reached a stable level.

To quantify Pc1a-induced persistent current, sodium currents were elicited by a 50-ms depolarizing pulse to -20 mV from a holding potential of -120 mV. Concentration-response relationships were determined using the ratio of the sustained current measured at 40-45 ms ($I_{40-45\text{ ms}}$) to the peak current (I_{max}). For analysis of Pc1a-dependent changes in voltage-dependent activation, currents were evoked by 100-ms depolarizing steps from -90 to +80 mV in 5-mV increments from a holding potential of -120 mV. Conductance (G) at each membrane potential (V) was calculated as $G = I/(V - V_{\text{rev}})$, where V_{rev} is the reversal potential. Normalized conductance-voltage relationships were fitted with a single Boltzmann function: $G/G_{\text{max}} = 1/(1 + \exp((V_{1/2} - V_m)/\text{slope}))$, where G_{max} is the maximal conductance, $V_{1/2}$ is the half-activation potential, V_m is the membrane potential, and slope is the slope factor.

Electrophysiological data were analyzed using FitMaster (HEKA Elektronik), Origin (OriginLab), and GraphPad Prism (GraphPad Software). Data are presented as mean $\pm$ SEM, and n denotes the number of cells analyzed.

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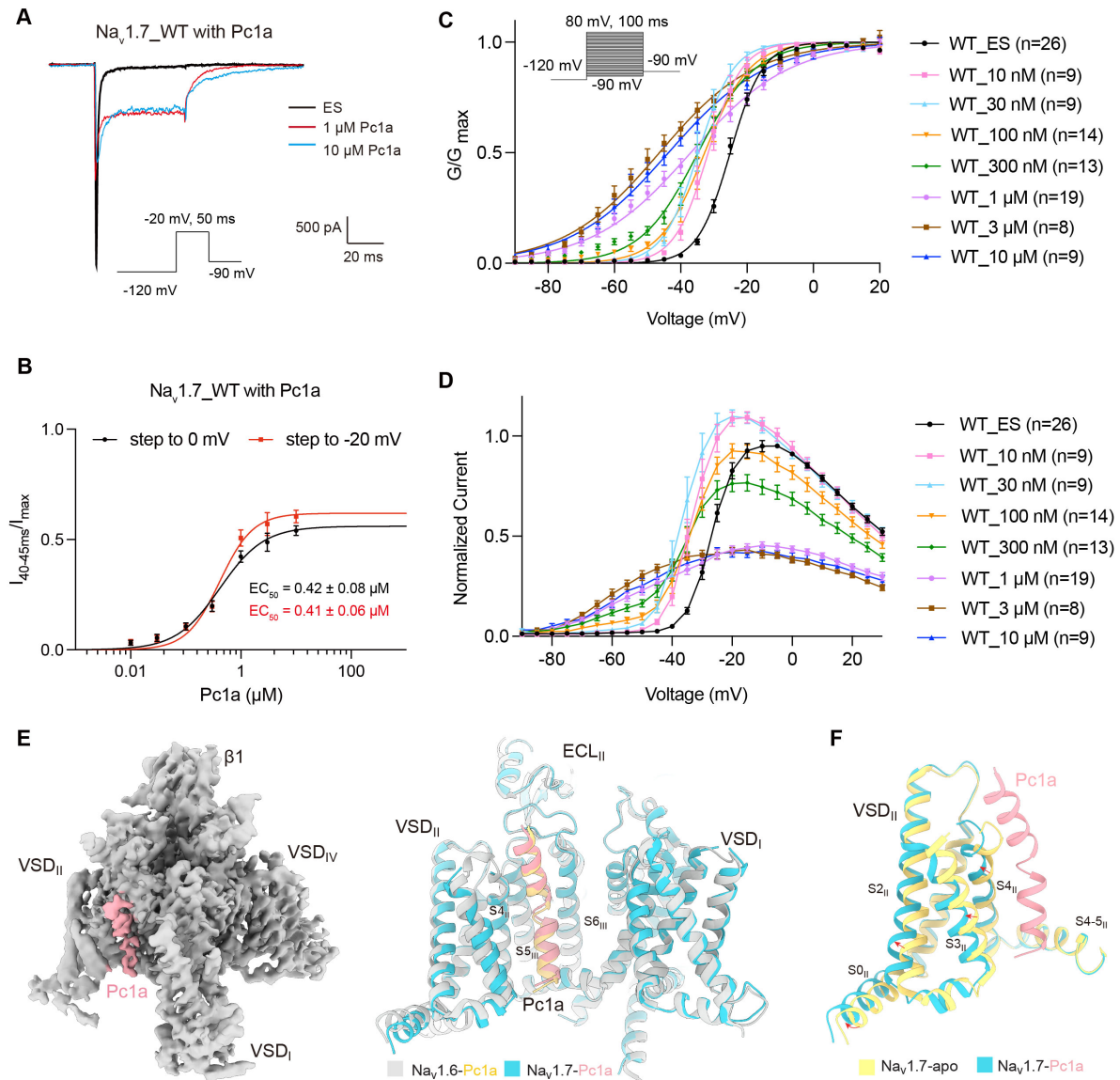


Figure Legends

Fig. 1 | Functional and structural characterizations of Pc1a-bound Na_v1.7. **A**,

Representative whole-cell sodium currents recorded from cells expressing human Na_v1.7 in the absence of Pc1a (ES) or in the presence of 1 or 10 μM Pc1a. Currents were elicited by a 50-ms depolarizing step from -120 mV to -20 mV, followed by repolarization to -90 mV. **B**, Concentration-response relationships for Pc1a-induced persistent current, quantified as the current remaining at 40-45 ms relative to the peak current ($I_{40-45\text{ms}}/I_{\text{max}}$). Currents were

elicited by voltage steps to 0 mV (black) or -20 mV (red). EC₅₀ values were $0.42 \pm 0.08 \mu\text{M}$ and $0.41 \pm 0.06 \mu\text{M}$, respectively. **C**, Voltage-dependent activation curves of Na_v1.7 in the absence or presence of increasing concentrations of Pc1a. The voltage protocol is shown in the inset. Pc1a progressively shifts activation toward more hyperpolarized potentials. Sample sizes (n) are indicated. **D**, Normalized current–voltage relationships of Na_v1.7 under the same Pc1a concentrations as in **C**. **E**, Cryo-EM structure of the Na_v1.7–Pc1a complex. Left, cryo-EM density map showing Pc1a (pink) bound at the interface between VSD_{II} and pore domain III. Right, structural superposition of Na_v1.7–Pc1a (cyan and pink) and Na_v1.6–Pc1a (grey and yellow), viewed parallel to the membrane. **F**, Structural comparison of VSD_{II} in apo Na_v1.7 (yellow) and Pc1a-bound Na_v1.7 (cyan). Pc1a is shown in pink. Pc1a binding is accompanied by rearrangement of VSD_{II}, including reorientation of the S3-S4 hairpin toward the VSD_{II} interior and displacement of the intracellular portions of S2_{II} and S0_{II}.

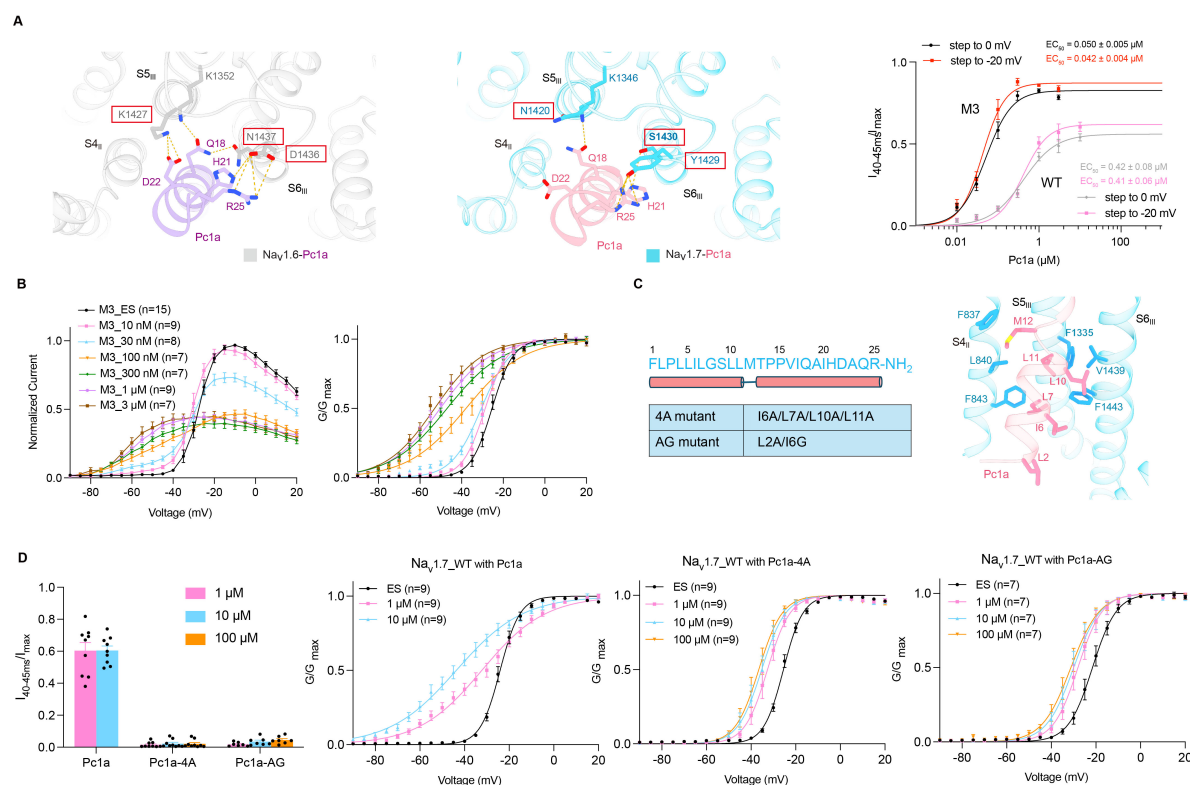


Fig. 2 | Molecular determinants of Pc1a recognition and gating modulation. A,

Structural basis for subtype-dependent Pc1a sensitivity. Left and middle, close-up views of the extracellular Pc1a-binding interfaces in Na_v1.6-Pc1a (grey and purple) and Na_v1.7-Pc1a (cyan and pink), respectively. Subtype-divergent residues are boxed. Dashed lines indicate polar interactions. Right, concentration–response relationships for Pc1a-induced persistent current in the Na_v1.7 N1420K/Y1429D/S1430N triple mutant (M3) compared with WT Na_v1.7. M3 yielded EC₅₀ values of 0.050 ± 0.005 μM and 0.042 ± 0.004 μM for voltage steps to 0 and –20 mV, respectively, compared with 0.42 ± 0.08 μM and 0.41 ± 0.06 μM for WT. **B,** Normalized current–voltage relationships (left) and voltage-dependent activation curves (right) of M3 in the absence or presence of increasing concentrations of Pc1a. Sample sizes (n) are indicated. **C,** Sequence and membrane-spanning architecture of Pc1a. Residues substituted in Pc1a-4A (I6A/L7A/L10A/L11A) and Pc1a-AG (L2A/I6G) are indicated. Right,

close-up view of the hydrophobic interface formed by Pc1a and S4_{II}, S5_{III} and S6_{III} of Na_v1.7.

D, Functional effects of hydrophobic Pc1a mutations. Left, persistent-current fractions ($I_{40-45\text{ms}}/I_{\text{max}}$) induced by WT Pc1a, Pc1a-4A and Pc1a-AG at the indicated concentrations.

Right, voltage-dependent activation curves of WT Na_v1.7 in the absence or presence of WT Pc1a, Pc1a-4A or Pc1a-AG. Both Pc1a variants induce little persistent current while retaining concentration-dependent hyperpolarizing shifts of activation, although with reduced efficacy compared with WT Pc1a. Sample sizes (n) are indicated.

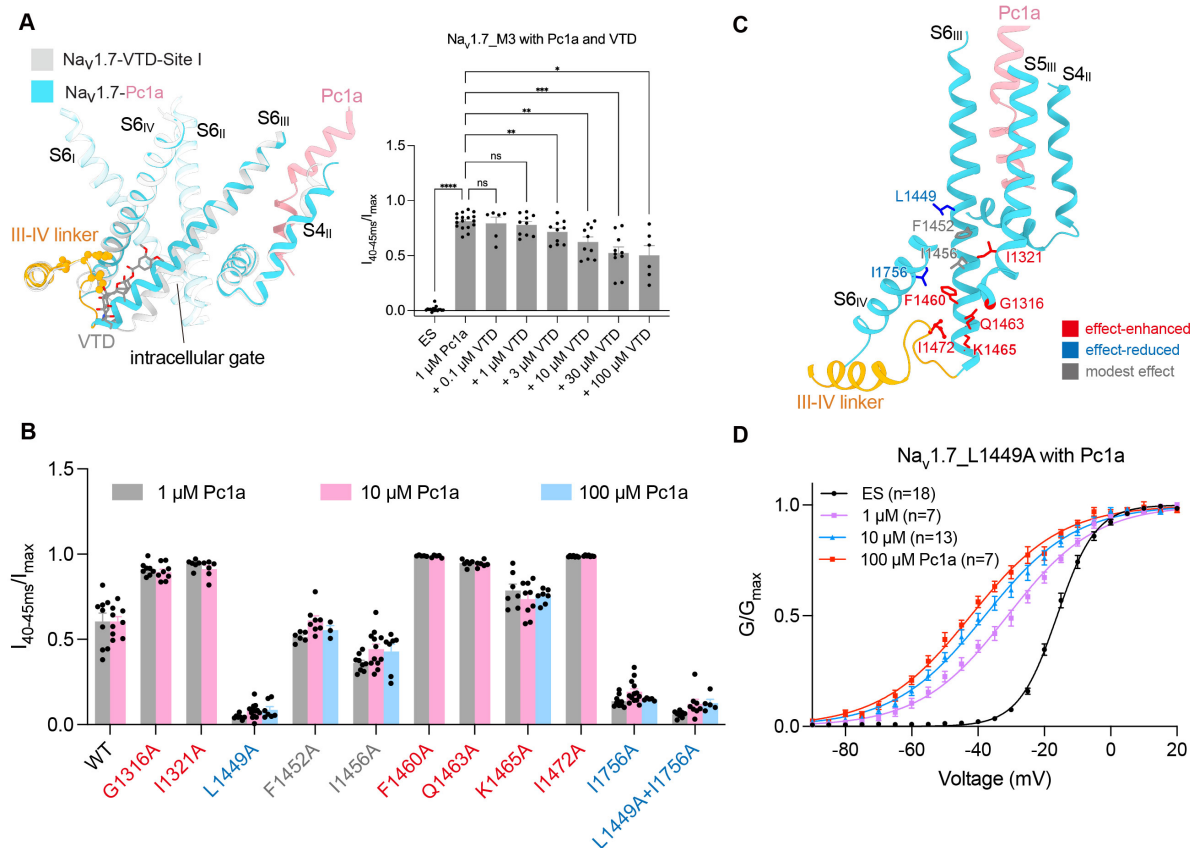


Fig. 3 | VTD antagonism and mutational mapping of Pc1a-dependent fast inactivation.

A, Functional antagonism between Pc1a and veratridine (VTD). Left, structural comparison of Pc1a-bound Na_v1.7 (cyan; Pc1a, pink) with the VTD site-I-associated Na_v1.7 structure (PDB: 21TQ, grey; VTD, grey), highlighting their spatial relationship to the intracellular gate and III–IV linker. Right, persistent-current fractions ($I_{40-45ms}/I_{max}$) of the sensitized Na_v1.7-M3 mutant under ES conditions, after application of 1 μM Pc1a, and following addition of the indicated concentrations of VTD in the continued presence of Pc1a. Higher concentrations of VTD progressively reduce Pc1a-induced persistent current. Statistical significance is indicated in the panel; ns, not significant. **B**, Mutational analysis of residues associated with the VTD site-I/fast-inactivation region. Persistent-current fractions induced by 1, 10 and, where tested, 100 μM Pc1a are shown for WT Na_v1.7 and the indicated single or double mutants. G1316A, I1321A, F1460A, Q1463A, K1465A and I1472A enhance Pc1a-induced

persistent current (red), whereas L1449A and I1756A markedly reduce it (blue). F1452A and I1456A show comparatively modest effects (grey). **C**, Structural mapping of residues examined in **B** onto the Nav1.7-Pc1a structure. Residues are colored according to their functional effects on Pc1a-induced persistent current: enhanced (red), reduced (blue) or modest effect (grey). Pc1a and the III-IV linker are shown in pink and orange, respectively. **D**, Voltage-dependent activation of Nav1.7-L1449A in the absence or presence of increasing concentrations of Pc1a. Despite the marked reduction in Pc1a-induced persistent current, Pc1a retains the ability to progressively shift L1449A activation toward more hyperpolarized potentials. Sample sizes (n) are indicated.

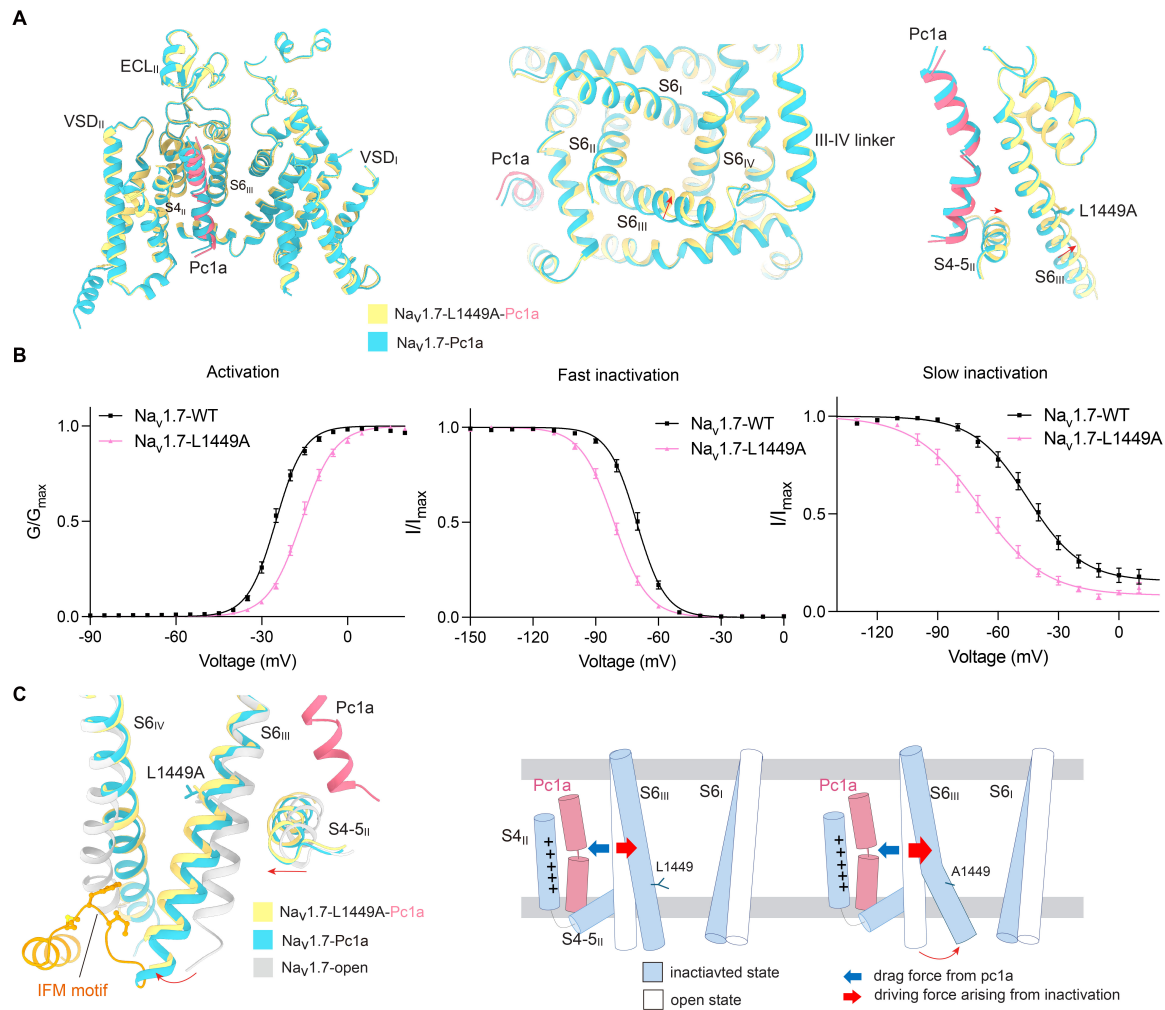


Fig. 4 | Structural basis for L1449A-mediated uncoupling of Pc1a modulation. A, Structural comparison of WT and L1449A Na_v1.7 bound to Pc1a. The Na_v1.7-L1449A–Pc1a structure (yellow; Pc1a, pink) is superimposed with the WT Na_v1.7–Pc1a structure (cyan). Side, intracellular and close-up views show that Pc1a retains an overall WT-like binding configuration in the L1449A mutant. Red arrows indicate local structural differences surrounding S4-5_{II} and S6_{III}. **B,** Intrinsic gating properties of WT Na_v1.7 and Na_v1.7-L1449A in the absence of Pc1a. Left, voltage-dependent activation; middle, steady-state fast inactivation; right, slow inactivation. L1449A shifts activation toward more depolarized potentials while shifting both fast and slow inactivation toward more hyperpolarized potentials, indicating an intrinsic redistribution of gating toward inactivation-prone states. **C,**

Structural interpretation and proposed mechanism for the effect of L1449A on Pc1a-dependent fast inactivation. Left, superposition of WT Na_v1.7-Pc1a (cyan), Na_v1.7-L1449A-Pc1a (yellow) and open-state Na_v1.7 (PDB: 21TP, grey), highlighting conformational differences involving S6_{III} and S4–5_{II}. The III-IV linker and IFM motif are shown in orange. Red arrows indicate the conformational changes associated with the transition toward the inactivated configuration. Right, schematic model illustrating the proposed balance of forces controlling S6_{III} movement. In WT Na_v1.7, Pc1a restrains the S6_{III} rearrangement associated with fast inactivation, thereby maintaining persistent current. L1449A intrinsically favors the inactivated configuration of S6_{III}, counteracting the restraining effect of Pc1a and allowing fast inactivation to proceed despite preserved Pc1a-dependent modulation of activation. Blue and red arrows indicate the restraining effect of Pc1a and the driving force toward inactivation, respectively.