Two-stage electro-mechanical coupling of a $K_V$ channel in voltage-dependent activation

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In voltage-gated potassium ($K_V$) channels, the voltage-sensing domain (VSD) undergoes sequential activation from the resting state to the intermediate state and activated state to trigger pore opening via electro-mechanical (E-M) coupling. However, the spatial and temporal details underlying E-M coupling remain elusive. Here, utilizing $K_V7.1$'s unique two open states, we report a two-stage E-M coupling mechanism in voltage-dependent gating of $K_V7.1$ as triggered by VSD activations to the intermediate and then activated state. When the S4 segment transitions to the intermediate state, the hand-like C-terminus of the VSD-pore linker (S4-SSL) interacts with the pore in the same subunit. When S4 then proceeds to the fully-activated state, the elbow-like hinge between S4 and S4-SSL engages with the pore of the neighboring subunit to activate conductance. This two-stage hand-and-elbow gating mechanism elucidates distinct tissue-specific modulations, pharmacology, and disease pathogenesis of $K_V7.1$, and likely applies to numerous domain-swapped $K_V$ channels.
Voltage gated K⁺ (KV) channels are homo-tetrameric proteins which sense changes in membrane potential and open the pore to conduct K⁺ ions through the membrane to regulate cellular excitability. KV channels contain the voltage-sensor domains (VSDs) formed by the transmembrane segments S1–S4 and the pore domain (PD) formed by the segments S5–S6.

The VSD activates in a stepwise manner from the resting to the intermediate state before finally arriving at the activated state, which triggers pore opening via electro-mechanical (E–M) coupling. The primary structural determinant underlying the E–M coupling process has classically been assigned to the linking helix between the VSD and the PD (S4-S5 linker or S4-S5L), with the physical movement of the VSD exerting an allosteric tug on key elements of the PD to induce pore opening. However, when and where the VSD activation couples to the pore opening still remains elusive. Here, we elucidate a two-stage E–M coupling mechanism in voltage-dependent gating of KV7.1 channels, triggered by the movement of VSD to the intermediate and then to the activated state. KV7.1, also known as KCNQ1, serves as a key player in regulating cardiac excitability. In the heart, KV7.1 associates with the auxiliary subunit KCNE1 to provide the slow delayed rectifier potassium current (I[subscript K1]), which is critical for terminating the cardiac action potential. The E–M coupling mechanism in voltage-dependent activation. Biophysical measurements and structure-based simulations show that each step of VSD activation evokes a distinctive set of E–M coupling interactions to the pore. When the S4 segment transitions to the intermediate state, the hand-like C-terminus of the S4-S5L interacts with the pore in the same subunit. When S4 then proceeds to the fully activated state, the elbow-like hinge between S4 and S4-S5L also engages with the pore to activate conductance. Because tissue-specific auxiliary subunits of Kv7.1 differentially modulate the IO and AO states to render either open state dominant in different tissues, our methods and results provide a foundation to understand physiological role, disease pathogenesis, and specific pharmacological modulation of Kv7.1 in distinct tissue contexts. The two-stage hand-and-elbow gating mechanism likely applies to numerous KV channels with the domain-swapped architecture.

**Results**

**Intermediate VSD activation engages classic E–M coupling.**

Previous studies suggested that interactions between the S4-S5L and the cytosolic end of S6 (S6c) are important for the E–M coupling. We tested this mechanism in the KV7.1 channel and found that it is also important for E–M coupling in KV7.1 channels (Fig. 1). To identify these E–M coupling interactions, we carried out voltage-clamp fluorometry (VCF) experiments, a technique in which a fluorophore is covalently attached to the S3-S4 linker of the pseudo-wild type KV7.1 channel (KV7.1-C214A/G219C/C331A, denoted as KV7.1*) to monitor VSD movements, while the ionic currents report pore opening. In KV7.1*, the fluorescence–voltage (F–V) relationship is well-fit by a double Boltzmann function (F[subscript I]–V and F[subscript O]–V), which corresponds to VSD activation from resting to intermediate (F[subscript I]–V) and intermediate to activated (F[subscript O]–V) states, respectively (Fig. 1a). The two steps VSD activation and two open states gating mechanism of KCNQ1 channels can be described by a simplified kinetic scheme without considering that KV7.1 channels are formed by four subunits with four identical VSDs.

We studied a LQT3-associated mutation V254M. The V254M mutant channel featured strong fluorescence signals associated with two-step VSD movement, with F–V curves exhibiting both F[subscript I]–V and F[subscript O]–V components (blue curves; Fig. 1c) comparable to pseudo-WT KV7.1* F–V curves (gray curves; Fig. 1c). However, the observed VSD movements no longer induce pore opening (Fig. 1c), suggesting that E–M coupling interactions in both IO and AO states are severely disrupted. The functional effects of V254M are illustrated in a cartoon in Fig. 1d. We undertook further site-directed mutagenesis in search for more residues with similar phenotypes as V254M. We identified four other mutations, including another LQT3 mutation A341V, that altered channel function similarly as V254M (Fig. 1e–g). These five mutants (V254M, H258W, A341V, P343A, and G345A) cluster near the C-terminal ends of S4-S5L and S6c in an arrangement consistent with the classic structural determinants for E–M coupling in other KV channels.
Some additional mutations in this region (E261K, L262A, I346A, and L347A) yielded no currents or fluorescence signal due to reduced channel expression (Fig. 1g), precluding functional analysis for E–M coupling. Critically, mutational disruption of the classic E–M coupling eliminates Kv7.1 opening at both the IO and AO states (Fig. 1c–h), suggesting that the classic E–M coupling interactions are already engaged upon VSD transition into the intermediate state and maintained at the activated state. Moreover, these interactions are necessary for pore opening at both IO and AO states.

**Fig. 1** Classic interactions are necessary for E–M coupling when the VSD transitions into the intermediate state. a VCF recordings of pseudo-WT Kv7.1⁺ (Kv7.1-C214A/G219C/C331A). Kv7.1+KCNE1 currents are shown at the same scale. The F–V relationships (blue circles) are fitted with a double Boltzmann function. F₁–V represents the VSD transition from resting to intermediate state; F₂–V represents the VSD transition from the intermediate to activated state. G–V represents channel opening with VSD transition at both the intermediate (IO) and the activated (AO) states. b Cartoon scheme illustrating the gating mechanism of two-step VSD movements and distinct two open states. c VCF recordings of V254M in Kv7.1⁺. V254M+KCNE1 currents are shown at the same scales. The F–V relationships of Kv7.1* and V254M are shown in gray and blue, respectively. d Cartoon scheme illustrating that V254M disrupts E–M couplings for both IO and AO. e Summary of data for WT and mutant Kv7.1. VSD activation (blue, percentage change in fluorescence) and pore opening (black, current amplitude) are normalized to the WT. n ≥ 3. Blank: cells not injected with channel mRNA. Data points are shown in small open circles. f V₅₀ values for the F₁–V and F₂–V. n ≥ 3. Data points are shown in small open circles. g Western blot results showing the membrane (top) and total (middle) expression of some mutants that eliminated both fluorescence and ionic currents. Gβt from total protein is shown as negative control. WT Kv7.1, H258W, and P343A are shown as positive controls. h Mapping the key residues V254, H258, A341, P343, and G345 (green) onto the S4-S5L/S6c interface in the Kv7.1 cryoEM structure (PDB: 5VMS)². All averaged data are shown in mean±SEM. Source data are provided as a Source Data file.
Fig. 2 LQTS mutation W248R specifically eliminates the E–M coupling when the VSDs adopt the activated conformation. a, b VCF recordings of W248R in Kv7.1. W248R+KCNE1 currents are shown at the same scale. The F–V relationships of W248R are shown in gray and blue, respectively. n ≥ 3. All averaged data are shown in mean±SEM. c Representative currents of W248R/E1R/R2E and W248R/E1R/R4E activated from −120 mV to 60 mV with 20 mV increments. Same scale for both currents. The inset shows western blot data for membrane expression of W248R/E1R/R4E. d VCF recordings of W248R/F351A. e Cartoon scheme illustrating that W248R specifically disrupts the AO–E coupling. f Mapping the key residues at the S4-SSL/S6c interface (green, V254, H258, A341, P343, and G345) in Fig. 1, and W248 and S338 (blue) onto the Kv7.1 cryoEM structure (PDB: 5VMS). Source data are provided as a Source Data file.

Then, are these classic interactions also sufficient for K\textsubscript{v}7.1 pore opening?

**Activated VSD activation engages AO specific E–M coupling.**

In studying another LQTS-associated mutation W248R\textsuperscript{33}, we found that the mutation specifically disrupted E–M coupling when the VSDs adopt the activated state, but left E–M coupling intact when the VSDs occupy the intermediate state, i.e., W248R selectively disables AO state E–M coupling, resulting in a channel that is conductive only in the IO state.

To determine whether a mutation specifically ablates the AO state, we utilized two extensively validated experimental strategies\textsuperscript{5,6,9,27,34,35}. The first tests the effect of the mutations when the VSDs are strongly biased to occupy the intermediate or in the activated states. To this end, the mutation is co-mutated on background intermediate VSD-locked (E160R/R231E, E1R/R2E) and activated VSD-locked (E160R/R237E, E1R/R4E) mutant channels\textsuperscript{5,6,9,27,34,35}. A mutation that specifically ablates the AO state should exhibit little ionic current when combined with the activated-VSD-locked (E1R/R4E) channel. On the other hand, when the same mutation is combined with the intermediate-VSD-locked (E1R/R2E) channel, robust IO state ionic current would be expected. The second strategy assays the effect of the mutation upon specific ablation of the IO state. This is achieved by two methods: KCNE1 co-expression and co-mutation with F351A. Both maneuvers suppress IO state conductance in Kv\textsubscript{7.1}\textsuperscript{5,8}. A mutation that specifically abolishes the AO state should exhibit no ionic current when co-expressed with KCNE1 or when co-mutated with F351A, as both IO and AO states are eliminated. If a mutation demonstrates consistent read-out across all four distinct tests (KCNE1 co-expression; co-mutation with E1R/R4E, E1R/R2E, and F351A), then the effect of the mutation will be attributed to selective disruption of the AO state.

W248R exhibited robust ionic current and VSD movement (Fig. 2a, b). When we applied the described four experiments to detect specific AO state disruption, a consistent picture emerges: W248R yields robust ionic current upon co-mutation with E1R/R2E (intermediate-VSD-locked), but co-mutation with E1R/R4E (activated-VSD-locked) results in little ionic current despite robust membrane expression (Fig. 2c). On the other hand, KCNE1 co-expression and F351A co-mutation (IO eliminated) with W248R strongly suppresses ionic current, despite robust fluorescence observed, indicating intact surface membrane expression and VSD activation (Fig. 2a, d). These results demonstrate that W248R specifically eliminates the AO state without eliminating VSD activation. VCF measurements indicate that the VSD of W248R still activates in two resolvable steps, with both F1–V and F2–V components (Fig. 2b), demonstrating that W248R does not affect the VSD transition to the activated state. These results are consistent with the mechanism that W248R specifically disrupts E–M coupling of the AO state, as illustrated in Fig. 2e.

The functional effects of W248R on Kv\textsubscript{7.1} gating are similar to another LQT5-associated mutation S338F\textsuperscript{5}. Structurally, W248 and S338 are located in the N-terminus of S4-SSL and the middle of S6, respectively, both of which are located generally outside of the region of the classical E–M coupling (Fig. 2f). Taken together, these results suggest that the classic E–M coupling interactions, while necessary, are not sufficient for pore opening when the VSD is at the activated state. Another set of E–M coupling interactions
are required for conduction when VSDs move from the intermediate to the activated state. Mutations such as S338F and W248R that disrupt these interactions result in selective loss of AO-current and are associated with arrhythmias. Encouraged by this finding, we set out to experimentally map this second set of E–M coupling interactions.

To identify residues involved in this second set of AO state E–M coupling interactions, we developed a pharmacological assay by utilizing a small molecule KV7.1 activator ML277, which increases KV7.1 current by specifically potentiating the AO state (Fig. 3a, b). This unique mechanism provides a straightforward assay: mutations that disrupt AO state E–M coupling (e.g. W248R) or ML277 binding (Supplementary Fig. 1) would result in loss of the ML277 potentiation of KV7.1 currents (Fig. 3b). Utilizing this strategy, we combined ML277 with scanning mutagenesis across the channel (Fig. 3c).

For sites of known disease mutations, it was the disease mutant forms that were examined, while for all other sites the mutations were examined. This strategy allowed us to systematically identify the residues involved in these second set of AO state E–M coupling interactions.
were to alanine or tryptophan. This strategy revealed 13 mutations, including W248R and S338F, that eliminated or reduced the ML277 potentiation effect (red bars; Fig. 3c). Of the thirteen residues, five are in the S4-S5L (W248, L250, L251, V255, and F256), four are in the S5 helix (Y267, I268, L271, and G272), and four are in the S6 helix (F335, S338, F339, and L342).

To confirm that these 13 mutations indeed selectively disrupted AO state E–M coupling, we performed the same functional assay afforded to W248R (Fig. 2) on the 11 mutations. For all these mutations, co-mutation with intermediate-VSD-locked (E1R/E2R) channels demonstrated robust current; while co-mutation with activated-VSD-locked (E1R/E4R) channels yielded only low ionic current (Fig. 3d, Supplementary Fig. 2). Suppressing the IO state with KCNE1 co-expression and F351A co-mutation also resulted in reduced current amplitude in all these mutants, although VCF experiments (S338F+KCNE1 and L251A+KCNE1) indicated membrane expression and VSD function (Fig. 3d, Supplementary Fig. 2). Altogether, these functional results provided a consistent read-out across four independent experiments, suggesting that these mutations indeed selectively disrupt the AO state. For all the mutant channels for which we were able to measure VCF signals, the results exhibit a two-step VSD activation with both F1–V and F2–V components (red and blue circles; Fig. 3e, Supplementary Fig. 2). Moreover, the voltage dependence of pore opening (G–V relation) tightly follows the F1–V relationship, consistent with the hypothesis that these mutant channels only conduct at the IO state (black and red circles; Fig. 3e). The VCF data indicate that these mutants do not prevent VSD transition into the activated states, suggesting that these residues are critical for the AO state E–M coupling when the VSD adopts the activated conformation.

Interestingly, we previously found four mutations (M238W, L239W, D242W, and R243W) at the C-terminal end of the S6 segment (S4c) that also showed robust currents, but the currents were severely suppressed by the co-expression of KCNE1. Further functional studies show that these S4c mutations produce similar phenotypes as the residues identified from the ML277 screen. S4c is also directly involved in E–M coupling for the AO state.

Mapping all these 17 residues onto the Kv7.1 cryo-EM structure reveals that they form two clusters in each subunit, which are spatially distinct from the classic E–M coupling interface (Figs. 1h, 3h). One cluster is located in S4c (cyan) and S4–SSL (blue), and the other is in S5 and S6 (blue; Fig. 3h). Within the VSD, key coupling residues at the S4c (cyan) are located adjacent to the hydrophobic plug or charge transfer center (CTC, orange; Fig. 3h). The CTC in the cryo-EM structure adopts the activated state with the fifth gating charge H240 (H5, cyan stick without surface) engaged in the CTC when the S4c adopts the activated conformation, suggesting that the movement of S4 to the activated state engages the S4c and S4–SSL into the CTC thereby initiating these AO-state specific E–M coupling interactions. The two clusters of sites are not in proximity within a single subunit, but instead form extensive contacts with clusters from the neighboring subunit, with the cluster in S4c and S4–SSL directly facing the cluster in S5 and S6 from an adjacent subunit (pink; Fig. 3h). This spatial arrangement maps network of residues stretching from S4 all the way to the pore, forming an E–M coupling pathway (Fig. 3h).

Interactions among these residues are exclusively for the AO state E–M coupling when the VSD adopts the activated conformation.

To experimentally confirm that the above E–M coupling residues indeed interact during Kv7.1 voltage-dependent activation, we utilized double mutant cycle (DMC) technique, a method extensively applied to residue interactions in proteins. DMC has previously been used to identify interacting residue pairs in ion channels, by measuring the net free energy change (ΔΔG) associated with activation that occurs when the interaction between two residues is disrupted. Specifically, DMC measures the energy changes of the WT channel (G0), two single mutants (G1 and G2), and the corresponding double mutant (G). The two residues are identified as interacting if the energy changes resulting from the two single mutants do not linearly sum to that observed in the double mutant (ΔΔG = |ΔG1 + ΔG2 − ΔG| > 1 kcal/mol). On the other hand, the two residues are classified as not interacting if |ΔΔG| < 1 kcal/mol.

We applied the DMC technique for the residue pair L251 (S4–SSL) and I268 (S5), which appear to be spatially close at the interface between two neighboring subunits. The activation energies of voltage-dependent activation of the WT (G0 = 1.7 kcal/mol), L251A (G1 = 0.1 kcal/mol), I268A (G2 = 1.2 kcal/mol), and L251A/I268A (G = +2.2 kcal/mol) channels were non-additive (Fig. 4a, b), with a net energy change ΔΔG = 1.8 kcal/mol (>1 kcal/mol). The result suggests a direct interaction between L251 and I268. Besides DMC, the gating kinetics also supports this interaction: I268A exhibits an obvious inactivation phenotype, suggesting a substantial change on the gating process. However, the double mutant L251A/I268A abolishes this inactivation phenotype from I268A, which indicates that the inactivation phenotype of I268A is not additive in L251A/I268A, and that L251A rescues the gating change seen for I268A (Fig. 4a).

DMC results also support the importance of S4–SSL/pore interactions (W248/I268 and L251/I339), and S4c/SSL interactions (M238/L271 and L239/L271) (Fig. 4c, d, Supplementary Fig. 4).

Two-stage E–M coupling mechanism. Our results so far indicate that Kv7.1 features two spatially distinct sets of E–M coupling...
interactions: (1) the classic set of interactions at the S4-SS5L/S6c interface (Fig. 1), which are engaged when the VSD moves to the intermediate state and maintained at the activated state, and (2) the set of interactions at the S4c/S5 and the S4-SS5L/pore interfaces (Figs. 2–4) which are specific to the VSD adopting the activated state. We next undertook molecular dynamic (MD) simulations to correlate VSD activation motion to these two sets of E–M coupling interactions. We performed MD simulation on four states of KV7.1 with different combinations of VSD-PD states: resting-closed (RC), activated-closed (AC), IO, and AO (see Methods, Supplementary Fig. 5). In order to quantify possible interactions between residue pairs in each model, we measured the frequency at which the distance between sidechains remain below the threshold values (see Methods) in all four subunits throughout the MD trajectories. Consistent with experiments, the pairs of possible interactions indicated in the MD trajectory include the residues found to be critical for E–M coupling (Figs. 1, 4, 5a–d). MD simulations found that the interactions among classic E–M coupling residues are maintained in all states (Fig. 5a–b, Supplementary Fig. 6), while interactions among the AO-state E–M coupling residues fall into three groups of state-dependent interactions. The first group includes interactions only present in the AO state model, where they may specifically stabilize AO state E–M coupling (Fig. 5c–d; top). The second group includes interactions only absent in the AO model, but present in other models (Fig. 5c–d; bottom). These interactions may need to be broken to enable AO state E–M coupling. The third group includes interaction L251/I268 which is present in both IO and AO states (Fig. 4a, b and Fig. 5c, d). Taken together, these MD simulation data are consistent with the experimentally identified interactions required for KV7.1 pore conduction. They indicate that the classic interactions are important in the E–M coupling when VSD is at both the intermediate and activated states, while the AO state E–M coupling interactions are engaged upon VSD transition into the activated state.

In sum, our findings in this study reveal a two-stage E–M coupling process and lead to a hand-and-elbow gating mechanism uncovering when and where E–M coupling interactions engage during voltage-dependent activation of KV7.1 channels (Fig. 5e). In this model, the S4 helix and S4-SS5L resemble a bent arm. The S4 (upper arm) moves in two resolvable steps, first to the intermediate state and then to the activated state. The sequential movements engage two stages of E–M coupling interactions through distinct mechanisms. (1) The motion of the S4 (upper arm) promotes channel opening through the S4-SS5L (hand) grip of the S6c of the same subunit at the first conductive IO state. (2) Subsequently, VSD transitions to the fully activated state, which engages the S4/S4-SS5L joint (elbow) in direct interactions with the S5 and S6 of a neighboring subunit to nudge the pore to adopt the second conductive AO state.

To further test if the two-stage hand-and-elbow E–M coupling mechanism is broadly conserved among KV channels, we performed a statistical coupling analysis (SCA) 44,45. SCA analyzes covariance in channel protein sequences and identifies groups of co-evolving residues termed protein sectors that correspond to networks of amino acid interactions critical for channel function 46 (Fig. 6). Since KV7.1 channel adopts the domain-
swapped architecture, we applied SCA to 1,421 domain-swapped Kv sequences (Fig. 6a–b) and identified two protein sectors utilizing SCA methodologies (Fig. 6b–c, see Methods, Supplementary Fig. 7). Sector 1 maps to two physically disconnected inward-facing clusters within the VSD and the PD (red; Fig. 6d). Notably, sector 1 demonstrates a lack of residues within the S4-S5L and a paucity of VSD-PD interactions (Fig. 6d). We interpret sector 1 as mainly including residues that are critical for maintaining the independent stabilities and functions of the VSD and PD domains. Sector 2 maps a network of residues that flows from the VSD to the PD through the S4-S5L reminiscent of classic E–M coupling interactions (blue; Fig. 6e). Moreover, when the same sector is mapped onto the neighboring Kv7.1 subunit, numerous inter-subunit interactions between S4, S4-S5L, and the neighboring S5 and S6 helices can be observed (blue and pink; Fig. 6e). As mentioned earlier, previous studies in Shaker K⁺ channels identified several pairs of residues that are involved in inter-subunit interactions and contribute to E–M coupling. Our experimental and SCA results indicate that the inter-subunit interactions are part of a chain of interactions including residues in S4c, S4-S5L, S5 and S6, and these interactions are specifically important for the AO state E–M coupling. We thus interpret sector 2 as an E–M coupling sector that contains residues involved in both stages of Kv E–M coupling. The SCA results suggest that the Kv7.1 E–M coupling interactions in this study may be functionally intact in all domain-swapped Kv channels, and the two-stage E–M coupling process may be broadly conserved.
Discussion

Elucidating the fundamental mechanism of voltage-dependent gating of Kv channels remains an important goal of basic biomedical research. After decades of structural and functional studies, processes such as VSD activation are now relatively well-understood. However, E–M coupling remains poorly understood, especially in regard to how each of the individual VSD transitions contributes to opening the pore. This is because E–M coupling is dictated by the energetics and dynamics of protein-protein interactions, which cannot be determined directly with structural studies and lack of exclusive approaches to investigate with functional studies. In this study, extensive mutagenesis, pharmacology, voltage clamp fluorometry experiments, and MD simulations provide the two-stage hand-and-elbow mechanism for how the stepwise VSD activation is coupled to the pore and its two distinct conductive states in Kv7.1 channels (Figs. 1–5). In this mechanism, the classic E–M coupling interactions at S4-SSL/Sc6 promotes channel opening upon VSD movement into the intermediate state, while another set of VSD/pore and S4-SSL/pore interactions engage only upon transition to the fully activated state. This work relied on unique alternative open states (IO and AO) in Kv7.1, which present unambiguous current readouts when the VSDs adopt the intermediate vs. activated state conformations. This unique trait also enables functional detection of the E–M coupling interactions explicitly associated with the intermediate and activated VSD states, a feat difficult to achieve in other channels, which only conduct when the VSD occupies the activated state (e.g. Shaker channels)38,39.

SCA performed in this study suggests that the hand-and-elbow gating model may extend to other domain-swapped Kv channels.
This result is consistent with recent discoveries in Shaker K⁺ channels that some non-canonical, inter-subunit interactions besides the classic E–M interactions also contribute to E–M coupling. The residues important for the non-canonical interactions identified in these recent studies are among the network of residues revealed in our SCA as being important for E–M coupling (Fig. 6). Although we defined sector 2 (Fig. 6e) in our SCA as an E–M coupling sector, we cannot exclude the possibility that sector 2 also includes residues important for VSD activation or gate opening. However, sector 2’s spatial pattern includes abundant residues within the S4-S5 linker which strongly suggests sector 2 contains significant number of E–M coupling residues. SCA was applied to the Kv7.1 channel superfamily in prior studies; however, the input sequence alignment consisted of both domain-swapped and non-domain-swapped Kv7 channels. These two architecturally-distinct ion channel super families likely feature distinct E–M coupling mechanisms. For example, the VSD of non-domain-swapped channels do not form inter-subunit contact with the PD of their neighboring subunit. The inter-subunit VSD-Pore E–M coupling mechanism discovered in this study is thus incompatible with the non-domain-swapped Kv7 architecture. In this light, including both channel architecture types in a single SCA may confound results for protein sector related to E–M coupling.

Kv7.1 and KCNE1 complexes form the IK, channels important in controlling cardiac action potential duration. More than 300 mutations of Kv7.1 are associated with LQTS, but so far only for a portion of these mutations the mechanism of altering channel function is understood. Our studies of the LQTS mutations that disrupt E–M coupling led to the discovery of two distinct sets of interactions that mediate E–M couplings in Kv7.1 channels (Figs. 1–3). Among these mutations, two (V254M, A341V) disrupt the classic E–M interactions, resulting in the total loss of IKᵢ due to the elimination of both IO and AO states (Fig. 1); while the other three (W248R, S338F, and F339S) disrupt the E–M interactions specific for the AO state, which also result in the total loss of IKᵢ (Figs. 2, 3) as the IKᵢ channel only conducts AO-state current.

Tissue-specific auxiliary KCNE subunits differentially modulate the Kv7.1 IO and AO states. In the heart, KCNE1 modulates Kv7.1 to only conduct at the AO state. Drugs such as ML277 that specifically enhance the AO state are candidates for specific anti-arrhythmic therapy. Our docking results show that ML277 binds at the S4-S5/porre interface where the residues interacting with ML277 are critical for the AO state E–M coupling (Fig. 3, Supplementary Fig. 1), serving as an excellent target for developing drugs with AO state specificity. On the other hand, patients who harbor one or more of LQTS-associated mutations that specifically disrupt AO state E–M coupling (Fig. 3d) are likely refractory to treatment with drugs that affect Kv7.1 similarly as ML277 (Figs. 2, 3). The two-stage hand-and-elbow model thus furnishes a framework to conceptualize Kv7.1 auxiliary subunit regulation, tissue-specific physiology, disease pathogenesis, and state-dependent pharmacological modulation.

Methods

Constructs and mutagenesis. Point mutations of the Kv7.1 channel were engineered using overlap extension and high-fidelity PCR. Each mutation was verified by DNA sequencing. The cRNAs of mutants were synthesized using the mMessage T7 polymerase kit (Applied Biosystems-Thermo Fisher Scientific). All primer sequences used in this study can be found in Supplementary table 1.

Oocyte expression. Stage V or VI oocytes were obtained from Xenopus laevis by laparotomy. All procedures were performed in accordance with the protocol approved by the Washington University Animal Studies Committee (Protocol # 20190030). Oocytes were digested by collagenase (0.5 mg/ml Sigma Aldrich, St Louis, MO) and injected with channel cRNAs (Drummond Nanoject, Broomall).

Each oocyte was injected with cRNAs (9.2 ng) of WT or mutant Kv7.1, with or without KCNE cRNAs (2.3 ng). The amount of injected cRNAs was doubled for the mutants to achieve the same peak surface expression. All oocytes were incubated in ND96 solution (in mM): 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, 2.5 CH₃COOC₅Na, 1:100 Pen-Strep, pH 7.6) for 18°C for at least 2 days before recording.

Western blot. Western blot experiments follow standard protocol. An antibody for KCNQ1 (conjugated with horseradish peroxidase HRP); KCNQ1 (G-8) mouse monoclonal IgG; Vendor: Santa Cruz Cat. No.: sc-365366; primary antibody for GJ: GJ (T-20) rabbit polyclonal IgG; Vendor: Santa Cruz Cat. No.: sc-378; secondary antibody for GJ: Goat anti-Rabbit IgG (H+L); Vendor: Thermo. Cat. No.: A16110. Source data of western blot are provided in Source Data file.

Electrophysiology experiments. Microelectrodes (Sutter Instrument, item #: B150-117-10) were made with a puller (Sutter Instrument, P-97), and the resistances were 0.5–3 MΩ when filled with 3 M KCl solution. Ionic currents were recorded by two-electrode voltage clamp (TEVC) in ND96 bath solutions. Whole-oocyte currents were recorded using a CA-1B amplifier (Dagan, Minneapolis, MN) with Patchmaster (HEKA) software. The currents were sampled at 1 kHz and low-pass-filtered at 2 kHz. All recordings were carried out at room temperature (21–23°C). For voltage-clamp fluorometry (VCF) experiments, oocytes were incubated for 30 min on ice in 10 μM Alexa 488 C₃-naleimide (Molecular Probes, Eugene, OR) in high K⁺ solution in mM (98 KCl, 1.8 CaCl₂, 5 HEPES, pH 7.6) for labeling. Cells were washed three times with ND96 solution to remove the labeling solution, and recordings were performed in ND96 solution on the CA-1B amplifier self-calibrated. Excitation and emission lights were filtered by a 405-nm filter cube (Leica, Germany, for Alexa 488) and the fluorescence signals were collected by a Poin20a photodiode (OSI Optoelectronics). The signals were then amplified by an EPC10 (HEKA, analog filtered at 200 Hz, sampled at 1 kHz) patch clamp amplifier and controlled by the CA-1B amplifier to ensure fluorescence signals were recorded with high signal-to-noise ratio. Other chemicals were as described previously. Each electrophysiology experiment was performed on at least three individual cells to avoid possible outliers. These experiments are consistently reproducible as shown in the repeated recordings. The number of recordings of each experiment was based on the convention of the field.

Electrophysiology data analysis. Data were analyzed with IGOR (Wavemetrics, Lake Oswego, OR), Clampfit (Axon Instruments, Inc., Sunnyvale, CA), Sigmaplot (SPPS, Inc., San Jose, CA), and custom MATLAB (MathWorks, MA) software. The instantaneous tail currents following test pulses were normalized to the maximal current to calculate the conductance–voltage (G–V) relationship. Because of photobleaching, fluorescence signals were baseline subtracted by fitting and extrapolating the first 2 s signals at the ~80 mV holding potential. ΔF/F was calculated after baseline subtraction. Fluorescence–voltage (F–V) relationships were derived by normalizing the ΔF/F value at the end of each four-seconds test pulse to the maximal value. F–V and G–V curves were fitted with either one or the sum of two Boltzmann equations in the form G=U/(1+exp(−z′F(V–V½)/RT)) where z is the equivalent valence of the transition, V½ is the voltage at which the transition is half maximal, R is the absolute temperature, T is the Faraday constant, and V is the voltage. For double mutant cycle analysis, the activation energy was given by ΔG=−z′F(V½–F) is the Faraday constant (23.06 kcal/mol). Both z and V½ were obtained by fitting the G–V relation with the Boltzmann equation.

MD simulations conducted on Kv7.1 models. The AO, IO, RC, and AC molecular models of Kv7.1 channel used for MD simulations were initially built by homology modeling with MODELLER software, using the Kv1.2 channel refined crystal structure (PDB: 3ULT) as a template for the AO model. For IO model, the intermediate conformation obtained in previous unbiased MD simulations of Kv1.2 refined structure in hyperpolarizing conditions was used. For the RC and AC models, the conformation obtained in biased MD simulations of Kv1.2 channel in hyperpolarizing conditions was used as a template. The uncoupled Kv7.1 CryoEM structure was used as a template for the AC model. These models were embedded in a 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) bilayer and surrounded by two slabs of a 150 mM KCl solution. Considering the importance of phosphatidylinositol-4,5-bisphosphate (PIP₂) in Kv7.1 function, we incorporated a PIP₂ molecule at the bottom of each VSD, in the inner leaflet of the POPC bilayer for RC, IO and AO models, in accordance to previous MD simulations conducted on the Kv7.1 channel. As the absence of the VSD-pore coupling in the Kv7.1 CryoEM structure is assumed to be due to the absence of PIP₂ lipids, we did not add this lipid in the MD model, in order to not induce any conformational change within the membrane. The MD simulations were performed using the NAMD program along with the CHARMM force field. All simulations were conducted in the NPT ensemble, so we applied Langevin dynamics to keep the temperature (300 K) and the pressure (1 atn) constant. The time scale of the internal energy of our systems was monitored using the equations of motion on the atoms’ cartesian coordinates. These equations were integrated with a time-step of 2 fs, using a multiple time-step algorithm in which

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short and long-range forces were calculated every 1 and 2 time-steps, respectively. Long-range electrostatics were calculated using Particle Mesh Ewald. The cutoff distance to the membrane ions was 11 Å. Der Waals interactions were computed using a distance of 8 Å. Der Waals interactions were then to let the lipid molecules reorient themselves around the protein, and then to let the protein sidechains relax within the membrane. In the last step of our MD modelling of the RC model throughout its step of the production phase trajectory was used for further analyses. The analyses of resulting MD trajectories were conducted in two parts. The first part aimed at validating our structural models with respect to experimental restraints. Before investigating the molecular determinants of E–M coupling in the MD trajectories, all the relaxed MD models were validated rigorously against experimental data. In addition to the structural validation of the KV7.1 model, the average pore radii in KV7.1 models, and both AC and RC models present a large difference between their selectivity filter and the inner pore, located below the selectivity filter can be useful. Indeed, several studies shed light on the relation between the radius of a nanopore and the free energy associated to the translocation of an ion. The results we obtained (Supplementary Fig. 5c) present two major regions that correspond to top co-varying residue positions (Supplementary Fig. 7c). The input multiple sequence alignment (MSA) was derived from P00520 (ion_trans Family) MSA in the Pfam protein families database63. The full P00520 MSA contains 58,529 ion transport protein sequences aligned from S1 through S6. All sequences in the P00520 MSA were annotated using PFAM annotation based on the codes provided by the pySCA package64. The annotations were used to filter and select sequences for SCA. Exclusion keywords annotation were voltage-gated or potassium to select for all Kv and potassium channels. Exclusion keywords were unknown, uncharacterized, and the number of the correct input for SCA. The two architectures of KV channels likely participate in distinct E–M coupling mechanisms. Our SCA analysis aims to extract potential E–M coupling networks specifically within the domain-swapped Kv channels. The preliminary input MSA filtered from P00520 underwent further processing by pySCA with default parameters65 and then the final MSA set to the human Kv7.1 sequence. To brieferly reiterate the default parameters: gapped positions were truncated with a gap fraction cutoff of 0.2 based on position and sequence. Sequences with <0.2 fractional identity to human Kv7.1 were removed from analysis. Sequence weights were computed with max fractional identity of 0.8 to the reference sequence. The final processed MSA was truncated based on the available residues in a human Kv7.1 homology model of the Xenopus Kv7.1 crioEM structure2, resulting in a final MSA size of 1,421 sequences at 200 positions (Fig. 6a, Supplementary Fig. 7a). This final MSA yielded first order conservation consistent with the topology of Kc channels (Supplementary Fig. 7b). First order conservation was calculated at a domain-wide level and second order conservation was calculated with Kullback-Leibler divergence which describes deviation of the observed amino acid frequency within the MSA against the pre-defined natural background amino acid frequency. Protein sectors were defined based on the ICS-based submatrix derived from the SCA matrix (Fig. 6b) utilizing pySCA66 and the resulting matrix was truncated based on the human Kv7.1 sequence to the 200 positions. In this way, we selected the top eigenvectors corresponding to top co-varying residue positions (Supplementary Fig. 7c). Significant top eigenvectors from the SCA covariance matrix was determined by a threshold of 0.8 for the correlation coefficient. In this case, the correlation coefficient of the five eigenvectors to five ICs such that each IC contains residue positions which most strongly co-vary among themselves and most weakly co-vary with residue positions

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in other ICs. Key residues in each independent component (IC) were determined by top five percent of the cumulative density function of each IC after independent component analysis (default for pySCA). The five identified ICs are shown in the IC submatrix and mapped onto the KCNQ1 cryo-EM structure (Fig. 6b, Supplementary Fig. 7d), corresponding to top five sets of co-evolving amino acid residue positions in the domain-swapped Kᵥf family. In the IC submatrix, on-diagonal boxes report the strength of internal correlations for residues within an IC, while the off-diagonal boxes show the strength of external correlations for residues between two ICs. Strong external correlations between ICs indicate that these ICs can be grouped into a single protein sector. As an example in our analysis, IC3 featured strong external correlation with IC2, but weak external correlation with IC1, IC4 or IC5 (Fig. 6b, row 3), indicating that IC2 and IC3 together form one protein sector. The five detected ICs can be roughly grouped into two sectors: (1) IC2+IC3 and (2) IC1+IC4+IC5 (Fig. 6b). We accordingly defined two protein sectors within the domain-swapped Kᵥf family by grouping the two ICs (Fig. 6b–e).

**Homology models of Kᵥ7.1 and molecular docking.** There are two templates available for modeling human Kᵥ7.1 (Kᵥ7.1): the cryo-EM structure of frog Kᵥ7.1 (PDB entry: 5vms) and the crystal structure of rat Kᵥ1.2-Kᵥ2.1 chimera (PDB entry: 2r9y). These two template structures have significantly different conformations, particularly in the regions near the S4-SSL and the VSD-pore interface. Therefore, both templates were used, and (two) Kᵥ7.1 structures (5vms_model and 2r9y_model) were built using the program MODELLER. Both homology models of Kᵥ7.1 were used in the following molecular docking studies.

The compound ML277 was docked to each Kᵥ7.1 model structure using our previously developed method. Briefly, a modified version of AutoDock Vina was employed to sample possible binding modes of ML277 on hKᵥ7.1. In this modified version, the maximum number of output modes is user-specified, and was set to 500 in the present study. The exhaustiveness value was increased to 30 to ensure exhaustive sampling. The protein was treated as a rigid body, and the single bond in ML277 were considered as rotatable. The docking site was focused on the pocket near the S4-SSL and the VSD-pore interface based on the experimental data. The size of the cubic search box was set to 30 Å, which was sufficient to cover the whole binding pocket. Up to 500 putative, flexible binding modes were generated and then re-ranked with our in-house scoring function, ITCScore.

The predictions based on the 2r9y_model (i.e., the homology model of hKᵥ7.1 that was constructed using 2r9y as the template) achieved significantly better binding scores than the 5vms_model (i.e., the homology model of hKᵥ7.1 based on 5vms). Therefore, we focused on the 2r9y_model; the predicted binding mode is plotted in Supplementary Fig. 1, and is consistent with the mutagenesis data. Interacting residues are represented by both the stick model and the surface representation.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Data supporting the findings of this manuscript are available from the corresponding author upon reasonable request. A reporting summary for this Article is available as a Supplementary Information file. The source data for Figs. 1a,c,e, 2a–d, 3b–g, 4a, 5a–c, Figs. 2, 3, MD trajectories, and code for SCA are provided in the Source Data file.

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