Exploring structure-function relationships between TRP and Kv channels

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The molecular mechanisms underlying the activation of Transient Receptor Potential (TRP) ion channels remain poorly understood. In contrast, the voltage-activated potassium (Kv) channels display a well-characterized mechanism, with many of their structural and functional similarities being well understood. In this study, the authors explore the hypothesis that the structure-function relationships of TRP and Kv channels may have common features. They achieve this by replacing previously identified domains and critical structural motifs of the membrane-spanning portions of Kv2.1 with corresponding regions of two TRP channels, TRPM8 and TRPV1. Their results show that the S3b-S4 paddle motif of Kv2.1, but not other domains, can be replaced by the analogous regions of both TRP channels without abolishing voltage-activation. In contrast, replacement of portions of TRP channels with those of Kv2.1 consistently yielded non-functional channels. Taken together, these results suggest that most structural elements within TRP channels and Kv channels are not sufficiently related to allow for the creation of hybrid channels.

Transient Receptor Potential (TRP) channels have been the intense focus of research since the first member was cloned in 1989. Despite these efforts, the structural and mechanistic basis of TRP channel function remains poorly understood, in part because we currently have limited high-resolution structural information on these channels. In addition, TRP channels are modulated by a vast array of ligands possessing disparate physical and chemical characteristics, making it difficult to localize their binding sites and establish their mechanisms of activation. For example, TRPV1 is activated by stimuli as diverse as voltage, heat, protons, vanilloid compounds such as capsaicin and resiniferatoxin (RTX), and peptide toxins such as the double-knot toxin (DkTx) and vanillotoxins. Another thermosensitive TRP channel, TRPM8, is activated by voltage, cold, and the small organic compounds, menthol and icilin. Such a vast array of stimuli remains fascinating and poorly understood. Mutagenesis- and chimera-based approaches have identified regions of these channels that play critical roles in channel activation. For example, these approaches have been used to identify residues in TRPV1 that are critical for its activation by ligands such as capsaicin, RTX, DkTx, temperature, and pH. Similar studies on TRPM8 have identified channel residues that are important for its activation by voltage and its chemical agonists, icilin and menthol. Although this information is extremely valuable, it remains a challenge to discern whether the residues identified are directly involved in ligand binding or whether they influence an allosteric transition involved in channel gating. This task is especially non-trivial in the context of TRP channels because the gating elements in these channels remain largely unidentified.

In attempting to understand the principles underlying the activation of TRP channels, we sought to draw on our knowledge of voltage-activated potassium (Kv) channels, a family of ion channels that have been subject to extensive biophysical and structural investigation. Several lines of evidence suggest that Kv channels and TRP channels may exhibit structural and functional similarities. First, members of both these channel families possess tetrameric architectures where each monomer consists of six transmembrane segments (S1–S6) forming the pore (Fig. 1). Second, both TRP and Kv channels display pharmacological similarities, such as modulation by isostuctural cystine knot peptide toxins—Kv channels are inhibited by voltage-sensor-binding toxins and TRPV1 is activated by double-knot toxin (DkTx) and vanillotoxins. Indeed, individual vanillotoxins have been reported to cross-react with TRPV1 and Kv2.1. Another TRP channel, TRPA1, is activated by the tarantula toxin, GsMtx-4. An additional pharmacological similarity between TRP and Kv channels is that members of both families are inhibited by internal quaternary ammonium ions. A third line of evidence supporting similarities between TRP channels and Kv channels is that residues important for ligand-modulation of these channels map to similar regions. For example, residues in the S3–S4 region of TRPV1 (Fig. 2, green residues) play important roles in its activation by capsaicin and RTX, and several S3 and S4 residues of TRPM8 are important for menthol and icilin-sensitivity of the channel (Fig. 2, orange and blue residues,
Results

We decided to focus our efforts on the Kv2.1 channel and on two TRP channels, TRPV1 and TRPM8. We chose Kv2.1 because the gating properties of this Kv channel can be modulated by an array of peptide toxins that interact with the S1–S4 voltage-sensing domain\(^2\), and because earlier studies have successfully used this channel to generate chimeras with other voltage-activated cation channels and voltage-sensitive proteins\(^{17,19,44}\). Our choice of TRPV1 was motivated by the availability of a large number of pharmacological tools targeting this channel, including vanilloid compounds and DKTX\(^{6,11,12}\). TRPM8 was an obvious choice for our studies because an earlier report suggested similar voltage-sensing mechanisms in this channel and Kv channels\(^{38}\).

Fig. 2 shows the primary sequence alignment used to generate chimeras, covering the S1–S6 transmembrane segments of TRPV1, TRPM8 and Kv2.1, along with a variety of other tetrameric cation channels and voltage-sensitive proteins, including those for which X-ray structures are available\(^{20,28,34,44}\). Fig. 3 summarizes all 50 chimeras that we generated, and provides the specific boundaries for regions within the S1–S6 segments that were transferred between Kv2.1 and either TRPV1 or TRPM8. Unless otherwise stated, channel constructs were investigated by injecting cRNA into oocytes and performing two-electrode voltage clamp recordings to investigate their functional properties.

**S3–S4 chimeras between TRPM8 and Kv2.1.** The S3b–S4 paddle motif in Kv channels is extremely tolerant to protein engineering because it is relatively structurally unconstrained, making few contacts with other parts of the protein\(^{20,28,3,34,44}\). Indeed, in earlier work, the paddle region of Kv2.1 was replaced by the paddle regions of other voltage-gated channels such as the prokaryotic Kv channel, KvAP, Nav channels, the voltage-activated proton channel Hv1, and the voltage-sensitive phosphatase Ci-VSP, without destruction of voltage-activation and with concomitant transfer of pharmacology\(^{14,44}\). Due to its functional and pharmacological importance, and its tolerance to replacement, we first focused on making chimeras by replacing the paddle region of Kv2.1 with the corresponding regions of TRPV1 and TRPM8.

We generated twelve chimeras in which different portions of the Kv2.1 paddle were replaced with portions of the putative S3–S4 region of TRPM8 (Fig. 3d; 1–12M8Kv). Seven of these chimeras gave rise to functional channels (Fig. 3d; green dots) that were activated by membrane depolarization and that were sensitive to the selective Kv channel blocker, agitoxin\(^2\). The voltage-activated currents observed for these chimeras exhibited a reversal potential of \(~2~mV\), consistent with the expected value for \(K^+\)–selective channels for the recording solution we used. All of these functional chimeras involved replacing regions within and immediately N-terminal to the paddle region (2M8Kv, 3M8Kv, 5M8Kv, 7M8Kv, 8M8Kv, 11M8Kv, and 12M8Kv), whereas those that failed to form functional channels (1M8Kv, 4M8Kv, 6M8Kv, 9M8Kv, and 10M8Kv) involved the transfer of regions extending beyond previously defined boundaries of the paddle motif\(^2\). Two of the largest functional paddle chimeras were 8M8Kv, a construct in which 31 residues of the paddle were replaced by 34 residues of TRPM8, and 3M8Kv, a construct in which 37 residues of the paddle region of TRPM8 (Fig. 4a). The energetics of gating were also perturbed in these chimeras; whereas 8M8Kv involved the transfer of regions extending beyond previously defined boundaries of the paddle motif\(^2\).
Kv2.1, 3M8Kv required stronger depolarizations to elicit voltage-activated currents (Fig. 4e; Table 1). The large rightward shift of the G–V relationship of 3M8Kv precludes utilization of agitoxin2 to subtract background currents because the toxin unbinds at the higher voltages required to activate this chimera. Interestingly, this chimera remains constitutively open and cannot be closed entirely by membrane hyperpolarization, giving rise to a steady holding current (Fig. 4d and f) and non-zero conductance values at negative voltages (G–V plot in Fig. 4e). To verify that this holding current arises from the chimera, we applied agitoxin2 and observed that the holding current was reduced to negligible values (not shown).

In addition to playing important roles in sensing voltage, the S4 helix of TRPM8 is thought to be important for menthol sensitivity16, raising the possibility that the transferred region of TRPM8 may confer ligand sensitivity to the chimeras. We therefore examined the sensitivity of the functional chimeras to menthol and in each case external application of the TRPM8 agonist was without effect. Voltage-activated currents before and after menthol treatment for our largest paddle chimera, 3M8Kv, are depicted in Fig. 4f.

If the S4 helix of TRPM8 serves as the voltage sensor of TRPM8, we might expect a TRPM8 variant containing a larger number of positively charged residues to display steeper voltage-dependent gating.
Figure 3 | Chimeras generated and characterized in this study. (a) Alignment of S1–S4 regions of TRPV1, TRPM8 and Kv2.1 used for generating most chimeras. (b) An alternate alignment of the S3–S4 regions of TRPV1 and Kv2.1. (c) Alignment of the S5–S6 regions for TRPV1, TRPM8 and Kv2.1. The numbers below specified residues in the alignment of the channels denote the start or end sites of the swapped regions. (d) Summary of chimeras generated in this study. Red dots indicate non-functional chimeras whereas green dots indicate functional chimeras. The chimeras are named using the code: (a, b) XC1C2, where numbers ‘a’ and ‘b’ correspond to the N-terminal residue and the C-terminal residue respectively of the transferred segment, ‘X’ is the serial number, ‘C1’ is the abbreviation for the donor protein and ‘C2’ is the abbreviation for the acceptor protein. Abbreviations for the proteins are: M8 for TRPM8, V1 for TRPV1 and Kv for Kv2.1.
To explore this idea, we replaced portions of the S4 helix of TRPM8 (which has 2 Arg and 1 His) with those of the S4 helix of Kv2.1 (which has 6 positively charged residues). The chimera replacing the largest portion of the S4 helix in TRPM8 added one Arg residue, one Lys residue, and replaced a His with an Arg residue. However, all chimeras involving replacement of S3–S4 regions of TRPM8 with segments of the Kv2.1 paddle failed to give rise to either voltage- or menthol-activated currents (chimeras 1-4KvM8; Fig. 3).

S3–S4 chimeras between TRPV1 and Kv2.1. The sequence similarity between TRPV1 and Kv2.1 in the S3–S4 region is lower than that between TRPM8 and Kv2.1 (22% compared to 31%). Consequently, there are several alignments with similar homology that can be constructed between Kv2.1 and TRPV1. We therefore explored two alignments in the S3–S4 region to design chimeras between TRPV1 and Kv2.1 (Fig. 3a,b; 5a). In the first, the loop between the S3 and S4 helices of TRPV1 is longer than in the alternate alignment. Chimeras generated using both alignments gave rise to functional channels (2V1Kv, 5V1Kv, and 6V1Kv), with the exception of 1V1Kv. In contrast, when the same portion of the Kv2.1 paddle was replaced by the S3–S4 region of TRPV1 using the alternate alignment to generate chimera 5V1Kv, a functional Kv...
channel was obtained (Fig. 5d and e), suggesting that the transferred region in this case was more compatible with the structure of Kv2.1 than for 1V1Kv. Similar to what was observed for the chimeras between Kv2.1 and TRPM8 discussed above, all functional chimeras were sensitive to agitoxin2, and had a reversal potential of ~20 mV.

Several of the functional paddle chimeras exhibited constitutive activity and could not be fully closed with membrane hyperpolarization even though they retained some voltage-sensitivity (for example, 2V1Kv and 6V1Kv; Fig. 5b, c, and e), resembling the 3M8Kv chimera discussed earlier. All these chimeras exhibited altered G–V relations, with slopes much lower than observed for Kv2.1 (Fig. 5e; Table 1). In the case of the 6V1Kv chimera, the G–V relation is so shallow and complex that it cannot be well-defined with a single Boltzmann function (Fig. 5e).

Residues in the S3–S4 region of TRPV1 shown in green in Fig. 5a have been demonstrated to be important for activation of the channel by capsaicin and RTX10. If these residues contribute to forming the receptor for these ligands, transferring the S3–S4 region of TRPV1 into Kv2.1 might render the chimeras sensitive to capsaicin. However, the largest of these chimeras (5V1Kv) was not sensitive to high concentrations of capsaicin even though it gave rise to robust voltage-activated currents (Fig. 5f). Similar to what we observed with TRPM8 chimeras, all reverse chimeras where portions of the S3–S4 region of TRPV1 were replaced by those of the Kv2.1 paddle failed to give rise to either voltage- or capsaicin-activated currents (1–4Kv1, Fig. 3).

Previous studies suggest that capsaicin binds to the internal regions between the S2 and S3 helices of TRPV1. If this idea is correct, the lack of capsaicin sensitivity of the S3–S4 chimeras 2V1K, 5V1Kv, and 6V1Kv is not surprising as they do not contain any portion of the S2–S3 linker region of TRPV1. In an effort to render the Kv channel sensitive to capsaicin, we swapped the internal regions of the S2 and S3 helices in Kv2.1 with those of TRPV1 to generate the 8V1Kv chimera (Fig. 3). This chimera did not give rise to either voltage- or capsaicin-activated currents, suggesting that it is non-functional.

**S1–S4 chimeras.** The S1–S4 domain of Kv channels can be transferred to channels that are not voltage-activated, endowing them with voltage-sensitivity41–43, demonstrating that the voltage sensor is an independent modular domain. Moreover, other voltage sensing proteins have been discovered that contain an S1–S4 domain without a separate pore domain, such as Gi-VSP44, and Hv145–49. Taken together, these observations suggest that nature utilizes the S1–S4 domain as a general scaffold to sense voltage. To test whether the S1–S4 regions of TRP channels have similar modular characteristics, we swapped the S1–S4 of TRPV1 and TRPM8 with that of Kv2.1, and also generated the reverse chimeras. All these chimeras (13M8Kv, 14M8Kv, 9V1Kv, 10V1Kv, 5KvM8, 6KvM8, 5KvV1 and 6KvV1 depicted in Fig. 3) did not give rise to voltage- or ligand-activated currents and were judged to be non-functional. We reasoned that these chimeras may have disrupted critical interactions between the S1 helix and pore helices of Kv2.1, resulting in a loss of channel function. To address this possibility, we created several S2–S4 chimeras, all of which were also non-functional (chimeras 15M8Kv, 11V1Kv, 7KvM8, and 7KvV1).

**S5–S6 pore chimeras.** In addition to serving as the ion permeation pathway, the pore region of TRP channels plays critical roles in channel gating and pharmacology. For example, the outer pore domains of TRPV1 and TRPV3 have been implicated in temperature sensing, and DkTx and the vanillotoxins are believed to activate TRPV1 by binding to its pore region10,22,23. Motivated by the putative functional importance of the pore domain in TRP channel function, we replaced the S5–S6 pore region of Kv2.1 with the pore regions of TRPV1 and TRPM8 (chimeras 16–18M8Kv and 12–14V1Kv; Fig. 3). We also generated the reverse chimeras (8–9KvM8 and 8–9KvV1; Fig. 3) where the pore regions of TRPV1 and TRPM8 were replaced by the Kv2.1 pore domain. However, none of these chimeras gave rise to voltage-activated currents, even for 17M8Kv, 18M8Kv, 13V1Kv and 14V1Kv, where the boundaries of the transferred region should not disrupt critical interactions between the S4–S5 linker and S6 helix defined for Kv channel47,42. We also investigated the DkTx-sensitivity of the three chimeras where the pore domain of Kv2.1 was replaced by that of TRPV1 (12–14V1Kv), but in each instance we could not observe measurable currents in response to application of 1 μM DkTx to the external recording solution, even when testing over a wide range of membrane voltages. Because heterologous expression of TRPV1 is more efficient in mammalian cells compared to oocytes, we transfected HEK-293 cells with a few of the chimeras (4Kv1, 5KvV1, 8KvV1, and 12V1Kv) and used whole-cell patch clamp recordings to look for evidence of functional channels. In these experiments, none of the chimeras gave rise to voltage-, capsaicin- or DkTx-activated currents that were distinguishable from non-transfected cells, confirming that they are non-functional.

### Table 1 | Voltage-activation relationships for Kv2.1 and chimeras with TRPV1 and TRPM8

| Channel     | z      | V1/2 (mV) |
|-------------|--------|-----------|
| Kv2.1       | 3.2 ± 0.3 | -4.9 ± 0.9 |
| 3M8Kv       | 1.5 ± 0.1 | 54.2 ± 1.5 |
| 8M8Kv       | 1.7 ± 0.1 | -23.4 ± 1.1 |
| 2V1Kv       | 0.9 ± 0.1 | 79.9 ± 0.7 |
| 5V1Kv       | 1.3 ± 0.1 | 70.3 ± 0.9 |

A single Boltzmann function was fit to G–V relations to obtain z and V1/2 values. n = 3 in all cases.

### Discussion

The primary objective of the present study was to establish structural relationships between TRP channels, for which little structural information is available, and Kv channels, for which a variety of X-ray structures have been solved42,43,44. One of the interesting findings in the present study is that transfer of S3–S4 regions of TRPV1 and TRPM8 into the paddle motif of Kv2.1 resulted in functional voltage-activated channels (Fig. 3, 4, and 5), even though this region of Kv2.1 and the two TRP channels has low sequence homology. When compared to the other “paddle chimeras” that have been generated and tested23,44, our constructs possess the lowest sequence homology between the two proteins within the transferred region. Indeed, TRPM8 and Kv2.1 have a sequence similarity of 31% in the paddle region and TRPV1 and Kv2.1 have a sequence similarity of 22%, as compared to 40–45% between KvAP, Hv1, Nav2.1, Ci-VSP and Kv2.1. Our results strengthen the notion that Kv channel paddles lie in a remarkably unconstrained environment and probably make few critical contacts with the rest of the protein.45,46,47,48,49,50,51,52.
Figure 5 | Chimeras of S3–S4 region of TRPV1 transplanted into Kv2.1. (a) Alignments of TRPV1 and Kv2.1 and design of chimeras. (b) Families of current traces for 2V1Kv after subtraction of capacitive and leak currents using agitoxin2. Holding voltage was $-20$ mV, tail voltage was $-100$ mV and depolarizations were from $-100$ mV to $+80$ mV in 10 mV increments. (c) Families of current traces for 6V1Kv after subtraction of capacitive and leak currents using agitoxin2. Holding and tail voltage was $-100$ mV, and depolarizations were from $-80$ mV to $+30$ mV in 10 mV increments. (d) Unsubtracted families of current traces for 5V1Kv. Holding and tail voltage was $-80$ mV, and depolarizations were from $-60$ mV to $+60$ mV in 10 mV increments. (e) G–V plots of Kv2.1 and the chimeras. (f) Unsubtracted current traces for 5V1Kv before and after treatment with capsaicin. The voltage protocol was identical to the one used in d. Error bars indicate s.e.m. ($n = 3$). Dotted lines in current races represent zero current.
residues, both of which are neutralized in our constitutively active chimeras. Our chimeras also contain many other mutations in the transferred region, making it difficult to ascribe the constitutively activity to arginine neutralizations per se. Nevertheless, these collective results demonstrate that mutations in the voltage sensors, in addition to the S4–S5 linker and S6 gate can influence the coupling mechanism.

Although a strong indirect case can be made for structural similarities between TRP channels and Kv channels, as reviewed in the introduction, the vast majority of the chimeras we generated did not form functional channels. This outcome may help to explain why a majority of the functional chimeras involving TRP channels have been generated between orthologs of the same subtype. For example, chimeras between the rat and the avian orthologs of TRPV1 provided critical insights into the molecular determinants of vanilloid binding to the channel5, and chimeras between rat and Xenopus TRPV1 provided evidence implicating the pore region of the channel as the binding site of DkTx2. Similarly, chimeras created between rat and chicken TRPM8 led to the identification of specific residues of TRPM8 that are involved in icilin sensitivity17. In contrast to these examples of functional chimeras between TRP channel orthologs, there are few reports on chimeras between TRP channels belonging to different subtypes. The prominent outliers include reports on TRPV1–TRPV2 chimeras14,15, and those between TRPV1 and TRPM826. Collectively, these results lead us to believe that the transmembrane regions of TRP channels have more constraining packing interactions than have been observed in X-ray structures of Kv channels20,26, where S1–S4 domains are loosely associated with the central pore domain and the paddle motif is relatively unconstrained (Fig. 1b). We speculate the structures of transmembrane regions of TRP channels are more closely related to that observed in the X-ray structure of MloTm9, a prokaryotic tetrameric K channel containing a cytoplasmic cyclic nucleotide-binding domain, in which the helices within the S1–S4 domains exhibit extensive and tight packing interactions with each other and with the S5–S6 helices forming the central pore domain.

Methods
Channel constructs and chimera design. The Kv1.1A7 channel was used because this Kv1.1 construct is sensitive to agitoxin22, enabling the toxin to be used to identify currents associated with chimeras containing the pore region of this channel. The rat orthologs of TRPV120 and TRPM826 were utilized for all experiments, and were a generous gift from David Julius (UCSF). Chimeras were generated by utilizing an overlap PCR approach.

Chimeras between Kv1.2 and TRPV1/TRPM8 were designed based on the sequence alignments shown in Fig. 2 and 3. These alignments were generated by initially using the AlignX tool of the Vector NTI software (Invitrogen) to align the sequences of tetrameric six transmembrane cation channels and to S1–S4 containing proteins, including TRPV1, TRPM8, Kv2.1, Kv1.2, Shaker, KvAP, GiVSP, Hv1, rNav1.2, NavAb and MloTmK. The alignment thus generated was further adjusted manually to improve homology with transmembrane helices, in particular for residues known to be structurally and functionally critical in Kv channels. The start and end points of transmembrane regions for Kv1.2, KvAP, NavAb, and MloTmK were obtained by visualizing their respective high resolution crystal structures, and those for other channels shown in Fig. 2 were predicted based on their sequence alignments with these four proteins.

Electrophysiology. DkTx was produced recombinantly and agitoxin2 was synthesized by solid-phase methods as described previously22. Oocytes for chimera expression were obtained as previously described13. Two-electrode voltage–clamp recordings were performed using an OC-725C oocyte clamp amplifier (Warner Instruments). Data was filtered at 1 kHz (8 pole Bessel), and digitized at 10 kHz. Microelectrode resistances were between 0.1–1.2 MΩ when filled with 3 M KCl solutions for recording chimeras with the Kv channel pore contained (in mM) KCl (50), NaCl (50), MgCl2 (1), CaCl2 (0.3), and HEPES (20), at pH 7.4 (pH adjusted with NaOH). For recording currents from chimeras that contained TRP channel pores, CaCl2 was replaced with BaCl2. Unless otherwise stated, capacitive and background currents were identified by first blocking the Kv channel with agitoxin2, and then subtracting them to generate the currents shown in Fig. 4 and 5.

HEK-293 cells for whole-cell patch clamp recordings were split on glass coverslips in a six-well plate, transfected with 1 μg DNA per well and used 12–48 h after transfection. Whole-cell currents were recorded using an Axopatch 200 B patch clamp amplifier (Axon Instruments), filtered at 10 kHz (8 pole Bessel), and digitized at 50 kHz. Microelectrode resistances were between 1–4 MΩ when filled with pipette solutions. External solution for recording currents from the chimeras with the Kv channel pore (8Kv1) contained (in mM) KCl (45), NaCl (100), MgCl2 (0.5), CaCl2 (2), and HEPES (10) at pH 7.2 (pH adjusted with NaOH), whereas the pipette solution contained (in mM) KCl (160), EGTA (1), MgCl2 (0.5), and HEPES (10) at pH 7.4 (pH adjusted with NaOH). External solution for recording the chimeras with TRP channel pore (8Kv1, SKv1, and 12Kv1) contained (in mM) KCl (2.8), NaCl (150), MgSO4 (1), and HEPES (10) at pH 7.4 (pH adjusted with CaOH).

Conductance (G)–Voltage (V) relationships were obtained by measuring tail currents following depolarization to test voltages as indicated in Fig. 4 and 5. A single Boltzmann function was fitted to the data according to the equation, $G/G_{\infty} = \frac{1}{1 + \exp(-zF(V-V_{1/2})/RT)}^{-1}$.

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J. K. and K. J. S. designed the experiments, J. K. performed the experiments, and J. K. and K. J. S. wrote the paper.

Additional information
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