**COMMENTARY**

**Oddsballs in the Shaker family: Kv2-related regulatory subunits**

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In a large family, there are always one or two children who are different from the rest. For the Shaker family of voltage-gated K+ channels (Kv1–4), the oddsballs are the “silent” Kv2-related regulatory subunits: Kv5.1, Kv6.1–6.4, Kv8.1–8.2 and Kv9.1–9.3. Although their surnames imply that they are from different families, phylogenetic analysis places them squarely within the Kv2 subfamily (Li et al., 2015). Unlike their Kv2 siblings and Kv1, Kv3, and Kv4 cousins, however, the Kv2-related regulatory subunits do not form functional homotetramers (hence the name “silent”). Instead, they coassemble with Kv2.1 and Kv2.2 subunits to form heterotetrameric channels with various biophysical properties (Post et al., 1996; Patel et al., 1997; Salinas et al., 1997; Kramer et al., 1998; Zhu et al., 1999; Ottschytsch et al., 2002). Several studies have attributed the inability of Kv2-related regulatory subunits to form homotetramers to their “self-incompatible” tetramerization domains (T1; Ottschytsch et al., 2002, 2005). The T1 domain is not required for Kv channel tetramerization per se (Kobertz and Miller, 1999), but it coassembles into a water-soluble tetramer (Kreusch et al., 1998) that hangs below the cytoplasmic S6 activation gate (Kobertz et al., 2000), preventing subunits from the different Kv (1–4) subfamilies from intermingling (Shen and Pfaffinger, 1995). Because one T1 domain interacts with T1 domains of adjacent subunits, a Kv2-related regulatory (R) subunit with a self-incompatible T1 domain could potentially give rise to functional heterotetrameric Kv2 channels with two stoichiometries: 3:1R and 2:2R with diagonally opposed regulatory subunits (Fig. 1A). However, intersubunit FRET experiments (Kerschensteiner et al., 2005) and gating currents (Bocksteins et al., 2017) have shown that the predominant stoichiometry of the functional Kv2 heterotetramer is 3:1R, indicating that there is something else that makes these kids weird. In this issue of the *Journal of General Physiology*, Pisupati et al. investigate the mechanisms by which the stoichiometry of Kv2:Kv6 heteromers is restricted to 3:1.

**The S6 bundle crossing is a self-incompatibility domain**

Pisupati et al. (2018) take a genealogical approach to identify residues in Kv6.4 subunits that are responsible for the Kv2-related regulatory subunit phenotype. They look at two regions in Shaker Kv family members: the T1 domain and the conserved C-terminal S6 “bundle crossing,” which makes up the intracellular gate in homotetrameric Kv channels (Fig. 1B). They found nothing unusual in the T1 domain; however, 42 out of 43 Kv2-related regulatory subunits contained atypical substitutions in the bundle crossing. In a subset of Kv2-related regulatory subunits, the proline hinge (P3) is often a polar residue (S/T) and the downstream valine (V6) is an aromatic residue (Fig. 1B). Previous studies (Hackos et al., 2002; Sukhareva et al., 2003) on the *Drosophila Shaker* S6 bundle crossing have shown that similar mutations in these positions give rise to leaky and nonconducting channels, respectively. Could these bundle-crossing residues in Kv2-related regulatory subunits be responsible for the functional stoichiometry of the Kv2 heterotetramers?

Because multiple stoichiometries were a real possibility (Fig. 1A), a TIRF microscopy single-molecule photobleaching assay was used to determine the stoichiometry of the Kv2.1/Kv6.4 heterotetramers at the plasma membrane. To calibrate their system, they first tagged Kv2.1 with an N-terminal GFP and used its photobleaching predictability to demonstrate that coexpression of Kv6.4 subunits could reduce the number of Kv2.1-GFP photobleaching steps in *Xenopus laevis* oocytes. Placing the GFP tag on the Kv6.4 N terminus enabled the authors to determine the number of Kv2.1/Kv6.4 heterotetramers that contain one (3:1R) or two (2:2R) regulatory subunits. Stacking the deck to favor the 3:1R heterotetramer (15:1 Kv2.1:Kv6.4 mRNA injection ratio) surprisingly resulted in 13% of the channels at the cell surface with two regulatory subunits (2:2R). Similarly confounding, injecting an mRNA ratio (1:50 Kv2.1:Kv6.4) to overwhelmingly favor the 2:2R heterotetramer only doubled the population of 2:2R hetero
tetramers at the cell surface, demonstrating that a simple bino
mial tetramerization mechanism in the ER cannot predict the stoichimetry of Kv2 heterotetramers at the plasma membrane. To determine whether there were specific regions of Kv6.4 that were influencing Kv2 heterotetramer stoichiometry, Pisupati et al. (2018) made Kv6.4-Kv2.1 chimeras and compared the percentage of 2:2R heterotetramers at the plasma membrane. As expected from previous studies (Ott schytzsch et al., 2005), placing the Kv2.1 T1 domain into Kv6.4 did not significantly change the percentage of the 2:2R heterotetramer at the cell surface. However, a chimera with the Kv2.1 C terminus (including the S6 bundle crossing) increased the 2:2R heterotetramer cell surface population such that it was the predominant species (60%). Further dissection of the Kv6.4-Kv2.1CT chimera revealed that just the six Kv2.1 bundle-crossing residues (Fig. 1B) were sufficient to favor the 2:2R heterotetramer, whereas reintroduction of either the Kv6.4 T3 or F6 bundle-crossing residue afforded a 2:2R proportion similar to WT Kv6.4.

One caveat of single molecule photobleaching is that it cannot
distinguish between functional and nonfunctional channels at the plasma membrane. Moreover, TIRF photobleaching is not easy and is prone to counting errors (Kobertz, 2014). The authors therefore turned to mRNA ratio injection experiments to determine whether the population of 2:2R heterotetramers were indeed functional. In the first set of experiments, mRNA injection ratios were varied and the resultant total and heteromeric currents were measured, the latter extracted from the data using the incomplete steady-state inactivation of Kv2.1:Kv6.4 hetero
tetramers. If the 2:2R heterotetramers conducted, then at very high Kv6.4 concentrations, where 2:2R heterotetramer cell surface expression is more abundant, a standing current should remain. For WT Kv6.4, the current dropped precipitously with increasing Kv6.4 mRNA concentrations and approached zero at the highest Kv6.4 mRNA concentration. In contrast, the current for the Kv6.4-Kv2.1CT chimera decreased much more slowly as Kv6.4 mRNA was increased, with nearly half of the current remaining at the highest mRNA injection ratio. Comparison of currents from the heteromeric channels revealed that the Kv6.4-Kv2.1CT chimera was passing three- to fourfold more current than WT. Together, these data supported the notion that a Kv6.4 regulatory subunit with a Kv2.1 bundle crossing gives rise to 2:2 heterotetramers that are more conducting than heterotetramers with two WT Kv6.4 regulatory subunits. The second set of experiments used Cd²⁺ block to isolate currents from 2:2R heterotetramers with diagonally opposed Kv2-related regulatory subunits. Kv2.1 I379C channels are blocked by Cd²⁺ 8 when two cysteines are located in adjacent subunits but not when diago
nally opposed (Krovetz et al., 1997). Repeating the RNA ratio injection experiments with mRNAs encoding the equivalent cysteine mutation in the Kv6.4 regulatory subunit, and then blocking the channels with Cd²⁺, confirmed that Kv6.4-Kv2.1CT chimeric 2:2R heterotetramers passed current whereas the WT 2:2R heterotetramers were minimally conducting and possi
bly nonconducting.

Assembly, architecture, and function of 3:1R and 2:2R heterotetramers

The discovery of residues in the S6 bundle crossing that influence the stoichiometry of Kv2.1-Kv6.4 channels raises several questions about Kv2 heterotetramer assembly. Homotetrameric Kv channels assemble in the ER via a dimerization of dimers mechanism (Tu and Deutsch, 1999). In vitro translation assays show that the N-terminal T1 domains in Kv1.3 subunits form T1–T1 interactions early, before polypeptide exit from the ER translocation channel (Lu et al., 2001). If these early T1–T1 inter
actions occur with nascent Kv2.1 and Kv6.4 subunits in cells, how does the C-terminal Kv6.4 S6 bundle crossing influence stoi
chiometry of the heterotetramer? One explanation is that the S6 bundle crossing is not involved in the initial tetramerization of the channel, but rather in the formation of a more stable mem
brane domain with a 2:2R stoichiometry that exits the ER and traffics to the plasma membrane. This is consistent with a previ
ous chimera study (Ott schytzsch et al., 2005), which showed that Kv6.4-Kv2.1 T1 chimeras become trapped in the ER and explained why adding the Kv2.1 T1 domain to the Kv6.4-Kv2.1CT chimera does not increase the population of 2:2R heterotetramers at the plasma membrane.

Loss of Cd²⁺ inhibition using a cysteine pore mutant demonstrated that some of the Kv6.4 subunits are diagonally opposed, but is the formation of 2:2 heterotetramers with adjacent sub
units not permitted? It makes sense from a steric hindrance perspective, yet there is no direct evidence that 2:2R heterote
tramers with adjacent Kv-2 related regulatory subunits cannot form. Using the Kv6.4-Kv2.1CT chimera, it may be possible to design single molecule experiments to survey the architecture of 2:2 heterotetramers at the plasma membrane. In addition to increasing the population of 2:2 heterotetramers at the cell surface, the Kv6.4-Kv2.1CT chimera could conduct, whereas currents...
from WT Kv6.4 were undetectable. Trying to prove that a channel is nonconducting is always a fool’s errand; however, it may be possible to differentiate the single channel conductances of the 3:1R and 2:2R heterotetramers of the Kv6.4-Kv2.1CT chimera using Cd²⁺ inhibition. A noticeable difference in single channel conductance between the two heterotetramers would provide evidence that the S6 bundle-crossing residues can throttle the single channel conductance of Kv-type and possibly other tetrameric cationic channels.

Are Kv6.4’s T1 domains differently abled?
Although the initial premise to search for self-incompatibility domains was based on the Kv6.4 T1 domain’s inability to yield a positive result in a yeast two-hybrid assay (Ottschytsch et al., 2002), the question remains whether the T1 domains from Kv2-related regulatory subunits are any different from their Kv2 brethren? Pisupati et al. (2018) did not find any “unusual” sequence variations in the T1 domains of Kv2-related regulatory subunits that would make them incompatible. Moreover, Kv6.4 chimeras with the Kv2.1 T1 domain are unable to increase the cell surface expression of either the homotetramer (Ottschytsch et al., 2005) or heterotetramers (Pisupati et al., 2018). Much of the Kv2 self-incompatibility hypothesis stems from the subunit’s inability to get past the protein quality control machineries in the ER. However, this failing only demonstrates that the Kv2-related regulatory subunits are unable to traffic to, and function at the cell surface, and does not cast light on their ability to form oligomers. To determine their true oligomerization potential, it is time to buckle down with some old fashioned in vitro biochemistry. To determine their true oligomerization potential, it would be possible to differentiate the single channel conductances of Kv-type and possibly other tetrameric cationic channels.

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