**BIOPHYSICS**

**Electro-mechanical coupling of KCNQ channels is a target of epilepsy-associated mutations and retigabine**

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KCNQ2 and KCNQ3 form the M-channels that are important in regulating neuronal excitability. Inherited mutations that alter voltage-dependent gating of M-channels are associated with neonatal epilepsy. In the homolog KCNQ1 channel, two steps of voltage sensor activation lead to two functionally distinct open states, the intermediate-open (IO) and activated-open (AO), which define the gating, physiological, and pharmacological properties of KCNQ1. However, whether the M-channel shares the same mechanism is unclear. Here, we show that KCNQ2 and KCNQ3 feature only a single conductive AO state but with a conserved mechanism for the electro-mechanical (E-M) coupling between voltage sensor activation and pore opening. We identified some epilepsy-linked mutations in KCNQ2 and KCNQ3 that disrupt E-M coupling. The antiepileptic drug retigabine rescued KCNQ3 currents that were abolished by a mutation disrupting E-M coupling, suggesting that modulating the E-M coupling in KCNQ channels presents a potential strategy for antiepileptic therapy.

**INTRODUCTION**

The KCNQ voltage-gated potassium (Kv) channel family (KCNQ1 to KCNQ5) serves diverse physiological functions, from regulating cardiac action potential duration (1, 2), maintaining epithelial salt homeostasis (3), to modulating neuronal excitability (4–6). Like other Kv channels, KCNQ channels adopt a canonical topology as tetramers, with each α subunit containing six transmembrane segments S1 to S6. S1 to S4 of each α subunit form the voltage-sensing domain (VSD) that senses changes in membrane potential, while S5 and S6 from all four α subunits comprise the central K⁺-selective pore (7, 8). Voltage-dependent gating of Kv channels involves three fundamental molecular processes: VSD activation, VSD-pore coupling, and pore opening. In response to membrane depolarization, the positively charged S4 segment moves toward the extracellular space and changes the conformation of the VSD, a process known as “VSD activation.” The conformational changes in the VSD then trigger a series of interactions between the VSD and the pore, a process known as “VSD-pore coupling” or “electro-mechanical (E-M) coupling.”

The VSDs of KCNQ1 activate in two steps upon membrane depolarization: first from the resting state to an experimentally resolvable intermediate state and then proceed to the fully activated state (9–15). Previous work showed that the KCNQ1 channel opens when its VSDs occupy both the intermediate and fully activated conformations, resulting in two conductive states, the intermediate-open (IO) and activated-open (AO) states (12–18). The IO and AO states have different properties including channel modulation (12), pharmacology (12, 14, 17, 19), and regulation by cell signaling (20–22). Distinctive regulation of the IO and AO states by various KCNE auxiliary subunits causes the channel complex to adopt different balances of IO- and AO-associated properties, underlying tissue-specific KCNQ1 current phenotypes (12, 14).

Our previous studies demonstrated that stepwise VSD activation of KCNQ1 induces pore opening at the IO and AO states via distinctive sets of E-M coupling interactions (15). When S4 moves to the intermediate state, the S4-S5 linker interacts with the bottom of the S6 in the same subunit, which is essential for the channel to open to both the IO and AO states. When S4 then transitions to the fully activated state, the S4 and S4-S5 linker of one subunit interact with S5 and S6 of the neighboring subunit. These interactions are important specifically for triggering the pore to open to the AO state. The specific mechanisms of E-M coupling for the two open states provide the basis for some of the IO- and AO-specific properties. For instance, ML277, a KCNQ1 activator, was found to only enhance the AO state occupancy by perturbing the E-M coupling specific for the AO state (17). In addition, some disease-associated mutations in KCNQ1 could specifically disable the E-M coupling of individual KCNQ1 open states (12, 15, 16, 23).

KCNQ2 and KCNQ3 form the heterotetrameric M-channels (KCNQ2/3) that primarily mediate the M-current in neurons (4, 24). KCNQ4 and KCNQ5 also contribute to M-currents (5, 6). The M-current activates at subthreshold potentials to regulate action potential firing of neurons (25, 26). Reflecting its key role in controlling neuronal excitability, congenital mutations in KCNQ2 and KCNQ3 that alter M-current function are commonly associated with forms of early-onset epilepsy (27), including benign familial neonatal convulsions (BFNC) (28–31) and the more severe early infantile epileptic encephalopathy (EIEE) (32–34). Activation of M-current hyperpolarizes neurons and suppresses membrane excitability, making the neuronal KCNQ channels prime therapeutic targets against epilepsy. As a result, numerous synthetic compounds specifically targeting neuronal KCNQ channels have been developed (35, 36), such as zts240 (37), ICA069673 (38), retigabine (RTG) (37, 39), and ML213 (40, 41). On the other hand, naturally occurring compounds
such as polyunsaturated fatty acids (42) and endocannabinoids (43) have been recently found as potent KCNQ activators. Among all the compounds, RTG is a first-in-class antiepileptic drug approved for human use as an adjunctive treatment for refractory epilepsy (44). A recent study showed that RTG stabilizes both the open conformation of the pore and the activated conformation of the VSD, suggesting an activation mechanism via E-M coupling (45). Recent RTG-bound KCNQ structures also revealed that RTG binds at a pocket between adjacent pore segments of the channel and interacts with residues important for E-M coupling (37, 39). These findings make RTG a suitable candidate drug to study the E-M coupling in neuronal KCNQ channels.

KCNQ2 to KCNQ5 share sequence and structure homology as well as many important mechanisms of function with KCNQ1 (7, 8, 37, 39, 41). However, whether the neuronal KCNQ channels also feature two open states in voltage-dependent gating remains elusive, which hinders further understanding of the molecular mechanism for E-M coupling. Furthermore, the link between E-M coupling in neuronal KCNQ channels and human pathophysiology has not been established. Despite extensive studies, whether antiepileptic compounds such as RTG modulates KCNQ channels by altering the E-M coupling has not been fully explored. A deeper understanding of the open state and E-M coupling mechanisms in different KCNQ channels will provide fresh insights into how these channels function and how inherited mutations affecting KCNQ function lead to disease.

In this study, we combined site-directed mutagenesis, electrophysiology, fluorescence measurements, and pharmacology to elucidate the E-M coupling mechanism in neuronal KCNQ2 and KCNQ3 channels. On the basis of the sequence and structure homology between KCNQ1, KCNQ2, and KCNQ3, we designed a set of functional experiments and revealed key similarities and differences in their E-M coupling during voltage-dependent gating. We then took advantage of the antiepileptic prototype drug RTG to demonstrate the impact of modulating E-M coupling in loss-of-function KCNQ2 and KCNQ3 mutants. Our approach and results extend the established framework for KCNQ1 gating mechanism, and build a foundation for understanding the physiological significance, disease pathogenesis, and pharmacological modulation of E-M coupling in neuronal KCNQ channels.

RESULTS

KCNQ2 and KCNQ3 only conduct with fully activated VSD conformation

KCNQ1 to KCNQ3 show high conservation of the VSD including residues critical for voltage-dependent activation (fig. S1A) and are predicted from the sequence alignment to have similar VSD structures. A recent cryo–electron microscopy (cryo-EM) structure of human KCNQ2 (37) displays similar S2-S4 registrations within the VSD as the previously published human KCNQ1 cryo-EM structure (8), with the charge transfer center residues E2 (E140) (E170 in KCNQ1) and F0 (F137) (F167 in KCNQ1) on S2 and D172 (D202 in KCNQ1) on S3 interacting with gating charge R5 (R210) (H5, H240 in KCNQ1) on S4, indicating conservation of the fully activated VSD conformation (Fig. 1A). The KCNQ3 VSD structure, although not available, is expected to be similar based on sequence homology. Previous structure-function studies have shown that the KCNQ1 VSD sequentially occupies two functionally stable and detectable states during voltage-dependent activation: from the resting to the intermediate and then to the fully activated state (Fig. 1B) (9–15). KCNQ1 can open when its VSDs adopt either the intermediate or fully activated states, resulting in two open states, the IO and the AO (Fig. 1C) (12–18). The two open states of KCNQ1 display distinct properties in their activation, pharmacological modulation, and tissue-specific auxiliary subunit regulation (12–18). However, whether KCNQ2 and KCNQ3 also feature a two-step VSD activation and open states similar to KCNQ1 is unknown. Throughout our study, we used the expression-optimized KCNQ3A315T mutant (46) for homomeric expression of KCNQ3 channels (see Materials and Methods). To track VSD transitions, we used the voltage clamp fluorometry (VCF) technique in KCNQ3, in which the S3-S4 linker of the channel is labeled by a fluorophore, to allow monitoring of VSD movement and pore opening simultaneously during voltage-dependent activation. We developed the KCNQ3 VCF construct KCNQ3A315T/Q218C/L150W/L157T (denoted as KCNQ3*) using aromatic substitutions guided by sequence differences with KCNQ1 that generates larger fluorescence signals compared to previous reports (Fig. 1D and fig. S1, B and C) (45) and has minimal effects on channel gating (fig. S1D). We found that KCNQ3* fluorescence-voltage (F-V) relation is well fit by a single Boltzmann function and closely overlaps with its conductance-voltage (G-V) relation (Fig. 1E). This is consistent with previous KCNQ3 VCF studies (45, 47), although we note that VCF signals from KCNQ3 remain challenging to record, likely underlying slight differences between studies, and challenges with understanding the exact relationship between activation of the VSD and pore opening. In addition, KCNQ3* shows double-exponential current and fluorescence activation kinetics with a small contribution of a slow component (Fig. 1D). A recent VCF study of KCNQ2 showed a similar result (48). These results differ from the VCF recordings of KCNQ1, which exhibit two well-separated components in the F-V relation (F1 and F2) as the result of the two-step VSD activation (Fig. 1E) (12–18). Thus, these results suggest that KCNQ2 and KCNQ3 may differ from KCNQ1 by lacking a stable intermediate state of the VSD or two open states. While the intermediate and fully activated VSD conformation have been experimentally determined in KCNQ channels, the resting VSD state remains poorly understood. Despite some functional and modeling studies, the precise conformation of the resting VSD state in KCNQ channels is unclear as it has not been structurally defined. Nevertheless, this did not affect our measurements of VSD movements to the intermediate and fully activated states.

To test whether KCNQ2 and KCNQ3 have only one open state and whether the single open state represents the AO state, we applied a double charge-reversal mutagenesis strategy (9, 12, 14, 49, 50) to arrest KCNQ2 and KCNQ3 VSDs in the fully activated VSD conformation and probed the pore opening of the channels. We generated a series of KCNQ2 and KCNQ3 charge-switching mutants designed to promote electrostatic interactions between specific residue pairs found in the activated VSD structure of KCNQ2 (Fig. 1, A and B) (37): F0R (Q2-F137R, Q3-F167R) paired with R5E (Q2-R210E, Q3-R239E) and D172N (Q2-D172N, Q3-D202N) (Fig. 1, G and J). These engineered activated VSD-stabilizing (AO-locked) KCNQ2 and KCNQ3 mutants generated constitutively open channels with minimal voltage dependence (Fig. 1, G and J, and fig. S2, B and D) compared to wild-type KCNQ2 and KCNQ3A315T channels (Fig. 1, F and I, and fig. S2, A and C). This is consistent with the idea that these mutations strongly stabilize the fully activated VSD state for conduction at the constitutive AO state. To also probe whether KCNQ2 and
KCNQ3 can conduct at the IO state, we generated another class of charge-switching mutants designed to promote electrostatic interactions between specific residue pairs found in the KCNQ1 intermediate VSD structure (14): F0R paired with Q3E (Q2-Q204E, Q3-Q233E) and D172N (Fig. 1, H and K). These intermediate VSD–stabilizing (IO-locked) KCNQ2 and KCNQ3 mutants yielded negligible currents despite robust surface membrane expression as detected by Western blot (Fig. 1, H and K, and fig. S3A), suggesting that the mutant channels could not open. Additional pairs of KCNQ3 IO-locked E2R/R4E (E170R/R236E) and AO-locked E2R/R5E (E170R/R239E) mutants also produced nonfunctional and constitutively open channels, respectively (fig. S2, E and F). Together,
these results suggest that KCNQ2 and KCNQ3 have one steady-state VSD activation from the resting to the fully activated state and conduct only at the AO state. This contrasts with KCNQ1, which features both conductive IO and AO states associated with a two-step VSD activation (Fig. 1C).

**Residues important for the AO state E-M coupling are conserved in KCNQ channels**

It has been shown that KCNQ1 VSD activation to the intermediate and the fully activated states engages two distinct sets of E-M coupling interactions to induce pore opening, resulting in the IO and AO states (15). In particular, our previous studies have identified the E-M coupling interactions specific for the KCNQ1 AO state, which involve residues in the C terminus of S4, the N terminus of the S4-S5 linker, and part of S5 and S6 (15). Sequence alignment reveals that most of these residues are conserved in KCNQ2 and KCNQ3 (Fig. 2A), suggesting the conservation of the E-M coupling mechanism specifically for the AO state. In previous studies, we found two disease-associated KCNQ1 mutations W248R and S338F that selectively eliminate the AO state by disabling E-M coupling when the VSDs occupy the fully activated state; however, these mutants remain conductive at the IO state (15–17). These tryptophan and serine residues are conserved in KCNQ2 and KCNQ3 as well (Fig. 2A), located in the N terminus of the S4-S5 linker and the middle of S6, respectively (Fig. 2B). We therefore made the identical mutations as in KCNQ1 to examine the effects on currents of homomeric KCNQ2 and KCNQ3A131T channels. W218R and S303F mutations in KCNQ2, and W247R and S342F mutations in KCNQ3A131T abolished currents, despite robust expression of the channel proteins in the membrane as detected by Western blot (Fig. 2, C to H, and fig. S3B). In addition, KCNQ2S303F and KCNQ2W218R mutants exerted a dominant-negative effect by strongly suppressing homomeric KCNQ2 currents when coexpressed with wild-type KCNQ2 (fig. S4, A to D). We further carried out VCF experiments and observed robust voltage-dependent fluorescence signals from the KCNQ3*S342F mutant despite diminished current expression (Fig. 2I), demonstrating that the S342F mutation specifically ablates pore opening without eliminating VSD activation. These results together showed that residues specific for the AO state E-M coupling are conserved in KCNQ1 to KCNQ3 channels, and suggest that, in KCNQ2 and KCNQ3, there is no open state other than the AO state when the VSD reaches the fully activated conformation.

**Fig. 2. Residues specific for the AO state E-M coupling are conserved in KCNQ1 to KCNQ3 channels.** (A) Sequence alignment of KCNQ1 to KCNQ3 S4-S5 linker and S6. Residues important for the KCNQ1 AO state E-M coupling are colored red and blue. Conserved AO state E-M coupling residues in KCNQ2 and KCNQ3 are bolded. (B) Conserved AO state E-M coupling residues W218 (red) and S303 (blue) in the human KCNQ2 cryo-EM structure (PDB: 7CR3). S5 and S6 from a neighboring subunit are shown in gold. (C to E) Representative current traces for KCNQ2WT, KCNQ2W218R, and KCNQ2S303F. (F to H) Representative current traces for KCNQ3A131T, KCNQ3A131T/W247R, and KCNQ3A131T/S342F. Insets: Western blot results for membrane expression of the mutants. (I) Top: Representative current (black) and fluorescence (green) traces for KCNQ3*S342F (KCNQ3* denotes the VCF construct) recorded using the voltage protocol described in Fig. 1D legends. Bottom: F-V relation of KCNQ3*S342F (green filled circle). $V_{1/2}$ and $k$ for KCNQ3*S342F: $-3.3 \pm 2.1$ mV and $16.9 \pm 1.7$ mV ($n = 6$). Dashed line is the Boltzmann fit for KCNQ3*S F-V reproduced from Fig. 1E.

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KCNQ2 and KCNQ3 exhibit permeant ion regulation and pharmacology homologous to the AO state of KCNQ1

Previous studies in KCNQ1 have shown that IO and AO states have distinct pharmacological sensitivity and modulation by permeating ions (12, 14, 17). For example, KCNQ1 channels predominantly open to the IO state and exhibit a large Rb⁺/K⁺ inward tail current ratio of ~3, whereas Iᵦᵣ channels (KCNQ1 + KCNE1) exclusively open at the AO state and show a much smaller Rb⁺/K⁺ tail current ratio of ~1, attributed to altered rapid gating transitions in the presence of Rb⁺ (12, 51). We compared the ion permeation of KCNQ2 and KCNQ2 + KCNQ3 with the KCNQ1 IO and AO states. By measuring the instantaneous tail current amplitudes of the channels at −60 mV after a +60-mV prepulse for 5 s in 100 mM K⁺ or Rb⁺ solutions (Fig. 3, A and B), we found that both homomeric KCNQ2 and heteromeric KCNQ2 + KCNQ3 exhibited a small Rb⁺/K⁺ tail current ratio (Fig. 3E), resembling the KCNQ1 AO state (Fig. 3D) (12, 51). We also tested the ion permeation of a KCNQ2 AO-locked mutant (Fig. 3C) and found that the Rb⁺/K⁺ tail current ratios were not different from wild-type channels (Fig. 3E). These comparisons suggest that the AO state of KCNQ2 and KCNQ2 + KCNQ3 channels features permeant ion regulation resembling that of the KCNQ1 AO state.

A recent study revealed that the compound C28 differentially affects the IO and AO states in KCNQ1 (19). C28 was found to promote KCNQ1 opening by two mechanisms. First, C28 induces a hyperpolarizing shift in the voltage dependence of VSD activation. Second, C28 selectively enhances the E-M coupling of the AO state to increase Iᵦᵣ currents, but not the IO state so that it does not
Disruptions of the AO state E-M coupling in KCNQ2 or KCNQ3 subunits result in loss-of-function phenotypes in the heteromeric M-channel

KCNQ2 and KCNQ3 subunits are important contributors to native “M-current” in neurons (4, 24). When expressed in Xenopus oocytes, KCNQ2 coassembles with KCNQ3 to form heteromeric “M-channels” that result in larger currents compared to KCNQ2 homomers alone (Fig. 4, A and B) (4). We have previously shown in KCNQ1 channels that the AO state–specific E-M coupling involves interactions between the S4 and S4-S5 linker from one subunit with the S5 and S6 of a neighboring subunit (15). In a heteromeric M-channel, the VSD of each KCNQ2 (or KCNQ3) subunit may make substantial contacts with the pore of a neighboring KCNQ2 (or KCNQ3) subunit (37). Therefore, the AO state E-M coupling in M-channels is an intersubunit interaction between KCNQ2 and KCNQ3 subunits with the tools that can selectively disable the AO state E-M coupling in homomeric KCNQ2 or KCNQ3 channels (Fig. 2), we sought to dissect the E-M coupling in heteromeric M-channels. We first examined the impact of KCNQ2 by coexpressing wild-type KCNQ3 with KCNQ2 carrying the AO state–eliminating (AO-less) mutations. Notably, coexpression of the KCNQ2S303F or KCNQ2W218R mutant strongly suppressed M-current amplitude (Fig. 4, C and D). On the other hand, coexpression of wild-type KCNQ2 with the KCNQ3 AO-less mutant KCNQ3S342F or KCNQ3W247R led to weaker suppression of M-current amplitude (Fig. 4, E, F, and I) but shifted the G-V relation to more positive voltages compared to wild-type KCNQ2 + KCNQ3 (~+21 and ~+14 mV, respectively) or wild-type KCNQ2 (+19 and +12 mV, respectively) (Fig. 4I). These results suggest that in heteromeric M-channels, the KCNQ2 AO-less mutant subunits strongly abolished channel function, whereas KCNQ3 AO-less mutants modified channel function by reducing current amplitude and shifting the G-V relation.

However, the wild-type KCNQ3 and the KCNQ2 AO-less mutants, when expressed alone, all show negligible currents (Fig. 2, D and E) (46). Therefore, it was not clear if the KCNQ2 AO-less mutant subunits abolished M-currents (Fig. 4, C and D) by a dominant-negative effect, or instead by failing to associate with wild-type KCNQ3 (which gives rise to little or no current in the absence of KCNQ2 (46)). To

![Image](https://example.com/image.png)

**Fig. 4. Mutational disruptions of the AO state E-M coupling in KCNQ2 or KCNQ3 subunits impair the heteromeric M-channel function.** Representative current traces for (A) KCNQ2WT, (B) KCNQ2 + KCNQ3, (C) KCNQ2S303F + KCNQ3WT, (D) KCNQ2W218R + KCNQ3WT, (E) KCNQ2WT + KCNQ3A315T, (F) KCNQ2WT + KCNQ3A315T, (G) KCNQ2WT + KCNQ3A315T, and (H) KCNQ2WT + KCNQ3A315T. (I) Steady-state currents normalized to KCNQ2 + KCNQ3 (100 ±10.1%, n = 6) were 64.3 ± 14.3% (n = 7) for KCNQ2WT + KCNQ3S342F and 39.3 ± 14.7% (n = 7) for KCNQ2WT + KCNQ3A315T. (J) G-V relations of KCNQ2WT (black open circle), KCNQ2WT + KCNQ3 (black open square), KCNQ2WT + KCNQ3S342F (gray open circle), and KCNQ2WT + KCNQ3A315T (gray open triangle), and KCNQ2WT + KCNQ3S342F (gray open inverted triangle). V_{1/2} for KCNQ2WT: −41.5 ± 0.7 mV (n = 5), for KCNQ2 + KCNQ3: −43.2 ± 1.0 mV (n = 6), for KCNQ2WT + KCNQ3S342F: −22.2 ± 1.4 mV (n = 7), and for KCNQ2WT + KCNQ3A315T: −29.4 ± 0.9 mV (n = 7). (K) Steady-state currents normalized to KCNQ3A315T (100 ±11.9%, n = 5) were 10.3 ± 24.6% (n = 5) for KCNQ2WT + KCNQ3A315T and 19.5 ± 18.6% (n = 6) for KCNQ2WT + KCNQ3A315T. (L) G-V relations of KCNQ3A315T (black open circle), KCNQ2WT + KCNQ3A315T (black open square), KCNQ2WT + KCNQ3A315T (gray open triangle), and KCNQ2WT + KCNQ3A315T (gray open inverted triangle). V_{1/2} for KCNQ3A315T: −48.1 ± 0.6 mV (n = 5), for KCNQ2WT + KCNQ3A315T: −38.9 ± 1.0 mV (n = 5), for KCNQ2WT + KCNQ3A315T: −26.3 ± 2.1 mV (n = 5), and for KCNQ2WT + KCNQ3A315T: −21.9 ± 1.6 mV (n = 6). Statistical significance was determined by Student’s t test. **P < 0.01.
answer this question, we coexpressed KCNQ2 AO-less mutants with KCNQ3A315T to examine their effects on the currents of KCNQ3A315T. We found that coexpression of the KCNQ2S303F or KCNQ2W218R mutant suppressed the KCNQ3A315T currents (Fig. 4, G, H, and K) and shifted the G-V relation to more positive voltages compared to KCNQ3A315T (ΔV = +22 and ΔV = +26 mV, respectively) or wild-type KCNQ2 + KCNQ3A315T (ΔV = +13 and ΔV = +17 mV, respectively) (Fig. 4L). The altered voltage dependence (compared to homomeric channels) after mutant subunit coexpression demonstrates that the AO-less mutants of both KCNQ2 and KCNQ3 can coassemble with their counterpart, leading to suppression of heteromeric M-channel function along with altered gating properties. In summary, our findings suggest that disruptions of the AO state E-M coupling by mutations in KCNQ2 or KCNQ3 subunits affect the intersubunit E-M coupling interactions in heteromeric channels to impair M-current function.

Epilepsy-linked mutations in KCNQ2 and KCNQ3 subunits disrupt E-M coupling

Inherited mutations in KCNQ2 and KCNQ3 are frequently associated with neonatal epilepsies (27–34). To explore whether the AO state E-M coupling in KCNQ2 and KCNQ3 has direct implications for human pathophysiology, we searched the National Center for Biotechnology Information (NCBI) ClinVar database for conserved AO state E-M coupling residues in KCNQ2 and KCNQ3 (Fig. 2A and fig. S5A) identified in patients. Of the ten conserved coupling residues in KCNQ2 (W218, L220, L221, V225, Y237, I238, L241, S303, F304, and L307), we found three mutations [Y237C (52), I238V, and F304S (53, 54)] that are associated with EIEE and one mutation (I238L) that is likely pathogenic (fig. S5A). On the other hand, of the nine conserved coupling residues in KCNQ3 (W247, L249, L250, Y266, I267, L270, S342, F343, and L346), we only found one mutation (Y266C) (homologous to Y237C in KCNQ2) (55) that is likely pathogenic (fig. S5A). These mutations on S5 and S6 of the channel are in close proximity to each other and to the AO-less mutations (fig. S5B). The KCNQ2F304S mutant, when expressed alone (Fig. 5A) or coexpressed with wild-type KCNQ3 (Fig. 5B), suppressed current amplitude (Fig. 5C) and shifted the G-V relation to more positive voltages (shifted ΔV = +31 mV for KCNQ2F304S + KCNQ3WT) compared to the wild-type controls (Fig. 5D). On the other hand, the KCNQ2Y237C mutant with or without the coexpression of

![Fig. 5. KCNQ2 and KCNQ3 mutations associated with EIEE disrupt the E-M coupling.](image-url)

(A and B) Representative current traces for KCNQ2F304S with and without coexpressed KCNQ3WT. (C) Steady-state currents normalized to KCNQ2WT (100 ± 24.1%, n = 5) and KCNQ2 + KCNQ3 (100 ± 4.4%, n = 6), respectively, were 8.5 ± 25.4% (n = 5) for KCNQ2F304S and 20.2 ± 11.2% (n = 7) for KCNQ2F304S + KCNQ3WT. (D) G-V relations of KCNQ2 + KCNQ3 (black open square) and KCNQ2F304S + KCNQ3WT (gray open inverted triangle). V1/2 for KCNQ2WT + KCNQ3WT: −41.8 ± 1.2 mV (n = 6) and for KCNQ2F304S + KCNQ3WT: −10.8 ± 1.5 mV (n = 7). Currents from KCNQ2F304S alone were too small to obtain reliable G-V relations. (E to G) Representative current traces for KCNQ2Y237C alone, or coexpressed with KCNQ3WT, or KCNQ3A315T. Insets: Western blots for membrane expression of the mutants. (H) Steady-state current amplitudes were 17.8 ± 1.4 μA (n = 4) for KCNQ3A315T and 0.3 ± 0.1 μA (n = 10) for KCNQ2WT + KCNQ3A315T. (I and J) Representative current traces for KCNQ3Y266C alone or coexpressed with KCNQ2WT. Inset: Western blot results for membrane expression of the mutant. (K) Steady-state currents normalized to KCNQ2 + KCNQ3 (100 ± 21.2%, n = 6) were 67.6 ± 27.8% (n = 5) for KCNQ2WT + KCNQ3Y266C. (L) G-V relations of KCNQ2 + KCNQ3 (black open square) and KCNQ2WT + KCNQ3Y266C mutant (gray open inverted triangle). V1/2 for KCNQ2WT + KCNQ3WT: −41.0 ± 0.9 mV (n = 6) and for KCNQ2WT + KCNQ3Y266C: −26.9 ± 1.3 mV (n = 5). Statistical significance was determined by Student's t test. *P < 0.05; ***P < 0.001.
wild-type KCNQ3 resulted in near-complete suppression of currents despite robust expression of the channel protein detected in the membrane (Fig. 5, E and F, and fig. S3C). Coexpression of the mutant with KCNQ3A315T also strongly suppressed the currents of KCNQ3A315T channels (Fig. 5, G and H, and fig. S3C), producing a more severe loss-of-function phenotype than our designed AO-less mutants (Fig. 4, G, H, and K). With the homologous mutation on KCNQ3A315T, KCNQ3A315TY266C alone abolished the currents without preventing the expression of the channel protein to the membrane (Fig. 5I and fig. S3C), suggesting that it functions as an AO-less mutant (similar to Fig. 2, G and H). Coexpression of the KCNQ3Y266C mutant with wild-type KCNQ2 did not significantly affect the M-current amplitude (Fig. 5, J and K) but shifted the G-V relation to more positive voltages (shifted +14 mV) compared to the wild-type KCNQ2 + KCNQ3 (Fig. 5L), mirroring the effects of our designed AO-less mutants (Fig. 4, E, F, I, and J). Mutants KCNQ2F304S and KCNQ2S238V, however, were found to have mild effects on the current amplitude (Fig. S5, C to E and G to I) and the G-V relation when expressed alone (shifted +12 and +5 mV, respectively) or coexpressed with wild-type KCNQ3 (shifted +4 and +7 mV, respectively) (Fig. S5, F and J). To mimic heterozygosity of disease-linked mutations, we coexpressed the KCNQ2 mutants with wild-type KCNQ2 and KCNQ3 subunits in a 0.5:0.5:1 ratio. In this configuration, KCNQ2F304S moderately suppressed M-current amplitude (Fig. S4, E, F, and I) and shifted the G-V relation (−12 mV) compared to the wild-type control (Fig. S4J), whereas KCNQ2S238V exerted a dominant-negative effect that strongly suppressed M-current amplitude (Fig. S4, E, G, and I). Coexpression of KCNQ3Y266C mutant with wild-type KCNQ3 and KCNQ2 in a 0.5:0.5:0.5 ratio produced a small shift (+6 mV) in G-V relation (Fig. S4I) and did not significantly affect the M-current amplitude (Fig. S4, E, H, and I). These results together demonstrate the physiological importance of the AO state and suggest that disrupting the AO state E-M coupling in KCNQ2 and KCNQ3 is a mechanism contributing to some neonatal epilepsies.

**Retigabine rescues the currents of KCNQ3 mutant by restoring the E-M coupling**

RTG is the first voltage-gated K⁺ channel activator approved for clinical use as an antiepileptic drug (44), and it specifically activates KCNQ2 to KCNQ5 channels by shifting the voltage dependence of channel opening to more negative potentials (56, 57). Previous studies have suggested that RTG binds to the pore domain of the channels through interactions with several residues and demonstrated that a conserved tryptophan residue in the S5 helix (W236 in KCNQ2; W265 in KCNQ3) is essential for RTG effects (40, 58–60). Consistent with functional studies, a recent cryo-EM structure of human KCNQ2 in complex with RTG (37) revealed that RTG binds at the intersubunit interface of the channel pore domain, by mainly forming hydrogen bonds with the side chain of W236, S303, and the main-chain carbonyls of L299 and F305, as well as hydrophobic interactions with residues W236, F240, L243, L272, L299, F304, and F305. We found that several RTG-interacting residues overlap (or are in close proximity) with residues important for the AO state E-M coupling (such as S303 and F304) (Fig. 6A and fig. S5A), suggesting that RTG may directly modulate E-M coupling in KCNQ2 and KCNQ3. We therefore examined the effects of RTG on the E-M coupling in KCNQ3 using the AO-less mutant KCNQ3A315T/S342F (equivalent to KCNQ2S303Q). Application of a saturating concentration (100 μM) of RTG led to a partial rescue of KCNQ3A315T/S342F currents (Fig. 6B). For KCNQ3A315T channels, 100 μM RTG produced a large hyperpolarizing shift (~−52 mV) in the G-V relation; however, the voltage dependence of activation of the RTG-rescued KCNQ3A315T/S342F channels was notably right-shifted compared to KCNQ3A315T (Fig. 6C), suggesting that RTG partially restored channel function in the mutant. We also carried out VCF experiments in the presence and absence of RTG using a voltage protocol that highlights kinetics of VSD deactivation, which are prominently affected by RTG (Fig. 6D). Consistent with a previous report (45), KCNQ3* G-V and F-V relations closely overlapped and exhibited similar RTG-mediated shifts to negative potentials (~−47 mV), suggesting that RTG binding within the pore domain can be readily transduced to the VSD via the tight coupling between pore opening and VSD activation (Fig. 6E). The AO-less KCNQ3S342F mutant exhibited no currents under control condition but retained robust fluorescence signals (Figs. 2I and 6D). In the presence of 100 μM RTG, the F-V relation of KCNQ3S342F was slightly shifted by ~15 mV (Fig. 6E), consistent with partial restoration of the coupling between pore opening and VSD activation. The difference in the coupling between pore opening and VSD activation was also revealed by VSD kinetics. Similar to previous studies (45), RTG affected the KCNQ3* VSD kinetics by slowing the VSD deactivation rate (Fig. 6, D and F), which is consistent with RTG binding to the pore domain and altering the E-M coupling. Compared to KCNQ3*, KCNQ3S342F showed a much faster VSD deactivation kinetics (Figs. 6, D and F). This effect is comparable to previously reported proximal C terminus mutations in KCNQ3 that uncoupled the pore and the VSD (45) and may reflect the differences in VSD function when the influence of the pore was weakened by the S342F mutation. RTG also slowed the VSD deactivation rate of KCNQ3S342F but to a much lesser extent compared to KCNQ3* (Fig. 6, D and F), suggesting that RTG may restore some of the E-M coupling. Together, these results suggest that RTG interacts with critical residues to directly modulate the E-M coupling in KCNQ2 and KCNQ3. When the E-M coupling is intact (KCNQ3A315T), transduction of RTG binding from the pore to the VSD is robust and efficient. On the other hand, when the E-M coupling is abolished (KCNQ3A315T/S342F), RTG can restore the E-M coupling to partially rescue channel function, but fails to have the same influence on the voltage dependence of VSD activation and VSD deactivation kinetics.

**Retigabine rescues some epilepsy-associated KCNQ2 and KCNQ3 mutants by modulating the E-M coupling**

We then further explored if RTG can rescue disease-linked KCNQ2 and KCNQ3 mutants with compromised E-M coupling (Fig. 5). These epilepsy-associated mutations are located near or within the voltage-sensor domain (VSD) and may reflect the differences in VSD function when the influence of the pore was weakened by the S342F mutation. RTG also slowed the VSD deactivation rate of KCNQ3S342F but to a much lesser extent compared to KCNQ3* (Fig. 6, D and F), suggesting that RTG may restore some of the E-M coupling. Together, these results suggest that RTG interacts with critical residues to directly modulate the E-M coupling in KCNQ2 and KCNQ3. When the E-M coupling is intact (KCNQ3A315T), transduction of RTG binding from the pore to the VSD is robust and efficient. On the other hand, when the E-M coupling is abolished (KCNQ3A315T/S342F), RTG can restore the E-M coupling to partially rescue channel function, but fails to have the same influence on the voltage dependence of VSD activation and VSD deactivation kinetics.
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Fig. 6. Retigabine restores E-M coupling to rescue the currents of AO-less KCNQ3 mutant. (A) Mapping the key AO state E-M coupling residue S303 (blue) and the EIEE-associated residues Y237, I238, and F304 (magenta) onto the retigabine-bound human KCNQ2 cryo-EM structure (PDB: 7CR7). Brackets indicate amino acid numbers for KCNQ3. S5 and S6 from a neighboring subunit are shown in gold. (B) Representative current traces and (C) G-V relations of KCNQ3A315T/S342F before and after 100 µM retigabine. $V_{1/2}$ for KCNQ3A315T/S342F with 100 µM retigabine (blue filled circle): $-6.2 \pm 1.0$ mV ($n = 9$). Dashed lines are fits for KCNQ3A315T with $V_{1/2}$ for control (black): $-47.3 \pm 0.8$ mV ($n = 5$) and 100 µM retigabine (blue): $-99.2 \pm 1.9$ mV ($n = 5$). (D) Representative fluorescence deactivation traces of KCNQ3* and KCNQ3*S342F before (green) and after (cyan) 100 µM retigabine. (E) F-V relations of KCNQ3* and KCNQ3*S342F before and after 100 µM retigabine. $V_{1/2}$ for KCNQ3* control (green open circle): $-52.7 \pm 4.6$ mV ($n = 4$), for KCNQ3* with 100 µM retigabine (cyan open circle): $-99.8 \pm 5.9$ mV ($n = 3$), for KCNQ3*S342F control (green filled circle): $-11.5 \pm 0.2$ mV ($n = 6$), and for KCNQ3*S342F with 100 µM retigabine (cyan filled circle): $-26.4 \pm 1.4$ mV ($n = 8$). (F) Fluorescence deactivation time constants of KCNQ3* and KCNQ3*S342F before and after 100 µM retigabine. Dotted lines are semi-log line fits. VCF was recorded using the deactivation voltage protocol illustrated at the top of (D).

(53, 54) + wild-type KCNQ3 channels (Fig. 7E) by shifting the G-V relation to more negative potentials (~−43 mV) and increasing the maximum conductance (by >25%) (Fig. 7F). To estimate the outcome in heterozygous conditions, we performed RTG experiments in channel configurations that mimic the genetic balance of the disease-linked mutants. When KCNQ2F304S is coexpressed with wild-type KCNQ2 and KCNQ3 subunits in a 0.5:0.5:1 ratio, RTG enhanced the currents by shifting the G-V relation to more negative potentials (~−43 mV) (fig. S6A) and increasing the maximum conductance (~2-fold) (fig. S6B). Similarly, when KCNQ3S266C is coexpressed with wild-type KCNQ3 and KCNQ2 subunits in a 0.5:0.5:1 ratio, RTG potentiated the currents by shifting the voltage dependence of opening (by ~−41 mV) (fig. S6A) and increasing the maximum conductance (~2.5-fold) (fig. S6B). Despite the profound effects of RTG on some of the disease-linked mutants and KCNQ3 A315T/S342F (Fig. 6), RTG did not rescue the currents of KCNQ2W218R, KCNQ2Y237C, or KCNQ3S303F or KCNQ3A315T/W247R mutant (fig. S6C). Together, these results demonstrate that RTG can rescue KCNQ2 and KCNQ3 mutants with disrupted E-M coupling and suggest that other small-molecule KCNQ openers with similar mechanisms to modulate E-M coupling can benefit the treatment of some neonatal epilepsies.

DISCUSSION

Elucidating the molecular mechanism for E-M coupling is an important step toward understanding the voltage-dependent gating of Kv channels. Recent structure-function studies revealed that the KCNQ1 channel features a two-stage hand-and-elbow E-M coupling mechanism (15), where two steps of VSD activation, from the resting to intermediate and lastly to the fully activated state (9–15), trigger two sets of E-M coupling interactions that generate two distinct conductive IO and AO states (fig. S7A) (12–18). This E-M coupling mechanism and the regulation of the IO and AO states define the gating, pharmacology, physiology, and pathophysiology of KCNQ1 (fig. S7) (12–18). KCNQ2 to KCNQ5 are homologous to KCNQ1 in structure (7, 8, 37, 39, 41) and share important characteristics of gating mechanism with KCNQ1, such as dependence on phosphatidylinositol 4,5-bisphosphate (PIP$_2$) (23, 61, 62) and calmodulin (63–65). However, whether the KCNQ2–KCNQ5 channels share the same E-M coupling mechanism in their voltage-dependent gating with KCNQ1 remains unclear. In this study, we provided functional evidence that the neuronal homolog KCNQ2 and KCNQ3 channels are different from KCNQ1 in which they feature a single AO state but with a conserved E-M coupling mechanism specific for the AO state (Figs. 1 to 3 and fig. S7A). We showed that the AO
state E-M coupling of M-channels is physiologically important and is a target of epilepsy-associated mutations (Figs. 4 and 5 and fig. S7A). Last, we took advantage of a well-studied neuronal KCNQ channel opener RTG to demonstrate that modulating the AO state E-M coupling holds therapeutic potential against congenital epilepsy (Figs. 6 and 7). This work is built on the foundation of a well-established mechanistic framework for KCNQ1 gating (12–18) and strengthens our understanding of the role of E-M coupling in physiology, pathophysiology, and pharmacology of KCNQ channels.

Recent cryo-EM structures revealed that the neuronal KCNQ channels share similar VSD conformations with KCNQ1, representing the conserved fully activated VSD state (Fig. 1A) (7, 8, 37, 41). Consistent with other studies (45, 47, 48), our VCF results showed that KCNQ3, unlike KCNQ1, exhibits a one-step VSD transition based on steady-state voltage-dependent activation (Fig. 1, D and E). We then demonstrated with VSD-arresting charge-switching mutations that KCNQ2 and KCNQ3 only conduct in the AO state when the VSDs occupy the fully activated conformation (Fig. 1, F to K), contrasting the two open states of KCNQ1 (12–18). Mutations that target the AO state E-M coupling completely abolished the currents (Fig. 2), further supporting the idea that KCNQ2 and KCNQ3 only feature an AO state. This conserved single AO state defines the properties of KCNQ2 and KCNQ3 channels, including the mechanism of E-M coupling, sensitivity to permeant ions, and pharmacology (Figs. 2 and 3), which are similar to the AO state of the KCNQ1 channel (fig. S7) (12, 15–17).

Although KCNQ2 and KCNQ3 predominantly activate with a fully activated VSD conformation, some differences with AO state properties of KCNQ1 should be noted. First, the AO-only KCNQ2 and KCNQ3 channels have a negative $V_{1/2}$ (~−40 mV) of $G-V$ relations and fast activation kinetics that resemble KCNQ1, which is IO-predominant. In contrast, the KCNQ1 AO state exhibits a right-shifted $G-V$ relation ($V_{1/2}$ ~ +40 mV) and slower activation kinetics (12–18, 64) compared to KCNQ2 and KCNQ3 (Fig. 1, D and E) (45, 47, 48). While these differences in the AO state activation properties may have important implications for the primary physiological roles of KCNQ2/3 (regulation of neuronal excitability) (25, 26) versus KCNQ1 (cardiac action potential repolarization) (1, 2), it remains
unclear why the gating features of KCNQ2 and KCNQ3 resemble the KCNQ1 IO state, instead of the AO state. It will be interesting for future studies to elucidate whether and how the differences in AO voltage dependence and kinetics between KCNQ2/3 and KCNQ1 derive from the VSDs of these channels.

While the steady-state F-V relation only indicated one-step VSD movement for KCNQ2 and KCNQ3 (Fig. 1E) (45, 47, 48), it should be noted that the possibility of a transient “intermediate state” existing during VSD activation cannot be totally ruled out. It is possible that such a VSD state may be kinetically observable based on biexponential kinetics of VSD activation (Fig. 1D and fig. S7A), but not stable enough to be detected in the steady state F-V (Fig. 1E), nor sufficient to generate channel opening (Fig. S7A). Two-step VSD activation is a common feature in Kv channels. Besides KCNQ1, a similar example is the Drosophila Shaker channel that exhibited two steps in the kinetics and steady state of VSD activation (66, 67). Some mutations in the Shaker channel were found to separate the two VSD activation steps even more (68–70). However, unlike KCNQ1, the Shaker channel only has one open state and no pore opening at the intermediate VSD state (71). Here, we showed that KCNQ2 and KCNQ3 are homologous to KCNQ1 but only feature one open state as well (Figs. 1 to 3). These comparisons suggest that one open state at the fully activated VSD conformation is the canonical mechanism in Kv channels, whereas the two open states in KCNQ1 are an exception. The reason for the two KCNQ1 open states is not clear and remains an important subject for future studies. The E-M coupling interactions specific for the AO state are conserved in KCNQ2 and KCNQ3, suggesting that the mechanism likely applies to many other domain-swapped Kv channels.

Besides the protein-protein interactions investigated in this study, PI(3,4,5)P3-mediated interactions are also important for E-M coupling. In KCNQ channels, depletion of PI(3,4,5)P3 closes channels (23, 45, 62). We have previously shown in KCNQ1 that this is due to the disruption of PI(3,4,5)P3-mediated VSD-pore coupling (23). On the other hand, in the presence of PI(3,4,5)P3 mutations that target the protein-protein E-M coupling also render nonfunctional channels (15). These results suggest that both classes of interactions are important for E-M coupling. Previous functional studies in KCNQ1 and KCNQ3 (23, 45, 61) as well as recent PI(3,4,5)P3-bound KCNQ1 and KCNQ4 structures (8, 41) have revealed a conserved PI(3,4,5)P3 binding pocket responsible for PI(3,4,5)P3-mediated VSD-pore coupling in KCNQ channels (fig. S8A). Mapping the PI(3,4,5)P3 binding sites and the sites important for protein-protein E-M coupling onto the human KCNQ2 structure revealed that they are spatially distinct (fig. S8B). However, the close proximity between the two sites (fig. S8B) suggests that the two E-M coupling mechanisms may interact allosterically to affect each other. Therefore, the possibility of allosteric effects of AO-disrupted mutations on PI(3,4,5)P3 binding cannot be totally ruled out (72). In addition, since RTG also regulates E-M coupling, it is conceivable that PI(3,4,5)P3 may modulate E-M coupling (45, 73).

The AO-only feature and its specific E-M coupling mechanism define not only the properties of homomeric KCNQ2 and KCNQ3 channels but also KCNQ2 in complex with KCNQ3, which is the major form of the M-channels in neurons (4). The sensitivity to permeant ions and pharmacology of the M-channels are similar to those of KCNQ2 and KCNQ3 (Fig. 3) (19, 74). The E-M coupling specific for the AO state is also physiologically important, as demonstrated by the coexpression of KCNQ2 or KCNQ3 subunits carrying the AO-less mutations with their wild-type counterparts (Fig. 4). Disrupting the E-M coupling in KCNQ2 versus KCNQ3 has differential consequences to the M-channel function (Fig. 4). This result may have some implications for why we observe far more disease-associated mutations in KCNQ2 compared to KCNQ3 and for the severity of disease manifestations. For instance, most BFNC mutations are found in KCNQ2 with less mutations identified in KCNQ3, whereas the EIIE mutations that cause a more severe form of childhood epilepsy are far more predominant in KCNQ2 (31, 75). It should be noted that differential expression of KCNQ2 and KCNQ3 during development (expression of KCNQ2 preceding that of KCNQ3) (76) may also underlie the higher pathogenic impact of KCNQ2 versus KCNQ3 variants. The AO state and its E-M coupling mechanism not only defines the fundamental properties of the M-channel (Figs. 3 and 4) but also is a target in disease pathology as several epilepsy-associated mutations were found to disrupt the E-M coupling (Fig. 5 and figs. S4 and S7A).

Last, we demonstrated with a small-molecule drug RTG that the E-M coupling could be specifically modulated to rescue the currents of a KCNQ3 AO-less mutant (Fig. 6 and fig. S7B) and the function of diseased M-channels (Fig. 7 and fig. S6), suggesting that targeting the conserved AO state E-M coupling is a potential antiepileptic strategy.

MATERIALS AND METHODS

Constructs and mutagenesis

Site-directed mutations were introduced into KCNQ2 and KCNQ3 channels using overlap extension and high-fidelity polymerase chain reaction (PCR). The presence of all desired mutations was verified by DNA sequencing. The complementary RNA (cRNA) of the channels was synthesized by in vitro transcription using the mMessage mMachine T7 or SP6 polymerase kit (Applied Biosystems–Thermo Fisher Scientific). In experiments involving homomeric expression of KCNQ3 channels, the A315T mutation was introduced to enable efficient trafficking and functional expression of KCNQ3 channels (46).

Xenopus oocyte channel expression

Stage V-VI oocytes were obtained from Xenopus laevis by laparotomy procedure in accordance with the protocols approved by the Washington University Animal Studies Committee (protocol #20190030) and by the University of Alberta Animal Care Committee (protocol #AUP0001752). Oocytes were digested with collagenase (0.5 mg/ml; Sigma-Aldrich, St. Louis, MO) to remove the follicle cell layer. A total of 9.2 ng of channel cRNA was then injected into each oocyte using Nanoinject (Drummond, Broomall, PA). For experiments involving coexpression of KCNQ2 and KCNQ3, cRNAs were coinjected at 1:1 (KCNQ2:KCNQ3) mass ratio, unless otherwise specified. For VCF experiments, all injected cRNA amounts were doubled to attain higher surface expression level. The injected oocytes were incubated at 18°C for at least 2 days in ND96 solution (36 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM Hepes, 2.5 mM Na pyruvate, and 1% penicillin-streptomycin (pH 7.6)) before recording.

Two-electrode voltage clamp and VCF

Microelectrodes were pulled from thin wall borosilicate glass (item #B150-117-10, Sutter Instrument, Novato, CA) by a micropipette puller (P-97 or P-1000, Sutter Instrument, Novato, CA) to a resistance of 0.5 to 3 megohms when filled with 3 M KCl solution. Whole-cell ionic currents were recorded from oocytes bathed in ND96 solution.
using a CA-1B amplifier (Dagan, Minneapolis, MN) in two-electrode voltage clamp (TEVC) mode. Signals were sampled at 1 kHz and low-pass–filtered at 2 kHz using the Patchmaster software (HEKA, Holliston, MA). Unless otherwise specified, currents were recorded at holding potential of −80 mV, followed by 4-s depolarizing pulses stepping from −120 to +60 mV in 10-mV increments, before returning to −40 mV for 2 s to measure tail currents. For ion permeation experiments, 100 mM K+ [100 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM Hepes (pH 7.6)] or 100 mM Rb+ [96 mM RbCl, 4 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM Hepes (pH 7.6)] solutions were perfused onto oocytes to steady state. For experiments comparing the current amplitudes, cRNAs encoding wild-type and mutant channels were injected on the same day. The experiments comparing the current amplitudes between wild-type and mutant channels. For VCF (pH 7.6) solutions were perfused onto oocytes to steady state. For experiments comparing the current amplitudes at the end of the +40-mV test pulse were used to compare the current amplitudes between wild-type and mutant channels. The G-V relationship (with and without drug application) was calculated by measuring the instantaneous tail currents following test pulses to various voltages and normalizing to the maximum tail current in control bath solution for each oocyte. The G-V relation was then fitted with a single Boltzmann equation in the form of $G = G_{\text{max}} / (1 + \exp(-(V - V_{1/2})) / k))$, where $G_{\text{max}}$ is the maximum conductance and is set equal to 1 in control condition, $V$ is the test pulse voltage, $V_{1/2}$ is the voltage where channels are at half-maximal activation, and $k$ is the slope factor reflecting the steepness of the curve. $k = RT / zF$, where $R$ is the universal gas constant, $T$ is the absolute temperature, $z$ is the equivalent valence, and $F$ is the Faraday constant. The change in $G_{\text{max}}$ after drug application was calculated as $(G_{\text{max,drug}} - G_{\text{max,ctrl}}) / G_{\text{max,ctrl}}$, where $G_{\text{max,ctrl}} = 1$. To correct for photobleaching, fluorescence signals were first baseline-subtracted by linear fitting and extrapolating the signals at the holding potential preceding each test pulses. The F-V relationship was derived after baseline subtraction by obtaining the relative fluorescence change ($\Delta F / F$) at the end of the test pulses to various voltages and normalizing to the maximum fluorescence change. The F-V relation was then fitted with a single-component Boltzmann equation (the same as G-V) or a two-component Boltzmann equation in the form of $F / F_{\text{max}} = A_1 / (1 + \exp(-(V - V_{1/2,1}) / k_1)) + A_2 / (1 + \exp(-(V - V_{1/2,2}) / k_2))$, where $A_1 + A_2 = 1$. The fluorescence deactivation time course was fitted with a single-exponential decay function to extract the time constant. Experiments were conducted on at least two separate batches of oocytes to confirm reproducibility. Statistical significance was determined by Student’s t test for pairwise comparisons and by one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test for multiple comparisons. All average data are presented as mean ± SEM.

Western blot
Membrane and cytoplasmic proteins of oocytes were extracted using the Mem-PER Plus Membrane Protein Extraction Kit (Thermo Fisher Scientific). Equal amounts of protein samples were prepared by adding 2x Laemmli sample buffer (Bio-Rad) and loaded on SDS–polyacrylamide gel electrophoresis gels. After gel electrophoresis, membrane and cytoplasmic proteins were probed by Western blot following standard protocols. KCNQ2 and KCNQ3 proteins were detected with primary antibodies rabbit anti-KCNQ2 (1:1000 dilution; Abb, ab22897) and rabbit anti-KCNQ3 (1:1000 dilution; Abcam, ab66640), respectively, followed by goat anti-rabbit secondary antibody (1:5000 dilution; Invitrogen, A16110), and visualized using enhanced chemiluminescence (ECL Western Blotting reagent, Thermo Fisher Scientific) and CL-XPosure Film (Thermo Fisher Scientific). β-Actin antibody (1:1000 dilution; Abcam, ab8227) was used to probe for control proteins.

Supplementary Materials
Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.aba3625

View/request a protocol for this paper from Bio-protocol.

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