

# An atypical $\text{Ca}_v1.1$ mutation reveals a common mechanism for hypokalemic periodic paralysis

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Hypokalemic periodic paralysis (HypoKPP) is a rare, dominantly inherited disorder of muscle that is characterized by recurrent and prolonged episodes of severe weakness. These episodes are associated with low serum  $\text{K}^+$  (often  $<3.0$  mM, normally 3.5–5.5 mM), often triggered by events that lower extracellular  $[\text{K}^+]$  such as carbohydrate-rich meals, and result in depolarization-induced loss of excitability. In 1994, genetic linkage and positional cloning established a HypoKPP locus in *CACNA1S*, which encodes the pore-forming  $\alpha$  subunit of the skeletal muscle L-type  $\text{Ca}^{2+}$  channel  $\text{Ca}_v1.1$  (Fontaine et al., 1994). The first missense mutations were identified soon after (Jurkat-Rott et al., 1994; Ptáček et al., 1994). Despite the tantalizing observation that the first three mutations were all missense substitutions of arginines in S4 segments, the identification of a functional defect with a plausible mechanistic connection to depolarization-induced loss of excitability and weakness in low  $\text{K}^+$  did not emerge until some 13 yr later. Two major challenges impeded functional studies of HypoKPP mutant channels. First, expression of  $\text{Ca}_v1.1$  at the plasma membrane is poor in model systems not derived from muscle. Second, the critical channel defect—an anomalous inward current—is of relatively small amplitude and difficult to distinguish from a nonspecific leakage current. In the current issue of the *Journal of General Physiology*, Fuster et al. use in vivo electroporation into mouse muscle as a system in which to achieve high expression of human  $\text{Ca}_v1.1$ . They express  $\text{Ca}_v1.1$  channels carrying an atypical HypoKPP mutation—V876E in S3 of domain III—and demonstrate an anomalous inward current at hyperpolarized potentials. This finding is an important advance in the field because it reveals that the one HypoKPP mutation that departs from the canonical pattern of R/X missense mutations in S4 segments still produces an anomalous inward current. Moreover, unlike all other  $\text{Ca}_v1.1$  HypoKPP mutations studied to date, V876E does not alter voltage-dependent gating of the conventional  $\text{Ca}^{2+}$  current, strongly supporting the notion that the anomalous inward current is the critical defect in HypoKPP.

The experimental insight on the source of the anomalous inward current in HypoKPP originated from the remarkable observation that missense mutations of *SCN4A*, encoding the  $\alpha$  subunit of the skeletal muscle sodium channel  $\text{Na}_v1.4$ , are also a cause of HypoKPP. The clinical HypoKPP phenotype is essentially identical, regardless of whether the underlying mutation is in  $\text{Ca}_v1.1$  or  $\text{Na}_v1.4$ . This genetic heterogeneity was initially puzzling, especially because the functional roles for  $\text{Na}_v1.4$  and  $\text{Ca}_v1.1$  in excitation-contraction coupling are very different. Moreover, the final common pathway for weakness in an episode of HypoKPP is a failure to maintain the resting potential ( $V_{\text{rest}}$ ) in low  $\text{K}^+$ , and yet studies in human HypoKPP fibers showed this paradoxical depolarization is not prevented by the channel blockers tetrodotoxin or nitrendipine (Ruff, 1999; Jurkat-Rott et al., 2000). The solution to the mystery of the depolarizing current in HypoKPP was gleaned from recent advances in understanding the conformational changes that occur in the voltage sensor domain of  $\text{K}_v$  channels. First, histidine-scanning mutagenesis in Shaker S4, as a tool to discern gating-dependent changes in aqueous accessibility of specific S4 residues to protons, also created a proton conduction pathway (Starace and Bezanilla, 2001). Other R/X substitutions even supported currents carried by  $\text{Na}^+$  or  $\text{K}^+$  (Tombola et al., 2005). These were initially called omega currents, to distinguish this conduction mechanism from ion flux through the conventional pore ( $\alpha$  current). In subsequent publications, the preferred term became the “gating pore current” to convey the view that the S4 helix undergoes a voltage-dependent translocation through a crevasse in the channel—the gating pore—and furthermore that mutations at this critical interface may permit ion flux as an anomalous gating pore current. Second, a consistent pattern emerged as more HypoKPP mutations were identified (Fig. 1). All 10 HypoKPP missense mutations in  $\text{Na}_v1.4$  are in the outer arginine residues of S4 (R1 or R2) in domains I, II, or III; and 8 of 9 HypoKPP mutations in  $\text{Ca}_v1.1$  are in R1

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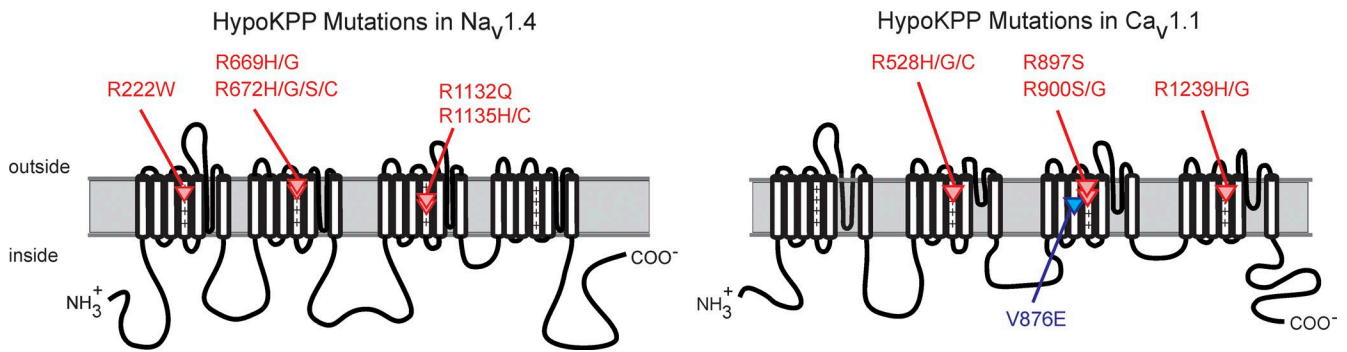


Figure 1. Mutations of  $\text{Na}_v1.4$  and  $\text{Ca}_v1.1$  associated with hypokalemic periodic paralysis occur predominantly at arginine residues in S4 transmembrane segments of the voltage sensor domains. The V876E mutation in S3 of domain III in  $\text{Ca}_v1.1$  is the only exception (blue highlight).

or R2 of domains II, III, and IV (Matthews et al., 2009). Only  $\text{Ca}_v1.1\text{-V876E}$ , the mutant channel now studied functionally for the first time by Fuster et al. (2017b), is an exception to the pattern.

The existence of gating pore currents caused by HypoKPP mutations was first established for  $\text{Na}_v1.4$  channels expressed in *Xenopus* oocytes. Compared with  $\text{Ca}_v1.1$ , mutations of  $\text{Na}_v1.4$  are a less frequent cause of HypoKPP (~20% of families), and the discovery of *SCN4A* mutations lagged the identification of calcium channel mutations by five years. Nevertheless, it was the ability to achieve high expression of  $\text{Na}_v1.4$  in oocytes that provided the first experimental insights on the defect responsible for HypoKPP. In the presence of TTX to block the conventional pore, an inward rectifying current was detected for all 8  $\text{Na}_v1.4$  HypoKPP mutant channels tested, but not wild-type channels (Sokolov et al., 2007; Struyk and Cannon, 2007; Struyk et al., 2008). The voltage-dependent translocation of the S4 helix is the basis for this rectification. In HypoKPP channels, the gating pore is permissive for ion conduction at hyperpolarized potentials because the S4 helix is in the inward conformation, which creates misalignment between the missense mutation of R1 or R2 and the narrow hydrophobic waist of the gating charge transfer center that normally occludes ion conduction. With depolarization, the S4 helix moves outward, taking the mutant R1 or R2 out of the hydrophobic waist and thereby shutting the gating pore leak. An anomalous inward current, consistent with a gating pore leak, has also been detected in muscle fibers from a HypoKPP knock-in mutant mouse model harboring  $\text{Na}_v1.4\text{-R669H}$  at R1 in domain II (Wu et al., 2011). The mouse model confirmed that an S4 R/X mutation for  $\text{Na}_v1.4$  expressed in a native context of skeletal muscle does conduct a gating pore current and also established the magnitude of this anomalous conductance as  $7 \mu\text{S}/\text{cm}^2$  or ~1% of the total resting conductance.

Once a gating pore current was identified in  $\text{Na}_v1.4$  HypoKPP mutant channels, the key question in the field

was whether HypoKPP mutations in  $\text{Ca}_v1.1$  would also support a gating pore current. If this were true, then it could explain why mutations of either  $\text{Ca}_v1.1$  or  $\text{Na}_v1.4$  might produce the same clinical phenotype. This hypothesis was plausible because S4 mutations cause gating pore currents in both  $\text{K}_v$  and  $\text{Na}_v$  channels, whose voltage sensor domains are highly conserved with  $\text{Ca}_v$  channels. The initial test of this hypothesis was performed on fibers biopsied from the quadriceps of HypoKPP patients. A larger slope conductance, by ~10–20  $\mu\text{S}/\text{cm}^2$ , was observed at negative potentials for HypoKPP fibers compared with wild type, consistent with a gating pore leak (Jurkat-Rott et al., 2009). In retrospect, a larger “depolarizing inward current” was observed in R528H fibers compared with wild type from the steady-state I-V relation eight years before the gating pore hypothesis (Ruff, 1999). It is remarkable that evidence of a gating pore conductance was detectable in these heroic studies on scarcely available human HypoKPP fibers because the anomalous increase in conductance was only ~10% of the total (in  $\text{Cl}^-$ -free solution to reduce  $G_{\text{Cl}}$  and 1 mM  $\text{K}^+$  to reduce  $G_{\text{Kir}}$ ) for these three-electrode voltage-clamp studies on 3.5-cm-long resealed cut fibers. The creation of an R528H knock-in mutant mouse model of HypoKPP provided an opportunity for more in-depth studies to confirm that a mutation of R1 in DIIS4 supports a gating pore current (Wu et al., 2012). An anomalous inward current was indeed detected in R528H mouse fibers, but even with a cocktail of blockers to reduce endogenous currents (TTX, TEA, 4-AP,  $\text{Co}^{2+}$ ,  $\text{Ba}^{2+}$ , and 9-AC), the increased conductance attributable to the mutation was only ~10% of the total residual conductance.

Upon this background of technical challenges to ascertain the presence of gating pore currents in mutant  $\text{Ca}_v1.1$  channels, Fuster et al. (2017b) has demonstrated that expression of exogenous human  $\text{Ca}_v1.1$  by in vivo electroporation in mouse muscle has sufficiently robust expression to screen for gating pore currents. This approach markedly increases the throughput of

screening  $Ca_v1.1$  HypoKPP mutations as sufficient expression occurs by 30 d rather than the time-consuming and expensive process of creating a knock-in mouse. Another improvement from their approach is the use of the silicone whole-cell clamp, which achieves a much more efficient dialysis of the myoplasm from a broken pipette tip and thereby enables better control of the intracellular ionic composition to reduce endogenous currents. Their initial study with this technique demonstrated a gating pore current for  $Ca_v1.1$ -R1239H at R2 of DIVS4 (Fuster et al., 2017a). The sensitivity for discerning a gating pore current distinct from the nonspecific background leak was improved, now being about a 35% increase above wild type, but a large number of fibers must still be sampled (50 in their case) to deal with the high variance of residual background currents in this preparation. Having established that this technique is able to confirm a gating pore current at a canonical R/X mutation in DIVS4 previously identified in human HypoKPP fibers, they now use this transient expression system in mouse muscle to test whether the atypical HypoKPP mutation V876E in S3 of DIII also supports a gating pore current. On average, the inward current was  $\sim 25\%$  larger at hyperpolarized potentials for V876E compared with wild type. This important result convincingly shows that V876E shares a common functional defect with all other HypoKPP mutant channels studied to date, for both  $Ca_v1.1$  and  $Na_v1.4$ . Moreover, the voltage dependence of activation and amplitude of the  $Ca^{2+}$  current for V876E-expressing fibers were indistinguishable from wild type, whereas the R/X HypoKPP mutations in S4 have reduced current density and altered activation. This further supports the notion that the essential defect that causes susceptibility to HypoKPP is the anomalous inward current that is active at the resting potential. An alternative technical advance provides an opportunity to characterize HypoKPP mutant  $Ca_v1.1$  channels in a membrane environment with much less interference from endogenous channels. Co-expression of Stac3 along with  $Ca_v1.1$ ,  $\alpha_2\delta$  and  $\beta_{1a}$  in *Xenopus* oocytes markedly enhances channel expression at the plasma membrane where gating pore currents have been observed for R528H and R528G (Wu and Cannon, 2017).

An important advantage of in vivo mouse model systems is that the HypoKPP  $Ca_v1.1$  channels are expressed in their native environment of fully differentiated and innervated adult skeletal muscle. In this context, it is reasonable to assume that the full complement of post-translational modification, accessory subunits, protein-protein interactions at the triad, and signaling events are intact, which strengthens the confidence and relevance of identified functional defects for discovering disease mechanisms. For example, the specific conductance of the gating pore leak is a critical parameter for simulating the defect of  $V_{rest}$  stability in HypoKPP

(Struyk and Cannon, 2008). The knock-in mouse is widely considered to be the best standard because transcript levels and trafficking to the membrane are under control of endogenous systems. Moreover, the R528H mice survive as homozygous mutants (Wu et al., 2012), from which we know the specific conductance of the gating pore current is  $28 \mu S/cm^2$  (in 100 mM  $Na^+$ ). Assuming equal abundance of wild-type and R528H  $Ca_v1.1$  subunits at the membrane in the heterozygous state, as occurs in humans with HypoKPP, the predicted specific conductance is  $14 \mu S/cm^2$ , which is remarkably consistent with the value of  $12 \mu S/cm^2$  estimated from limited studies on human HypoKPP R528H fibers (Jurkat-Rott et al., 2009).

In the transient expression system used by Fuster et al. (2017b), the specific conductance of the anomalous inward current was estimated to be  $19.6 \mu S/cm^2$  for R1239H (carried by protons in a TEA external solution) and  $68 \mu S/cm^2$  for the atypical HypoKPP mutation V876E (in 140 mM  $Na^+$ ). An interesting question is how these values translate to the heterozygous condition in HypoKPP patients. The endogenous  $Ca_v1.1$  was not ablated in the electroporation experiments, and remarkably, the peak  $Ca^{2+}$  current density ( $\sim 5$  A/F) was comparable with that in naive wild-type fibers. This implies that the total density of  $Ca_v1.1$  subunits (endogenous and transfected) at the membrane was comparable with naive fibers. Moreover, electroporation studies with a mutated  $Ca_v1.1$  to reduce isradipine sensitivity showed that up to 70% of the current was insensitive to block (DiFranco et al., 2011). This suggests that the majority of  $Ca_v1.1$  subunits at the membrane are from the transfected construct. If a similar ratio of V876E to wild-type channels occurred in Fuster's preparation, then the equivalent specific conductance in a heterozygous patient would be  $49 \mu S/cm^2 = (68 * 0.5/0.7)$ , which is notably higher than for the other HypoKPP mutant channels and may account for the more severe clinical phenotype associated with this particular mutation.

More studies are needed to determine whether the inward current for V876E actually passes through the same gating pore conduction pathway as the one created by R/X mutations in S4. For example, the gating pore currents from R/X mutations in S4 of  $Na_v$  or  $K_v$  channels, as well as I  $\rightarrow$  H mutations in S1 or S2 of Shaker K channels, all have strong rectification caused by movement of the voltage sensor (Campos et al., 2007). Conversely, V876E has minimal rectification over  $-120$  to  $0$  mV in the presence of a symmetrical gradient for the permeant ion. This discordance invites alternative models of the abnormal current demonstrated in this valuable new study.

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