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COMMENTARY

Evaluation of mutant muscle Ca^{2+} channel properties using two different expression systems

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Hypokalemic periodic paralyses are genetic diseases characterized by attacks of muscle paralysis that are associated with a decrease in blood K^+. Type 1 hypokalemic periodic paralysis (HypoPP1) arises from mutations in the gene encoding the main subunit of the skeletal muscle voltage-gated Ca^{2+} channel, Cav1.1, whereas type 2 (HypoPP2) results from mutations in the gene encoding the α subunit of the skeletal muscle voltage-gated Na^+ channel, Nav1.4 (Cannon, 2015). In both cases, the vast majority of mutations are of the arginines in one of the voltage sensor S4 segments, likely explaining common clinical phenotypes observed in HypoPP1 and 2 despite the different physiological roles supported by these channels. Whereas Nav1.4 expression in cell heterologous systems has facilitated extensive functional characterization of HypoPP2-mutated Nav1.4, the difficulty in expressing Cav1.1 in nonmuscle cells has considerably hampered the search for the pathogenic mechanisms involved in HypoPP1. Nevertheless, the recent use of different expression systems has allowed a thorough exploration of the biophysical properties of HypoPP1-mutated Cav1.1 (Fuster et al., 2017a,b), including one reported in a recent issue of this journal (Wu et al., 2018).

Apart from two mutations—one localized in the S3 segment of domain III (V876E) and one in the S4-S5 loop of domain III (H916Q)—all HypoPP1 mutations consist of a substitution of the first or second outermost basic residue with a less charged amino acid (histidine, serine, or glycine) in the voltage-sensing S4 segment of domain II (R528H/G), domain III (R897S, R900S), or domain IV (R1239H/G) (Cannon, 2010; Matthews and Hanna, 2010; Jurkat-Rott et al., 2012; Moreau et al., 2014; Allard and Fuster, 2018). Long before the identification of HypoPP1 mutations (Jurkat-Rott et al., 1994; Ptácek et al., 1994), early experiments performed in muscle fibers from HypoPP1 patients' muscle biopsies had shown that diseased muscle cells were abnormally depolarized in low K^+ external solution to the point of rendering cells inexcitable (Rüdel et al., 1984; Ruff, 1999). This critical finding was initially puzzling because the Ca^{2+} channel, although having a dual voltage-sensing role in controlling the opening of the SR Ca^{2+} release channel as well as itself, is not directly involved in muscle excitability. Key studies performed on voltage-gated K^+ and Na^+ channels expressed in heterologous systems, which possess comparable voltage-sensing domains, have revealed that the replacement of arginine residues in S4 segments with smaller uncharged residues created a hydrophilic pathway permeable to protons and/or cations (Starace and Bezanilla, 2004; Sokolov et al., 2005; Tombola et al., 2005; Struyk and Cannon, 2007). For mutations affecting the outermost arginine, this anomalous pathway was shown to be open at resting potentials, giving rise to an inward cationic current called gating pore current or ω current. The pathway shuts upon depolarization, when the outward-moving S4 segment transposes another arginine to occlude the mutation-induced pathway. Because the vast majority of HypoPP1 mutations also correspond to substitution of the outermost arginine in Cav1.1, it has been tempting to propose that a depolarizing gating pore current flows through the voltage-sensing domain of the HypoPP1-mutated Ca^{2+} channels at resting potentials, as observed in mutated K^+ or Na^+ channels.

As mentioned above, in contrast to voltage-gated K^+ and Na^+ channels, the search for a gating pore current in HypoPP1 Cav1.1 has been considerably restrained by the difficulty in expressing skeletal muscle Ca^{2+} channels in nonmuscle cells. An elevated leak current at hyperpolarized potentials has nevertheless been found in muscle fibers from HypoPP1 patients, in muscle fibers from a transgenic mouse expressing a HypoPP1 mutant Cav1.1, and in a dysgenic muscle cell line transfected with a HypoPP1 mutant Cav1.1 (Jurkat-Rott et al., 2009; Wu et al., 2012; Bednarz et al., 2016). Recently, three studies have implemented two different strategies to explore a gating pore current in HypoPP1-mutated Cav1.1. One strategy took advantage of the recent finding that the STAC3 protein, initially identified in the triad and involved in the excitation–contraction coupling process, promoted the trafficking of Cav1.1 to the membrane in heterologous expression systems (Polster et al., 2015, 2016). By coexpressing STAC3

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with human WT HypoPP1 R528H or R528G mutant Cav1.1 channels, plus the α2-δ1b and β1a subunits, Wu et al. (2018) were able to obtain a high level of expression of the Ca^{2+} channels in *Xenopus laevis* oocytes. Using the cut-open oocyte voltage clamp technique, Wu et al. (2018) found a reduced conductance and a left-shifted voltage-dependence of activation in Ca^{2+} channels with HypoPP1 R528H and R528G mutations. More importantly, they revealed a very significant increase in the leak current over the –120 to –20 mV voltage range in R528H and R528G mutants with no apparent rectification, consistent with the development of an anomalous gating pore conductance that is open at hyperpolarized potentials. Ion substitution experiments also indicated that the R528H gating pore current was primarily carried by Na^+, although in most cases R to H substitutions were reported to lead to proton-permeable gating pores (Starace and Bezanilla, 2004; Struyk and Cannon, 2007). The R528G mutant gating pore was found to be permeable to guanidinium, but ion selectivity was not determined in the presence of physiological saline. These experiments provided convincing evidence that HypoPP1 R528H/G mutant Cav1.1 channels support a pathogenic gating pore current at negative voltages.

A different strategy consisted of transfecting GFP-tagged human WT and HypoPP1 mutant Cav1.1 into adult mouse hind limb muscles by in vivo electroporation. All these transfected Cav1.1 isoforms were found to properly localize in the t-tubule membrane, allowing reliable measurements of Cav1.1 transmembrane currents under voltage clamp (Fuster et al., 2017a,b). In a first paper, Fuster et al. (2017a) showed a shift toward negative potentials of voltage-dependent activation and a reduced conductance of Ca^{2+} channels for the HypoPP1 R1239H mutant. Using a combination of voltage-clamp and intracellular pH measurements, they also demonstrated that the R1239H mutation induced an elevated leak H^+ current for membrane potentials more negative than –40 mV, likely flowing through a gating pore created by the mutation. Using the same expression strategy in combination with voltage-clamp, intracellular pH, and intracellular Na^+ measurements, Fuster et al. (2017b) investigated a HypoPP1 Cav1.1 mutation at V876 that has the peculiarity of not being located in an S4 segment, raising the question of whether such a mutation could induce a gating pore current. They first showed that Ca^{2+} current properties were the same in V876E and WT fibers, demonstrating that HypoPP1 pathogenesis is not related to any change in the Ca^{2+} channel function of Cav1.1. Surprisingly, as for mutations located in S4 segments, the V876E mutation also induced a very large inward leak current at negative voltages carried by H^+ in the absence of other external permeant cations, as noted by Chahine (2018). However, Chahine (2018) did not comment on the additional finding that the leak current in V876E-expressing fibers was carried by Na^+ in the presence of physiological saline. This result is reminiscent of histidine scanning mutagenesis studies in *Shaker* K^+ channels, in which mutated residues in S2 or S1 segments are able to create a gating pore lined by these mutated residues and the most extracellular arginine of the S4 segment, in the same manner as for arginine substitution (Campos et al., 2007). Thus, the effect of the V876E mutation is perhaps not as surprising as it might first seem. It is thus very likely that, in the Ca^{2+} channel too, valine 876 and one of the most external arginines in the S4 segment form a narrow hydrophobic plug separating the internal and external solutions, which is disrupted when valine is replaced by glutamate and results in a gating pore. It is also not surprising that the V876E gating pore current is reduced with depolarization in the same voltage range that leads to activation of intramembrane charge movements (Collet et al., 2003). Indeed, as described in the *Shaker* K^+ channel, intramembrane charge movements reflect the translocation of S4 segments in the Ca^{2+} channel in response to depolarization that should carry another arginine to occlude the pore.

Presently, four of the eight HypoPP1 mutations in the Ca^{2+} channel (R528H/G, R1239H, and V876E) have been found to generate a gating pore. The two experimental strategies described above have thus proven complementary to demonstrate that the generation of a gating pore likely constitutes a pathogenic mechanism common to all HypoPP1 disorders. Both have their own advantages and drawbacks. STAC3-enhanced membrane expression of Cav1.1 in oocytes undoubtedly offers a very robust and reliable system that is easily implemented to investigate the function of Cav1.1 mutations while minimizing the contribution of contaminating conductances (Chahine, 2018; Wu et al., 2018). The main drawback is that the channel is not expressed in the highly organized region of the triad in fully differentiated adult skeletal muscle, which may alter the biophysical properties of the channel. Along this line, the STAC3-coexpressed Cav1.1 L-type Ca^{2+} current in oocytes was not found to inactivate in response to maintained depolarization, whereas a well identified voltage-dependent inactivation mechanism is known to occur in fully differentiated muscle fibers (Collet et al., 2003; Ursu et al., 2004). The presence of a native muscle environment for the transfected channel is the main advantage of the in vivo mouse model system (Fuster et al., 2017a,b; Allard and Fuster, 2018). Inversely, in this system, the endogenous mouse Ca^{2+} channels are not entirely replaced by the transfected channels so that the biophysical properties of the Ca^{2+} channel inevitably correspond to a mixture of the biophysical properties of endogenous and transfected channels. Despite this limitation, the use of a physiological external Ca^{2+} concentration (2.5 mM) to assess the Ca^{2+} channel current; external and internal solutions that can be dialyzed within the fiber; good voltage control provided by the silicone clamp technique (Lefebvre et al., 2014); and the opportunity to combine fluorescence measurements of H^+ and Na^+ flux with voltage clamp readily allow the investigation of Ca^{2+} currents as well as the voltage-dependence and selectivity of mutation-dependent gating pore currents. It is also valuable that the human WT Cav1.1 Ca^{2+} current was found to display comparable properties to the endogenous mouse muscle Ca^{2+} current. The large number of fibers tested in these studies has caused concern and has led to the suggestion that this large number was required to detect a gating pore current (Cannon, 2017; Wu et al., 2018). However, a statistical effect size test (Cohen's d test) in both studies indicated that the data were poorly influenced by the sample size. Moreover, testing of 11–17 fibers in mutant and WT Cav1.1-expressing fibers was sufficient to reveal a statistically significant elevation of H^+ and Na^+ influx in R1239H and V876E mutant fibers and a Na^+ gating pore in V876E expressing fibers (Fuster et al., 2017a,b).

In the near future, implementation of STAC3-enhanced membrane expression of Cav1.1 and Cav1.1 transfection in mouse muscle should noticeably accelerate the biophysical characterization of gating pore currents through HypoPP1 Cav1.1 mutants. These two experimental strategies have demonstrated how relevant and, above all, complementary they are. Applying both will mutually ensure that their drawbacks do not introduce any bias in the data.

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