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COMMENTARY

Improving the characterization of calcium channel gating pore currents with Stac3

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$Ca_V1.1$ channels are organized into four homologous domains (domains I–IV), each composed of a nonpermeable voltage sensor domain (VSD) and a calcium-permeable pore domain. Mutations in the VSD may create a leak and render the VSD permeable to ions. Recording of such a leak current, known as a gating pore current, was hampered by the low expression of these channels in a heterologous expression system. In this issue, Wu et al. show for the first time the biophysical properties of this current using Stac3 to boost the expression of the $Ca_V1.1$ channel in *Xenopus laevis* oocytes.

Early studies

The first evidence of a current flowing through a mutated voltage sensor domain (VSD) was reported by Starace and Bezanilla (2004). This was a somewhat fortuitous discovery, as the authors' studies at the time were focused on the structure and operation of the VSD and the accessibility of the S4 segment arginine residues during gating. They showed that substitution of the first positive charge (R1) of the S4 segment of the *Drosophila melanogaster* Shaker VSD to a histidine residue (R1H) led to the appearance of a specific proton (H^+) leak. The current that the authors observed was sensitive to changes in extracellular pH, and they proposed that H^+ ions pass through the channel protein using a pathway distinct from the physiological K^+ ion permeation pathway. The H^+ leak was not sensitive to agitoxin II, a toxin known to obstruct the pore of the channel. However, the current did pass through the VSD via a histidine factor, which can bind a proton from the extracellular medium and then release it into the intracellular medium.

The R1H substitution revealed the presence of a permeation pathway across the VSD of the Shaker K^+ channel, most probably involving a proton wire, through which H^+ ions could translocate into the cell in a way similar to that proposed for gramicidin channels (Starace and Bezanilla, 2004). Proton conduction through gramicidin channels has been described as occurring via a hop and turn mechanism in which H^+ ions hop between water molecules, accounting for the high H^+ selectivity observed.

Subsequent studies by Tombola et al. (2007) showed that the substitution of Shaker R1 with smaller uncharged amino acid residues generated a gating pore current that the authors called the "omega current." In this case, the current was selective for cations rather than H^+ ions and conducted guanidinium efficiently. To date, three pathologies have been associated with the creation of a gating pore: periodic paralysis, peripheral nerve hyperexcitability, and cardiac arrhythmias associated with dilated cardiomyopathy.

Periodic paralysis

Periodic paralysis manifests in two forms: hypokalemic periodic paralysis (HypoPP) and normokalemic periodic paralysis (NormoPP). HypoPP is characterized by paralytic attacks that occur in the context of low serum K^+ concentrations (<3 meq/liter). These paralytic episodes are triggered by various factors including exercise, emotional stress, cold, fever, and high-fat meals (Cannon, 2006). HypoPP has been associated with mutations in $Ca_V1.1$, the skeletal muscle Ca^{2+} channel encoded by the *CACNA1S* gene (60% of cases), and $Na_V1.4$, the skeletal muscle Na^+ channel encoded by the *SCN4A* gene (10% of cases). However, initial studies reported only minor impairments of the biophysical properties of mutant channels. In addition, divergent biophysical properties have been reported for some mutations in the *SCN4A* gene, which manifest as either increased or reduced inactivation.

Paralytic attacks occur in response to the presence of two stable resting membrane potentials (VRest) of muscle myocytes. The value of the first stable VRest is approximately −75 mV, and the value of the second stable VRest in pathological myocytes is approximately −60 mV (−85 mV being the normal resting potential of a myocyte; Struyk and Cannon, 2007; Struyk et al., 2008; Jurkat-Rott et al., 2009; Cannon, 2010; Wu et al., 2011). When myocytes adopt the second stable VRest, they become nonexcitable or paralytic (Jurkat-Rott et al., 2009). However, the HypoPP biophysical defects initially reported were of different

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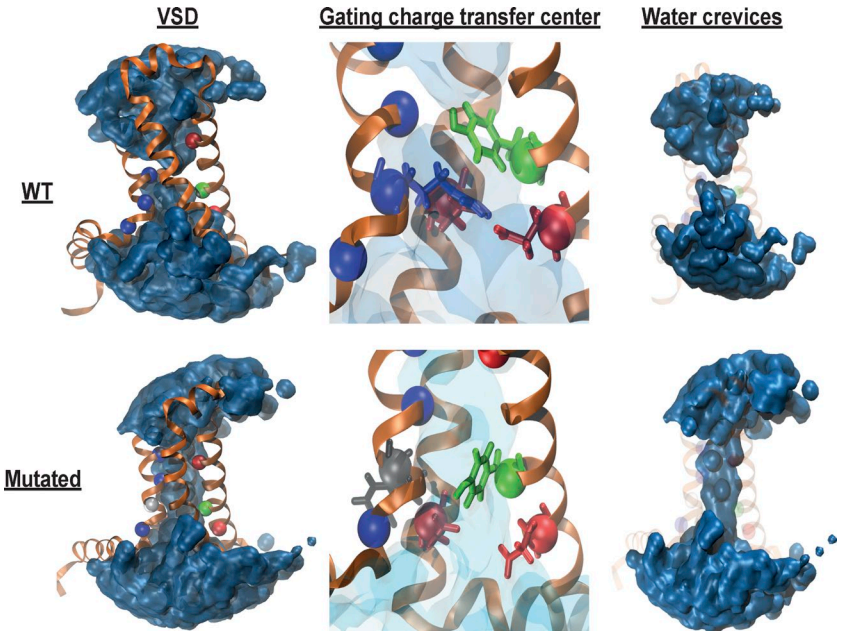

Figure 1. **3-D structure of the voltage-sensing domain and creation of a gating pore.** 3-D structure of the domain I VSD structure of WT (top) and mutated (R225W, bottom) $Na_V1.5$ channels in a partially activated state (β state). The protein segments are shown in orange and the volume occupied by water in blue. The amino acids participating in the gating charge transfer center (GCTC) are shown as blue spheres for positive charges on the S4 segment, red for negative charges on the S2 and S3 segments, and green for positive charges on the S4 segment. A conserved aromatic amino acid is shown on the S2 segment. The left panels show the entire VSD, the S1 segment has been deleted in the middle, and right panels are shown for the sake of clarity. The middle panels highlight the close interactions between the positive charges on the S4 segment and the GCTC. These interactions allow the formation of a hydrophobic septum in the center of the VSD, isolating the water crevices on both sides of the membrane (right). In the context of a mutation (here, R225W), the interactions between S4 and GCTC are broken, causing the junction of the water crevices, creating a continuous aqueous pathway, and opening a gating pore (adapted from Moreau and Chahine, 2015).

magnitude and thus did not explain the elevation of VRest values (Cannon, 2010). This led to several studies aimed at identifying new underlying molecular mechanisms to explain the biophysical properties.

Because the reported HypoPP mutations of $Ca_V1.1$ and $Na_V1.4$ channels are located on the S4 of various domains, several studies focused on identifying a mutation-dependent gating pore. Gating pore currents are ionic currents that do not flow through the permeation pore of the channel, but instead flow through the VSD, which is composed of the S1–S4 segments of the channel (Fig. 1). The highly charged S4 segments in these complexes are surrounded by water crevices that extend deep into the membrane from both the extracellular and intracellular surfaces. Only a narrow proteinaceous region separates the inner cavity from the outer cavity. Normally, ions do not cross the voltage sensor complex. Rather, charged residues on the S4 segments (Arg or Lys) are translated from the inner crevice to the outer crevice during gating. However, several investigators have shown that mutations of these charged residues can induce gating pores (Sokolov et al., 2007; Struyk and Cannon, 2007). Gating pore currents represent ∼1% of the permeation pore current in Na^+ channels, but can be large in K^+ channels because of the fourfold symmetry of the subunits (∼6% of the permeation pore current).

NormoPP is a variant of HypoPP that is characterized by episodes of paralysis at physiological levels of serum K^+ (Vicart et al., 2004). The gating pore has been identified as being responsible for the development of this pathology. Unlike HypoPP, the creation of an open gating pore at depolarized (and not hyperpolarized) potentials is likely the pathological cause of the paralytic episodes in NormoPP (Sokolov et al., 2008). The pathological mechanism involved may be related to the well-known immobilization phenomenon of the S4, which would cause a temporarily permeable gating pore when the potential is hyperpolarized. This temporary immobilization would lead to the passage of Na^+ ions through the gating pore after each action potential, which could result in an overload of intracellular Na^+. The pathological mechanism would thus be similar to that described for HypoPP; however, the primary effect of these mutations would still be ion leakage in the depolarized state (Sokolov et al., 2008; Fan et al., 2013; Groome et al., 2014). Given the ionic selectivity, K^+ should be the most permeating physiological ion when the gating pore is open. However, the pathological consequences of such an ion leak have not been clearly elucidated.

Peripheral nerve hyperexcitability

Mutations in the VSD of the $K_V7.2$ channel (R207Q and R207W) have been associated with the development of peripheral nerve hyperexcitability (Dedek et al., 2001; Wuttke et al., 2007). $K_V7.2$ channels, which are expressed in the brain and spinal cord, oligomerize with $K_V7.3$ channels to generate the M current, which primarily functions to maintain the resting membrane potential. It is interesting to note that $K_V7.2$ channel mutations are usually located in the pore domain and are associated with the development of neonatal epilepsies, which suggests a role in maintaining the resting potential. Because of experimental difficulties with $K_V7.2$ channel expression, studies have focused on mutations in the $K_V7.4$ channel that are equivalent to R207Q and R207W (Miceli et al., 2012). Two such mutations result in the creation of a gating pore that remains open at depolarized potentials. The appearance of this new conductance would cause a slight depolarization of the membrane potential of motor neurons, facilitating the generation of action potentials and ultimately leading to cellular hyperexcitability.

Cardiac arrhythmias and dilated cardiomyopathy

Several similar mutations in the S4 segments of the $Na_V1.5$ channel (the cardiac homologue of the $Na_V1.4$ channel) have been identified. Patients with these mutations have an atypical clinical phenotype that associates complex cardiac arrhythmias with dilated cardiomyopathy. Although the clinical phenotypes

observed in patients with these mutations are similar, the first biophysical studies of these mutations have described, surprisingly, divergent biophysical alterations. The biophysical characterization of the R219H mutation, identified in a patient with cardiac arrhythmia and dilated cardiomyopathy, revealed the existence of a proton-selective gating pore current activated by hyperpolarization (Gosselin-Badaroudine et al., 2012). This alternative permeation pathway was subsequently identified in the context of two other mutations (R222Q and R225W; Moreau et al., 2015) that cause the appearance of a selective pore through which cations permeate after depolarization. The creation of a gating pore could thus be a new pathological mechanism for causing cardiac dysfunctions. However, the cascade of events linking the creation of such a permeation pathway and the clinical phenotype is still a matter of debate.

Gating pore currents in calcium channels

Although gating pore currents have been reported in Na^+ channels, the detection of such currents in Ca^{2+} channels has been hampered by the low expression levels of Ca^{2+} channels in heterologous expression systems. Therefore, in the case of HypoPP, although most mutations affect Ca_v channels, the majority of studies concern Na_v mutations. However, recent progress has been made with recordings from muscle fibers from knockin mutant mice using the three-microelectrode voltage-clamp approach (Wu et al., 2011) and, after the overexpression of these channels by in vivo local electroporation, with currents recorded using the silicone-clamp method. However, even with these approaches, the presence of other ion channels has made the characterization of gating pore currents in Ca^{2+} channels very difficult.

In this issue of the *Journal of General Physiology*, Wu et al. (2018) took advantage of the fact that $Ca_V1.1$ channels are highly expressed when coexpressed with Stac3. Stac3 is a skeletal muscle–specific protein that localizes to the triad and is a component of the excitation–contraction coupling machinery. Mutations in human Stac3 cause myopathy (Horstick et al., 2013). Stac3 coexpression has been shown to enhance the levels of $Ca_V1.1$ at the cell surface (Horstick et al., 2013; Polster et al., 2015). The authors coexpressed Stac3 in *Xenopus laevis* oocytes and found that the 200-fold increase in Ca^{2+} currents was sufficient to ascertain whether HypoPP mutant $Ca_V1.1$ channels are leaky because of missense mutations of arginine residues in the S4 segments of the VSD. Using the high-resolution, cut-open oocyte voltage-clamp method to record currents, the authors showed that R528H and R528G (R1H/R1G) in the S4 of domain II both support gating pore currents. However, unlike other R/H HypoPP mutations, R528H does not selectively conduct an H^+ current.

This is an interesting advance in terms of recording gating pore currents from mutated Ca^{2+} channels. It does, however, raise several questions that warrant further studies, such as developing a structural model to investigate why R528H displays mixed Na^+ and H^+ selectivity and why it is impermeable to guanidinium ions; determining the efficacy of the histidine in that position as a H^+ transporter; and determining the position of R528 in the charge transfer center. Such results would indicate whether the R528H-dependent gating pore has a size and permeation pathway different from other R/H mutations. However, it should be noted that the authors did not use any offline linear leak subtraction (Fig. 7 in Wu et al., 2018). Such a process is usually used to get rid of inherent nonspecific leak and would allow an easier way to assess the gating pore properties.

The majority of $Ca_V1.1$ mutations identified in HypoPP patients are located in the S4 segment of the VSD. Recently, a new mutation (V876E), which is located in the S3 segment of domain III VSD rather than in the S4 segment, has been identified in patients with severe HypoPP outcomes (Ke et al., 2009). Functional studies using electroporated muscle fibers showed that muscle fibers expressing the V876E mutation exhibit a leak current at negative voltages, which is increased by external acidification, suggesting that the leak current is carried by H^+ ions (Fuster et al., 2017). This constitutes a highly intriguing advance because all positive S4 charges could still prevent the permeation of cations. Furthermore, the authors also described a voltage dependence of the current that remains to be understood. Coexpressing the $Ca_V1.1$ channel carrying the V876E mutation with Stac3 could be used to confirm whether such a mutation induces a gating pore current in a heterologous expression system and to study its biophysical properties (ion selectivity and voltage dependence) in greater detail. Most voltage-gated K^+, Na^+, and Ca^{2+} channels are built in a similar way; VSDs, comprising four transmembrane segments, drive the opening of the 4 × 2 transmembrane pore domain. Because of previous experimental difficulties, the intimate biophysical properties of Ca_v channels were often extrapolated from the knowledge acquired from the study of other voltage-gated ion channels. A recent study (Capes et al., 2012) has reported that gating pores cannot be opened by single S4 arginine mutations in domain IV of Na_v channels. In contrast, similar mutations do create gating pores in Ca_v channels, illustrating profound differences between these two channels besides structural and functional similarities.

The coexpression of Stac3 used by Wu et al. (2018) thus brings the opportunity to access and study the specific biophysical properties of Ca_v channels.

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