Specific contributions of the four voltage-sensing domains in L-type calcium channels to gating and modulation

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Voltage-gated cation channels contain a single pore surrounded by four voltage-sensing domains (VSDs), each containing a critical component of voltage sensing, the S4 transmembrane segment. In response to membrane depolarization, the VSDs undergo a conformational change that results in positively charged residues within each of the S4 segments to move across the plane of the lipid bilayer, causing the channel's activation gate to open. However, many questions about this mechanism remain. Why are there four VSDs per channel? Are all four VSDs needed to activate the channel? If not, how many, and which ones, need to move for channel activation? Do all VSDs contribute equally to the gating properties of the channel, or do different VSDs perform specialized tasks? In the past, these questions have been subject to intensive investigations in the potassium and sodium channel fields. However, pertinent evidence was scarce and mostly indirect for voltage-gated calcium channels (Ca_Vs), until the research team of Riccardo Olcese at the University of California at Los Angeles introduced the voltage-clamp fluorometry method to the study of Ca_V channels (Pantazis et al., 2014). In this issue, Savalli et al. apply this powerful approach to determine which VSDs are involved in the modulation of $Ca_V1.2$ gating properties by the auxiliary $\alpha_2\delta$-1 channel subunit (Savalli et al., 2016). Their results demonstrate that the VSDs that principally govern channel gating are also subject to modulation by $\alpha_2\delta$-1.

Ca_Vs transduce membrane depolarization into cellular functions such as secretion of hormones and neurotransmitters, contraction of striated and smooth muscles, and gene regulation. Currents through Ca_Vs contribute to pacemaking and action potential shape in nerve and muscle cells, and calcium influx through Ca_V channels regulates the different signaling pathways involved in cell functions as diverse as fertilization, cell division, metabolism, differentiation, learning and memory, and even cell death. Accordingly, the different isoforms and splice variants of Ca_V channels operate within a much wider range of membrane potentials than voltage-gated sodium channels (Lipscombe et al., 2013), hence their subdivision into low-voltage– and high-voltage–activated channels (Ca_V3 and Ca_V1-2 channels, respectively). High-voltage–activated Ca_Vs are multisubunit ion channel complexes composed of a pore-forming α_1 subunit ($Ca_V1.1$-4 and $Ca_V2.1$-3) and several auxiliary channel subunits: $\alpha_2\delta$1-4, β1-4, calmodulin, and, in skeletal muscle, the γ1 subunit and STAC3 (Campiglio and Flucher, 2015). The auxiliary subunits are involved in targeting the channel complexes to specific membranes and, in addition, different combinations of auxiliary subunits endow calcium channels with specific functional properties.

The α_1 subunits of Ca_V (and Na_V) channels are pseudotetrameric channels (Fig. 1). Each of the four homologous repeats contains six transmembrane helices (S1–S6), the first four of which (S1–S4) form a functional VSD, whereas S5 and S6 plus the connecting P-loop of all four repeats together form the channel pore containing the ion selectivity filter and the activation gate (Catterall, 2011). Previous work on the sodium channel, and isolated findings from the analysis of natural and experimentally introduced mutations in Ca_V channels, suggested that the four VSDs (I, II, III, and IV) contribute differently to activation of these cation channels. In sodium channels, combined evidence from classical biophysical work, mutagenesis, pharmacology, and voltage-clamp fluorometry indicated that VSDs I–III control channel activation, whereas VSD IV determines voltage-dependent inactivation (Ahern et al., 2016). For Ca_V channels, most of the information pertinent to this question comes from studies of L-type calcium channels, specifically $Ca_V1.1$ and $Ca_V1.2$. The latter is widely expressed in the nervous system, the cardiovascular system, and endocrine cells. In contrast, $Ca_V1.1$ is specifically expressed in skeletal muscle and, because of its unique properties, represents a striking example of the division of labor among the four VSDs of a pseudotetrameric channel.

The skeletal muscle $Ca_V1.1$ isoform is unique in the sense that its voltage sensors independently activate two distinct functions: excitation-contraction (EC) coupling and current conduction through the L-type calcium channel itself (Melzer et al., 1995). With physiological

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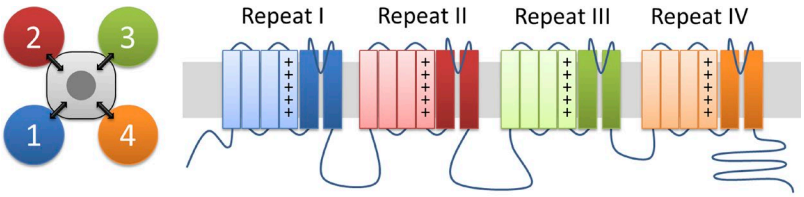

$Ca_V1.2$					
VCF:	kinetics, (V½)	V½	V½	--	(1)
Mutation:	kinetics	--	--	--	(2)
$\alpha_2\delta$-1					
siRNA, VSD:	kinetics	V½	V½	--	(3,4)
$Ca_V1.1$					
Mutation:	kinetics	(ECC)	(ECC)	V½	(5,6,7)
$\alpha_2\delta$-1					
siRNA:	kinetics	--	--	--	(8)
Structure:	L1-2, L5	L5	L5	--	(9)

Figure 1. **Pseudotetrameric domain structure of Ca_Vs with functions associated to each VSD.** Each of the four homologous repeats contains a functional VSD (first four transmembrane helices) and contributes one fourth of the channel pore. Voltage-clamp fluorometry (VCF), mutagenesis, siRNA knockdown, and structure studies provide evidence for distinct functions and interactions of each of the four VSDs of $Ca_V1.2$ and $Ca_V1.1$ channels and their modulation by $\alpha_2\delta$-1. Kinetics and $V_{1/2}$ refer to kinetics and voltage dependence of activation, respectively. L1-2 and L5 refer to extracellular loops connecting transmembrane helices S1 and S2 and helix S5 with the pore, respectively. References: (1) Pantazis et al. (2014); (2) Nakai et al. (1994); (3) Tuluc et al. (2007); (4) Savalli et al. (2016) in this issue; (5) Tuluc et al. (2016a); (6) Eltit et al. (2012); (7) Tuluc et al. (2009); (8) Obermair et al. (2005); (9) Wu et al. (2015).

depolarizations, movement of the $Ca_V1.1$ gating charges directly activates the physically associated calcium release channel (type 1 ryanodine receptor) in the sarcoplasmic reticulum. In contrast, nonphysiologically long and strong depolarizations are required to activate relatively small calcium currents in skeletal muscle. These limitations on the speed and voltage dependence of $Ca_V1.1$ calcium current activation are controlled by VSD I and IV, respectively. Early chimera studies, in which sequences of VSD I were exchanged between $Ca_V1.1$ and $Ca_V1.2$, demonstrated that VSD I is important for determining the specific activation kinetics of these two Ca_V1 isoforms (Nakai et al., 1994; Tuluc et al., 2016a). Furthermore, alternative splicing of exon 29 in VSD IV of $Ca_V1.1$ was shown to give rise to two channel variants with greatly different voltage dependence of activation and reduced current amplitude (Tuluc et al., 2009), identifying VSD IV as a rate-limiting factor for the voltage dependence of current activation. These experiments clearly demonstrated that, in $Ca_V1.1$, VSDs I and IV are necessary for the activation of L-type calcium currents but contribute differentially to the kinetics and voltage dependence of activation. Conversely, because activation of EC coupling is faster than current activation and occurs at physiological voltages at which the current is not activated, it can be reasoned that activation of the slow VSD I and the voltage-insensitive VSD IV of $Ca_V1.1$ are not necessary for activation of EC coupling (Tuluc and Flucher, 2011). This conclusion is further supported by a disease mutation in VSD I (R174W) of $Ca_V1.1$, which ablated L-type calcium current without affecting EC coupling (Eltit et al., 2012).

The recent voltage-clamp fluorometry study of $Ca_V1.2$ provided for the first time a direct analysis of the kinetics and voltage dependence of individual VSDs in calcium channels (Pantazis et al., 2014). This technique had been used previously and extensively to analyze VSD movement in K_V and Na_V channels (Priest and Bezanilla, 2015; Ahern et al., 2016). To allow fluorescent labeling of the VSDs of $Ca_V1.2$, a cysteine was introduced into the extracellular flank of the S4 transmembrane helix of each of the four VSDs, one at a time. After expression of these $Ca_V1.2$ constructs in *Xenopus* oocytes, the cysteine was labeled with a thiol-reactive fluorophore. Upon depolarization, the positively charged S4 helix moves across the membrane in an outward direction, and this structural rearrangement of the VSD results in an altered extent of quenching of the fluorophore. Thus, any change (up or down) in the fluorescence signal provides a readout of conformational changes in the respective VSD. The observed rapid response of the fluorescence signal indicated that the conformational changes indeed reflect the response of the individual VSD to the changed membrane potential. Although the fluorometry signal does not provide information about the nature and absolute magnitude of the structural rearrangement, the kinetics and voltage dependence of the individual VSD can be faithfully recorded in parallel to the voltage-clamp recording. Finally, the slope of the voltage sensitivity curves is a measure of the effective charge moved across the electric field. Changes thereof can arise either from altered length and slope of the S4 trajectory across the membrane or from changes in the electric field resulting from a different distribution of hydrophilic and hydrophobic regions within the channel (Priest and Bezanilla, 2015).

The interpretation of voltage-clamp fluorometry data regarding the effects of VSD movement on pore opening is greatly facilitated by mathematical gating models. In the earlier study, Olcese's group examined two types of models based on different assumptions (Pantazis et al., 2014). The obligatory models assume that each VSD is either necessary or not for activation of pore opening. The allosteric model assumes that all VSDs contribute

to pore opening to different degrees, and therefore this model provides quantitative information on the contribution of each VSD.

In the human $Ca_V1.2$, all VSDs moved in response to depolarization, excluding the possibility that one or more VSDs remain locked in the activated or inactivated state during depolarization. The kinetics of VSDs I, II, and III were faster or similar to that of current activation (Pantazis et al., 2014). However, VSD IV moved much slower, indicating that movement of this VSD is not required for channel opening. The voltage dependence of VSDs II, III, and IV was more negative than that of ion conductance, whereas the voltage dependence of VSD I coincided with that of ion conductance. This suggests that VSD I may be rate limiting for the voltage dependence of channel opening. The data were reasonably well fit by a gating model that assumes obligatory activation of only VSDs II and III for pore opening. The allosteric model indicated that VSDs II and III together contributed ∼85%, and VSD I ∼15%, to channel gating. Importantly, the model indicated virtually no contribution of VSD IV to pore opening. As this VSD moved at very low (left-shifted) depolarizations, but with kinetics much slower than channel activation, its movement is probably not coupled to gate opening. In analogy with the situation in Na_V channels (Ahern et al., 2016), it is tempting to speculate that the movement of this VSD might initiate voltage-dependent inactivation of $Ca_V1.2$ channels.

These experiments described the properties of the complete $Ca_V1.2$ α_1:$\alpha_2\delta$-1:β_3 channel complex expressed in *Xenopus* oocytes (Pantazis et al., 2014). In the present study, Savalli et al. (2016) examine the role of the $\alpha_2\delta$-1 subunit to channel gating by expressing $Ca_V1.2$:β_3 without $\alpha_2\delta$-1. First of all, the authors find that the absence of $\alpha_2\delta$-1 specifically slows down the kinetics of VSD I to about half of the activation speed of the complete channel complex, whereas the kinetics of the other VSDs remain unaltered. This effect is consistent with the primary function of $\alpha_2\delta$-1 in regulating the activation kinetics of $Ca_V1.2$ and $Ca_V1.1$ channels when expressed in a native muscle expression system. In reconstituted dysgenic ($Ca_V1.1$-null) muscle cells, siRNA knockdown of $\alpha_2\delta$-1 decelerated activation kinetics of $Ca_V1.2$ and accelerated activation kinetics of $Ca_V1.1$ (Obermair et al., 2005; Tuluc et al., 2007). Apparently, the $\alpha_2\delta$-1 subunit stabilizes the specific intrinsic activation properties of calcium channels as it makes the slow channel ($Ca_V1.1$) slow and the fast channel ($Ca_V1.2$) fast. Importantly, the voltage-clamp fluorometry experiments demonstrate that regulation of activation kinetics by $\alpha_2\delta$-1 is exclusively accomplished by VSD I (Savalli et al., 2016). This finding is consistent with the critical role of VSD I in regulating activation kinetics that has previously been demonstrated with $Ca_V1.1$/$Ca_V1.2$ channel chimeras (Nakai et al., 1994; Tuluc et al., 2016a). If, however, VSD I with the help of $\alpha_2\delta$-1 serves the critical role of determining activation kinetics, its movement/activation must be obligatory for pore opening. Therefore, these findings contradict the obligatory model III of Pantazis et al. (2014), in which only VSDs II and III are obligatory for channel activation. If its movement is irrelevant for pore opening, VSD I could not limit the speed of activation. In contrast, even if—as the allosteric model indicated—the energetic contribution of VSD I to activation may be minor, its activation can still be obligatory, and even rate limiting, for the speed of pore opening. The observation that the voltage dependence of VSD I most closely resembles that of ion conduction further supports this notion.

The most striking effect of the $\alpha_2\delta$-1 subunit on the biophysical properties of calcium currents is a 50-mV shift of the voltage dependence of activation ($V_{1/2}$) to less depolarizing potentials (Savalli et al., 2016). In the voltage-clamp fluorometry experiments, the dramatic right shift in $V_{1/2}$ of current activation in the absence of $\alpha_2\delta$-1 is accompanied by smaller but still substantial right shifts of VSD I, II, and III voltage dependence (Savalli et al., 2016). Also, the slope of the voltage dependence curves for VSDs I, II, and III decrease in the absence of $\alpha_2\delta$-1, indicating a decrease of the effective charge moved in each of these VSDs. This affects the energetic contribution of these VSDs to pore opening. According to the allosteric model, the presence of $\alpha_2\delta$-1 doubles the energetic contributions of VSDs I and III and triples that of VSD II.

Together, these findings demonstrate that the $\alpha_2\delta$-1 subunit exerts its effects on three of the four VSDs by facilitating their intrinsic functions. $\alpha_2\delta$-1 normalizes the speed of activation by increasing the activation kinetics of the one VSD (I) that determines kinetics, and it normalizes the voltage dependence of channel activation by left shifting the voltage dependence and increasing the voltage sensitivity of the three VSDs that govern pore opening (I, II, and III). Apparently, the $\alpha_2\delta$-1 subunit does not endow any of the VSDs with a particular function or property of its own, but the association of the $\alpha_2\delta$-1 with $Ca_V1.2$ appears to stabilize the channel complex in a conformation that brings about the most accurate movement of the VSDs in response to depolarization and coupling to pore opening.

Interestingly, in their earlier study (Pantazis et al., 2014), members of the Olcese group found that VSD IV does not at all contribute to pore opening (see discussion above), and here (Savalli et al., 2016) they show that the absence or presence of $\alpha_2\delta$-1 does not alter the properties of VSD IV. In agreement with a low-resolution structure of $Ca_V1.2$ (Walsh et al., 2009), the authors speculate that the largely extracellular $\alpha_2\delta$-1 protein might interact with the extracellular domain of VSDs I, II, and III but not of VSD IV. Indeed, the recent high-resolution structure of $Ca_V1.1$ revealed

that $\alpha_2\delta$-1 interacts with the L5 loops of repeats I–III and the loop connecting S1 and S2 of VSD I (Wu et al., 2015). This specific interaction with VSD I again is consistent with the unique role of VSD I in determining activation kinetics (Nakai et al., 1994; Tuluc et al., 2016a), as well as with the physiological role of $\alpha_2\delta$-1 in shaping the specific kinetic properties of $Ca_V1.1$ and $Ca_V1.2$ (Obermair et al., 2005; Tuluc et al., 2007). Surprisingly, the other interactions of $\alpha_2\delta$-1 with VSDs I, II, and III are with the L5 loops next to the pore-forming segments of the channel. These interactions likely affect the pore directly and only secondarily the movement of the VSDs. This suggests that the coupling between the pore region and VSD I, II, and III is capable of transmitting $\alpha_2\delta$-1 modulation in both directions. Although interactions of $\alpha_2\delta$-1 with the L1-2 loop of VSD I affect the speed of pore opening, interactions with the L5 loop in the pore-forming segments of repeats I–III might affect the coupling with the respective VSDs and thus their voltage dependence. Knowing the putative interaction domains, this hypothesis can now be tested by combining site-directed mutagenesis with voltage-clamp fluorometry.

How can the negligible contribution to pore opening of VSD IV, and the lack of its modulation by $\alpha_2\delta$-1 in $Ca_V1.2$ channels (Pantazis et al., 2014; Savalli et al., 2016), be reconciled with the critical importance of VSD IV in determining the voltage dependence of current activation and channel open probability found in $Ca_V1.1$ channels (Tuluc et al., 2009, 2016a,b)? The simplest explanation would be that the VSDs in the two L-type calcium channels serve fundamentally different roles in controlling channel gating. In light of the fact that the VSDs in the skeletal muscle isoform $Ca_V1.1$ serve the additional function of activating EC coupling, a different assignment of the VSDs to channel gating is plausible. If that is so, the nonhomologous roles of the four VSDs in $Ca_V1.2$ would be more like those in Na_V channels than those in its closest relative, $Ca_V1.1$. Another explanation would be specific interactions of this VSD with associated proteins. In fact, the high-resolution structure of the skeletal muscle $Ca_V1.1$ complex demonstrated that the γ_1 subunit interacts with the S3 segment of VSD IV (Wu et al., 2015). This interaction would occur in the skeletal muscle expression system but not with recombinant expression of $Ca_V1.2$ in *Xenopus* oocytes. Alternatively, it is possible that inclusion of exon 29 in the adult $Ca_V1.1a$ splice variant not only shifts the voltage dependence of VSD IV activation, but at the same time changes its functional link to the channel pore. In that case, the developmental isoform $Ca_V1.1e$ (lacking exon 29) might function like $Ca_V1.2$, where VSD IV moves at low voltages but remains idle with respect to pore opening. Its activation at very low voltages is required for pore opening but does not contribute any energy to it. In contrast, in the mature isoform $Ca_V1.1a$, inclusion of exon 29 might stabilize VSD IV in the closed position and thus put a brake on pore opening, unless it becomes activated by nonphysiologically strong depolarizations. Future studies will be necessary to solve this problem. The recent mutagenesis and voltage-clamp fluorometry studies will provide the tools to do so.

In conclusion, both mutagenesis and voltage-clamp fluorometry studies have demonstrated that the four VSDs of Ca_V1 channels are nonhomologous with respect to their biophysical properties and functions (Fig. 1). On the one hand, they display distinct voltage sensitivity and kinetics of activation; on the other hand, they appear to be differentially coupled to the channel pore and thus contribute different amounts of energy to pore opening. Only if activation of a given VSD is obligatory, or contributes significantly to pore opening, can its biophysical properties and modulation by $\alpha_2\delta$-1 influence the macroscopic current properties. VSDs II and III show intermediate voltage dependence and make the major energetic contribution to pore opening. VSD I makes a small contribution to pore opening, but because of the relatively right-shifted $V_{1/2}$, it can be rate limiting for current kinetics. Modulation of these three VSDs (I, II, and III) explains the described modulation of gating properties by the $\alpha_2\delta$-1 subunit. VSD IV is the first to respond to membrane depolarization but appears to make no contribution to pore opening in $Ca_V1.2$. However, upon insertion of exon 29 in adult $Ca_V1.1$, the voltage dependence of activation of this channel is substantially right-shifted, indicating that VSD IV becomes rate limiting and can prevent pore opening. Whether these distinct properties and functions of the individual VSDs are specific for the respective channel isoforms, or represent a general pattern for all voltage-activated calcium channels, remains to be shown.

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