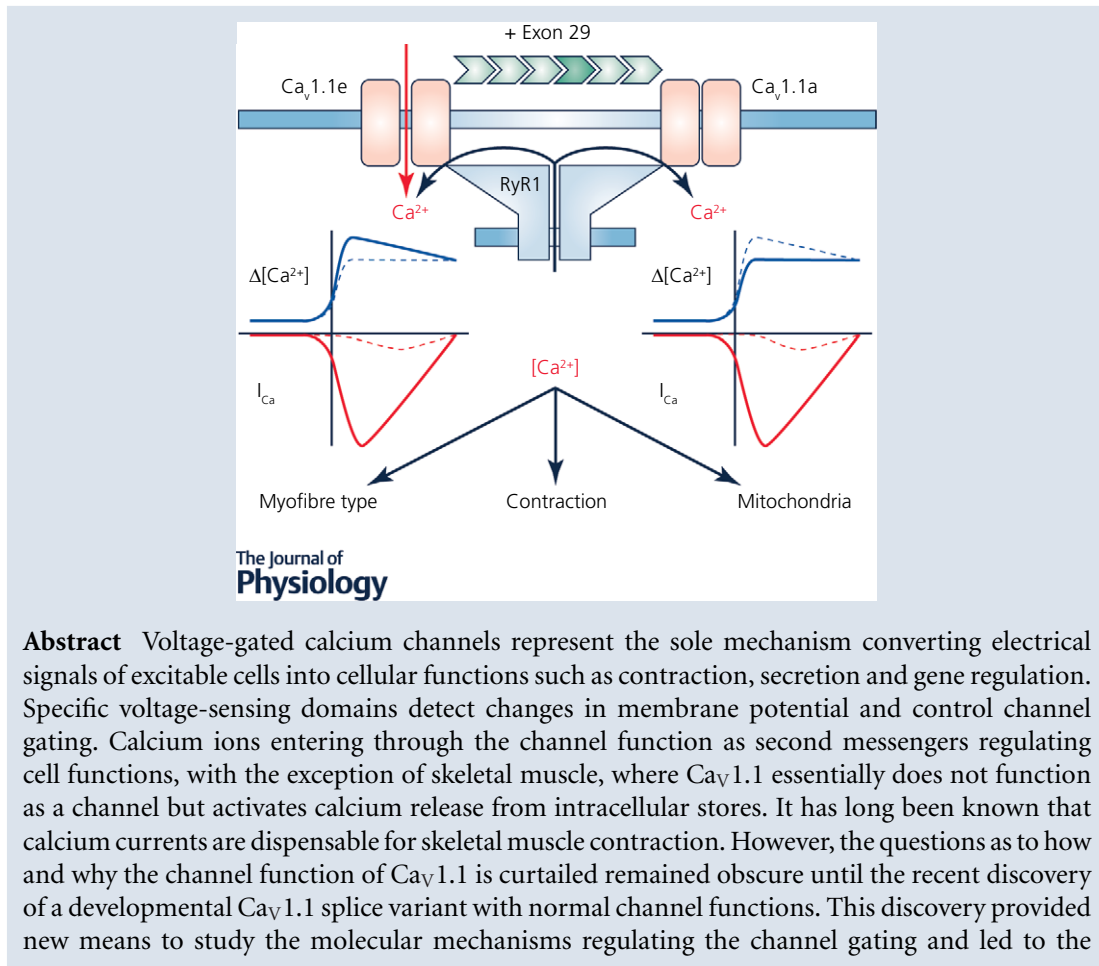TOPICAL REVIEW

How and why are calcium currents curtailed in the skeletal muscle voltage-gated calcium channels?

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Abstract Voltage-gated calcium channels represent the sole mechanism converting electrical signals of excitable cells into cellular functions such as contraction, secretion and gene regulation. Specific voltage-sensing domains detect changes in membrane potential and control channel gating. Calcium ions entering through the channel function as second messengers regulating cell functions, with the exception of skeletal muscle, where $Ca_V1.1$ essentially does not function as a channel but activates calcium release from intracellular stores. It has long been known that calcium currents are dispensable for skeletal muscle contraction. However, the questions as to how and why the channel function of $Ca_V1.1$ is curtailed remained obscure until the recent discovery of a developmental $Ca_V1.1$ splice variant with normal channel functions. This discovery provided new means to study the molecular mechanisms regulating the channel gating and led to the

understanding that in skeletal muscle, calcium currents need to be restricted to allow proper regulation of fibre type specification and to prevent mitochondrial damage.

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Abstract figure legend Curtailing calcium influx during excitation–contraction coupling is critical for fibre type specification and mitochondrial integrity. In mammalian skeletal muscle, developmentally regulated splicing of $Ca_V1.1$ virtually abolishes calcium influx through the voltage-gated calcium channel. In fetal muscle the $Ca_V1.1e$ splice variant lacking exon 29 activates L-type calcium currents (I_{Ca}) at physiologically depolarized membrane potentials and contributes significantly to the cytoplasmic calcium signals controlling contraction ($\Delta[Ca^{2+}]$). However, inclusion of exon 29 in the adult $Ca_V1.1a$ shifts the voltage dependence of activation by +30 mV and eliminates the contribution of calcium influx to the cytoplasmic calcium signal. Alternative splicing of exon 29 controls channel gating without affecting activation of the ryanodine receptor (RyR1) using a unique molecular mechanism in the fourth voltage-sensing domain of the $Ca_V1.1$ channel. When inclusion of exon 29 fails in knock-out mice or in muscles of myotonic dystrophy patients, the increased calcium influx perturbs fibre type specification and causes mitochondrial damage.

Abbreviations   CICR, calcium-induced calcium release; EC, excitation–contraction; LTCC, L-type calcium current; RyR1, type 1 ryanodine receptor; RyR2, type 2 ryanodine receptor; SR, sarcoplasmic reticulum; VSD, voltage sensing domain.

Introduction

As indicated by their name, voltage-gated calcium channels (Ca_Vs) are capable of sensing the depolarization of membrane potentials and in response to this electrical stimulus open a calcium-selective channel pore. Accordingly, their primary role is in conducting activity-regulated calcium influx in excitable cells. However, there is one notable exception: the skeletal muscle calcium channel $Ca_V1.1$. This member of the Ca_V channel family has the same structure as all the other Ca_Vs, with four voltage-sensing domains (VSDs) grouped around a single ion pore, but its activation by skeletal muscle action potentials does not result in substantial calcium influx into muscle cells. Instead, its voltage-dependent activation is directly transmitted to opening of the calcium release channel (type 1 ryanodine receptor; RyR1) in the sarcoplasmic reticulum (SR). Therefore, in skeletal muscle $Ca_V1.1$ primarily functions as a voltage sensor for excitation–contraction (EC) coupling, but not as a calcium channel (Melzer *et al.* 1995). Indeed, it has long been known that calcium influx through $Ca_V1.1$ is dispensable for EC coupling in skeletal muscles of most vertebrates (Armstrong *et al.* 1972), although during long-lasting tetanic stimulation a small L-type calcium current (LTCC) may contribute to refilling of the SR calcium stores and thus help to maintain muscle performance during prolonged activity (Robin & Allard, 2015). Nonetheless, it appears paradoxical that skeletal muscle contraction – the prototypical calcium regulated cell function – involves a member of the Ca_V family with limited channel function.

This raises the obvious question as to why the skeletal muscle $Ca_V1.1$ is such an ineffective calcium channel. Is there a physiological necessity for curtailing its channel function, or are its poor channel properties the result of an evolutionary accident that somehow remained without consequence because calcium release through the RyR1 compensated for the missing channel function? Recent findings by us and others indicate that curtailing voltage-dependent calcium influx is important for skeletal muscle differentiation, function and health. In particular the discovery of a $Ca_V1.1$ splice variant, which functions as a sizeable channel, demonstrated that during skeletal muscle development calcium influx is actively turned off by alternative splicing of $Ca_V1.1$ (Tuluc *et al.* 2009). This raises a second question: How is the calcium current function perturbed without affecting the voltage-sensing function of $Ca_V1.1$? Apparently $Ca_V1.1$ channels of different species utilize multiple mechanisms and even alternative strategies to curtail their currents. Importantly, recent structure–function studies on the two $Ca_V1.1$ splice variants revealed hitherto unnoticed intramolecular interactions, which are specific for the particular VSDs of Ca_V channels and provide control over the voltage sensitivity of the channel function without affecting EC coupling (Tuluc *et al.* 2015). Here we review the recent studies addressing the physiological importance and the biophysical mechanism of the unique gating properties of the skeletal muscle $Ca_V1.1$ calcium channel.

Structure and function of Ca_V1.1 in skeletal muscle

Voltage-gated calcium channels are multi-subunit complexes composed of a pore-forming α_1 subunit, an extracellular $\alpha_2\delta$ subunit, a cytoplasmic β subunit and, at least in skeletal muscle, another integral membrane protein, the γ subunit (Catterall, 2011) (Fig. 1). The mammalian genome contains 10 genes encoding α_1
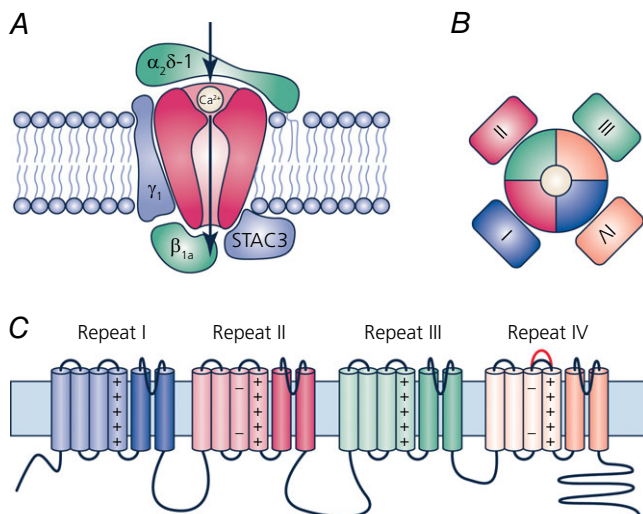
subunits and four each for $\alpha_1\delta$ and β subunits. The α_1 subunit contains four homologous repeats (I–IV) with six transmembrane helixes (S1–S6) each. S1–S4 of each repeat form a voltage-sensing domain (VSD); S5, S6 and the connecting P-loops of all four repeats together form the channel pore with its activation gate. The cytoplasmic loop between repeats I and II contains the binding site for the β subunit and the long cytoplasmic C-terminus contains important sites for current modulation, protein–protein interactions and channel targeting (Walker & De Waard, 1998; Striessnig, 2007). Unique for the skeletal muscle $Ca_V 1.1$ complex are the presence of the γ_1 subunit, the functional interaction of the cytoplasmic loop between repeats II and III with the RyR1, and the recently proposed interaction with the adapter protein STAC3 (Tanabe *et al.* 1990; Flucher *et al.* 2005; Horstick *et al.* 2013; Nelson *et al.* 2013). β subunits are involved in membrane targeting and endow the channels with specific gating properties (Buraei & Yang, 2010; Campiglio & Flucher, 2015). The $\alpha_2\delta$ subunits also contribute to normal membrane expression of the channels, but in muscle cells they are important determinants of the channel's specific activation kinetics

Figure 1. Structure of $Ca_V 1.1$

A, the subunit composition of $Ca_V 1.1$ with the pore-forming α_{1S} subunit, the auxiliary $\alpha_2\delta$-1, β_{1a} and γ_1 subunits and the essential, skeletal muscle-specific adaptor protein STAC3. *B*, the α_1 subunit of Ca_V channels has a pseudotetrameric structure with a central pore surrounded by four voltage-sensing domains (VSDs I–IV). *C*, the α_{1S} subunit consists of four homologous repeats of six transmembrane helices. The first four helices (S1–S4) of each repeat form a functional VSD with the positively charged S4 helix representing the primary voltage sensor. Helices S5–S6 and the connecting pore loops together form the channel pore. Note that the extracellular S3–S4 loop of the fourth VSD is subject to alternative splicing. Inclusion of exon 29 in this loop (red) has massive consequences for channel gating properties.

(Obermair *et al.* 2005; Tuluc *et al.* 2007; Savalli *et al.* 2016). Whereas the $\alpha_2\delta$-1 and γ_1 subunits are dispensable for skeletal muscle EC coupling, the β_1 subunit and STAC3 are essential components of the skeletal muscle EC coupling apparatus (Campiglio & Flucher, 2015; Polster *et al.* 2016).

In skeletal muscle these components of the calcium channel complex assemble with the RyR1 calcium release channel and several other proteins to form calcium release units (Flucher & Franzini-Armstrong, 1996). Depending on whether they form junctions between the SR and T-tubules or between the SR and the plasma membrane, the calcium release units are called triads or peripheral couplings, respectively, although functionally they are equivalent. The specific and probably physical interaction of four $Ca_V 1.1$ channels with a RyR1 homotetramer is reflected in the regular arrangement of $Ca_V 1.1$. In freeze-fracture electron microscopy these can be seen as *tetrads* of integral membrane particles (Block *et al.* 1988). This coordinated organization of the two calcium channels is considered to be the structural prerequisite for skeletal muscle EC coupling.

Upon depolarization of the plasma membrane and T-tubules $Ca_V 1.1$ senses the change in membrane potential and activates opening of the RyR1 release channel (Ríos & Pizarro, 1991). The exact mode of interaction between the two channels is still elusive. However, it is clear that the specific skeletal muscle sequences of the $Ca_V 1.1$ II–III loop and the specific skeletal muscle isoform β_{1a} are both essential for the signalling process (Grabner *et al.* 1999; Schredelseker *et al.* 2005; Sheridan *et al.* 2006). Perhaps the recently discovered STAC3 is also involved in the communication of the two channels (Horstick *et al.* 2013; Nelson *et al.* 2013; Polster *et al.* 2016). The functional coupling of $Ca_V 1.1$ and RyR1 is extremely efficient so that in response to an action potential SR calcium release is activated within milliseconds and repetitive RyR1 activation can be maintained at frequencies of several hundred hertz, resulting in tetanic contractions (Rome, 2006). As mentioned above this process is independent of calcium influx though the $Ca_V 1.1$ channel.

Upon unphysiologically strong and long depolarizations, $Ca_V 1.1a$ also produces slowly activating LTCC. Importantly, current activation requires up to 30 mV stronger depolarizations than activation of EC coupling by the same $Ca_V 1.1$ (Tuluc *et al.* 2009). Thus, the voltage sensors of $Ca_V 1.1$ trigger two distinct processes at different membrane potentials. In dysgenic ($Ca_V 1.1$-null) myotubes reconstituted with only the classical $Ca_V 1.1a$ isoform, half-maximal activation ($V_{1/2}$) of SR calcium release occurs near +10 mV whereas half-maximal activation of LTCC requires depolarization to approximately +40 mV (Tuluc *et al.* 2009). Depending on the cell type and experimental conditions, the absolute $V_{1/2}$ values vary considerably. For example in freshly isolated mouse muscle fibres, $V_{1/2}$ of gating charge

movement (Q_{on}) and current activation have been reported as low as –37 mV and 0 mV, respectively (Collet *et al.* 2003). Nevertheless, the pronounced difference in the voltage dependence of Q_{on} or SR calcium release on the one hand and of LTCCs on the other hand is seen in mature fibres and in myotubes solely expressing $Ca_V1.1a$. In primary cultures and C2C12 myotubes this difference is less pronounced, probably because of a significant contribution of the developmental $Ca_V1.1e$ splice variant (Garcia *et al.* 1994; Schuhmeier & Melzer, 2004). These, current gating properties make $Ca_V1.1a$ the slowest activating and the least responsive to physiological depolarizations of all voltage-gated calcium channels.

Curtailed calcium currents – necessary condition or accidental byproduct of skeletal muscle EC coupling?

Considering the central role of activity-dependent calcium signalling in the control of muscle contraction, it is unexpected and even counter-intuitive that $Ca_V1.1$ calcium currents should not contribute to it. In fact, in the heart the closely related $Ca_V1.2$ channel contributes to cardiac muscle EC coupling in a twofold manner. Voltage-dependent activation of $Ca_V1.2$ causes calcium influx into the nano-domain of the calcium release units of cardiac myocytes (peripheral couplings), where it acts as trigger-calcium to activate further calcium release through the type 2 ryanodine receptors (RyR2) by a mechanism termed calcium-induced calcium release (CICR; Bers, 2008). But in addition to this trigger function, the calcium entering the cytoplasm through $Ca_V1.2$ substantially contributes to the calcium signal which regulates contraction. Thus, the magnitude of the LTCC directly and indirectly determines the force of contraction, and modulation of LTCC, for example by cAMP-dependent phosphorylation of $Ca_V1.2$, is a central mechanism for regulating cardiac contraction. This example clearly demonstrates that LTCC can be useful in EC coupling.

Interestingly, this 'cardiac' EC coupling mechanism is also used by skeletal muscles of invertebrates and early chordates like amphioxus, indicating that in principle CICR is compatible with the requirements of skeletal muscle function (Schredelseker *et al.* 2010). This notion is further supported by experiments in myotubes of dysgenic ($Ca_V1.1$-null) mice, where the lacking $Ca_V1.1$ was replaced with other members of the Ca_V1 family. For example when dysgenic myotubes were reconstituted with $Ca_V1.2$ or with $Ca_V1.3$, cardiac-type EC coupling could be observed in skeletal myotubes (Kasielke *et al.* 2003; Tuluc *et al.* 2007). The only requirement was that the Ca_V channel needed to be colocalized with RyR1 in the triads. This indicated that expression of the skeletal muscle RyR1 isoform does not necessitate a direct coupling mechanism with the $Ca_V1.1$ voltage sensor: it can also be activated by CICR, provided that the trigger calcium is supplied by a Ca_V channel targeted into the nano-domain of the calcium release unit.

Thus, the direct coupling mechanism in skeletal muscle is not a necessity because CICR had been abolished, but direct coupling of $Ca_V1.1$ and RyR1 functionally replaced the CICR mechanism in skeletal muscle. Moreover, several lines of evidence support the view that suppressing calcium influx during skeletal muscle EC coupling was not only tolerable but even beneficial (Schredelseker *et al.* 2010). Different vertebrate species utilize distinct strategies to abolish or reduce LTCC through the skeletal muscle $Ca_V1.1$. In higher teleost fishes calcium conduction is completely abolished by amino acid substitution in the channel pore. Teleosts have two $Ca_V1.1$ genes due to gene duplication. Interestingly, both are non-conductive, although this is accomplished by distinct amino acid substitutions in and near the calcium selectivity filter (Fig. 2). Moreover, non-conductivity in one of the two fish $Ca_V1.1$ isoforms was achieved by different substitutions in the higher and lower teleost orders, exemplified by zebrafish and medaka, respectively (Schredelseker *et al.* 2010). Collectively, the use of three different amino acid substitutions to achieve non-conductivity in both isoforms of all higher teleost is a strong indicator that curtailing calcium currents in skeletal muscle is of physiological importance.

In tetrapods, $Ca_V1.1$ channels are principally calcium conductive, but under physiological conditions $Ca_V1.1a$ currents are curtailed by other mechanisms. As mentioned above, upon long-lasting depolarization mammalian $Ca_V1.1a$ channels conduct LTCC. However, activation requires strong depolarization, activation is very slow and current amplitudes are small. These limiting channel properties are not the consequence of a single mutation in the pore or even a single molecular mechanism. In contrast – as if suppressing the currents were an essential goal to achieve – multiple mechanisms contribute to this effect. First of all, two distinct VSDs determine the slow activation and the right-shifted voltage sensitivity of $Ca_V1.1a$. Sequence substitutions in VSD I affect the kinetic properties without influencing the voltage dependence of activation (Nakai *et al.* 1994; Tuluc *et al.* 2016). On the other hand, sequence substitutions and point mutations in VSD IV affect voltage dependence without influencing current kinetics (Tuluc *et al.* 2015, 2016; Fig. 3). Secondly, the auxiliary $\alpha_2\delta$-1 and γ subunits contribute to the poor current properties (Flucher *et al.* 2005; Andronache *et al.* 2007). Knock-down and targeted deletion of $\alpha_2\delta$-1 in skeletal muscle cells accelerates activation kinetics of $Ca_V1.1$ but not of $Ca_V1.2$, showing that in skeletal muscle this auxiliary subunit is an important determinant of the specific slow activation of LTCC (Obermair *et al.* 2005; Tuluc *et al.* 2007; Fuller-Bicer *et al.* 2009). Knockout of the γ subunit causes a right-shifted voltage dependence of inactivation, indicating that this auxiliary subunit limits

the window current of LTCC in skeletal muscle (Ursu *et al.* 2004). The cooperation of these multiple mechanisms to achieve a single goal, suppression of LTCC, again supports the notion that curtailing calcium currents in skeletal muscle might be physiologically important.

However, there is one notable exception to this trend. Skeletal muscle calcium currents are facilitated by specific interactions with the RyR1. In the absence of RyR1,

for example in myotubes of dyspedic mice, $Ca_V1.1$ currents are very small (Nakai *et al.* 1996). Only $Ca_V1.1$'s specific interaction with RyR1 substantially increases $Ca_V1.1a$ current density to the still comparably small, but measurable normal levels. This would suggest that the currents are important after all; however, the physiological relevance of the restoration of LTCC by this retrograde coupling mechanism is still elusive (Flucher, 2016).
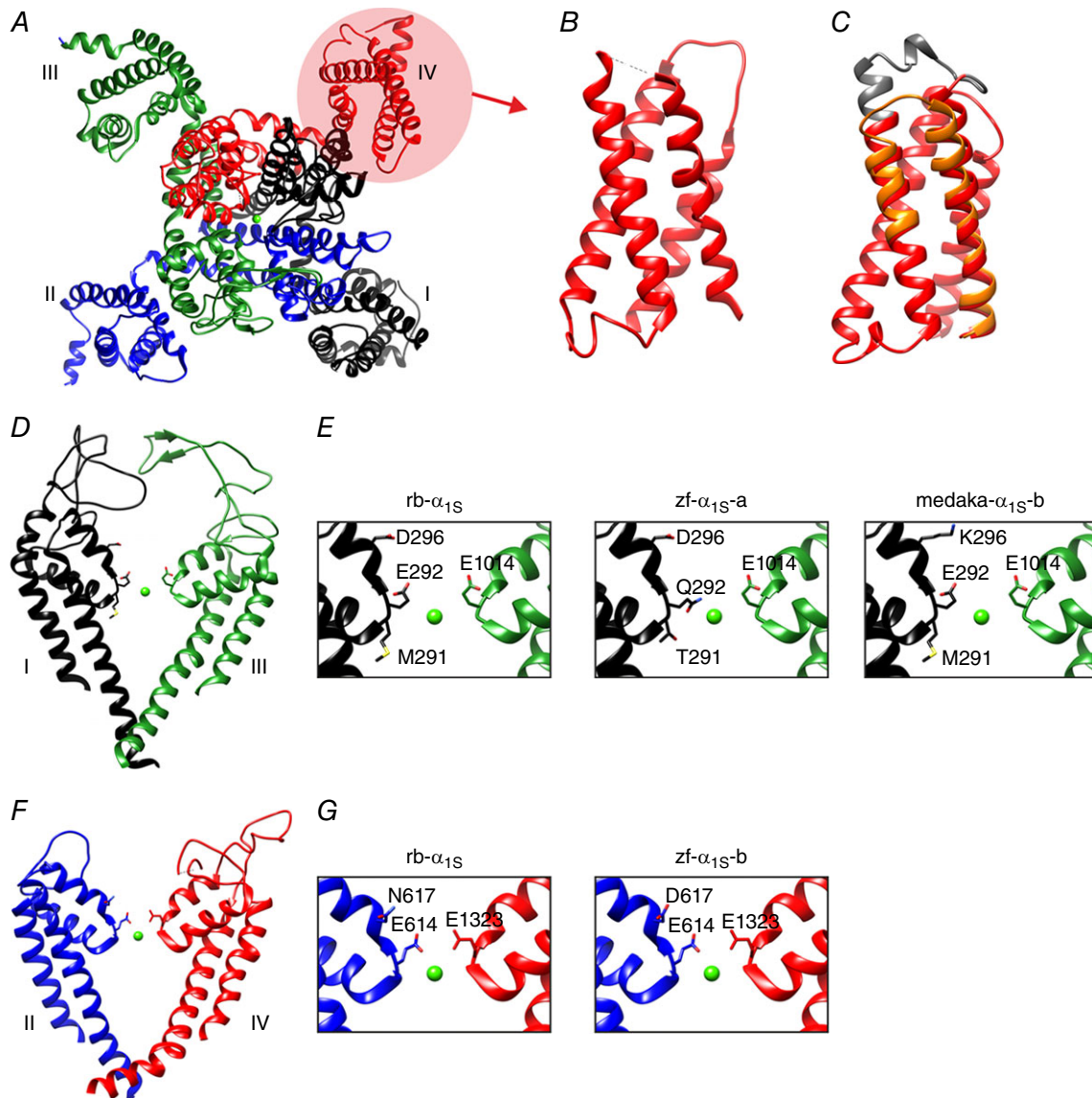

Figure 2. $Ca_V1.1$ structures involved in curtailing calcium currents
A, top view of the $Ca_V1.1$ channel with the four homologous repeats shown in different colours, using the high-resolution cryo-EM structure of Wu *et al.* (2015). *B* and *C*, side views of VSD IV models constructed of the high-resolution cryo-EM structure (*B*), and our *Rosetta* structure models (*C*), of the two splice variants with and without exon 29 (grey) (Tuluc *et al.* 2015). Note the overall similarity of the predicted and experimentally obtained structures. Inclusion of exon 29 slightly displaces IVS3 and IVS4 relative to each other, resulting in a channel which fails to activate during physiological depolarizations. *D* and *F*, side view structure of the channel pore. *E* and *G*, critical pore residues of the calcium selectivity filter of the rabbit channel (rb-α_{1S}) and the single amino acid substitutions which render the two fish $Ca_V1.1$ paralogues (zf-α_{1S}-a, -b, medaka-α_{1S}-b) non-conductive (Schredelseker *et al.* 2010).

Molecular mechanism for limiting voltage sensitivity of Ca$_V$1.1 activation

As mentioned above the classical Ca$_V$1.1a isoform can independently activate two voltage-dependent signalling processes. In dysgenic myotubes reconstituted with Ca$_V$1.1a, depolarization to about 0 to +10 mV rapidly activates RyR1-mediated calcium release (EC coupling), whereas activation of calcium currents through its own channel pore (LTCC) requires approximately +30 mV stronger depolarizations and is extremely slow. How can this be? Our recent discovery of a Ca$_V$1.1 splice variant with different current activation properties set off a series of studies which together unravelled this puzzle (Tuluc *et al.* 2009).
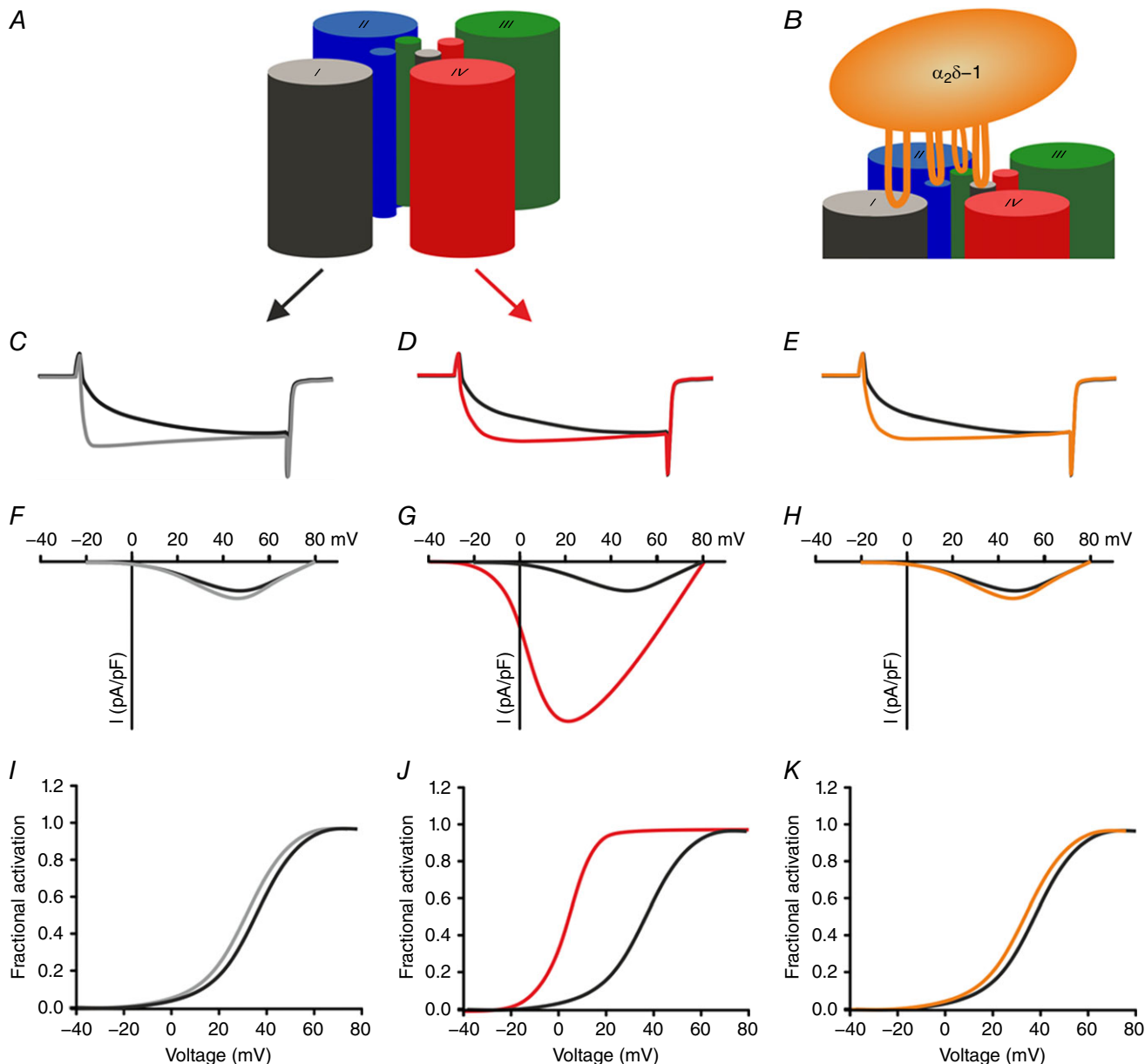
Figure 3. Distinct contributions of the four VSDs to determining the specific gating properties of Ca$_V$1.1
A and *B*, schematic models showing the role of VSD I in regulating activation kinetics (*C*) and its modulation by the $\alpha_2\delta$-1 subunit (*B* and *E*). Note that changes in current kinetics are not accompanied by changes in voltage sensitivity and current amplitude. *D*, *G* and *J*, VSD IV regulates the voltage dependence of activation (*D* and *J*) and current amplitude (*G*) by alternative splicing of exon 29. *C–E*, typical calcium currents of Ca$_V$1.1a (including exon 29; black), Ca$_V$1.1e (lacking exon 29; red), Ca$_V$1.1 with IS3+IS3–S4 linker from Ca$_V$1.2 (grey), and Ca$_V$1.1a without $\alpha_2\delta$−1 (orange). *F–K*, current–voltage relationship (*F–H*) and conductance–voltage relationships (*I–K*) of the currents shown above.

The alternatively spliced exon 29 encodes a 19 amino acid sequence in the extracellular linker connecting the transmembrane helices IVS3 and IVS4. Before birth the majority of $Ca_V 1.1$ transcripts lack exon 29. However, after birth expression of $Ca_V 1.1a$ is strongly increased and within 3 weeks the great majority of $Ca_V 1.1$ transcripts include exon 29 (Sultana *et al.* 2016). Importantly, the developmental splice variant lacking exon 29 activates EC coupling and LTCC at the same voltage and its current amplitude is comparable to that of $Ca_V 1.2$ (Tuluc *et al.* 2009). Thus, exclusion of exon 29 in the developmental $Ca_V 1.1e$ splice variant causes the –30 mV shift in voltage dependence of activation and the dramatic increase of current density, but without shifting the voltage sensitivity of gating charges or EC coupling (Tuluc *et al.* 2009; Fig. 3). Conversely, inclusion of exon 29 in VSD IV is sufficient to specifically right-shift the voltage dependence of LTCCs, suggesting that activation of this VSD is required for activation of LTCC but not for EC coupling. Parallel analysis of the gating charge movement (Q_{ON}) demonstrated that its voltage dependence coincided with that of EC coupling and was not significantly right-shifted by the inclusion of exon 29. This indicated that, although voltage dependent, the conformational changes of VSD IV leading to the opening of the channel pore contributed little to the total charges moved upon $Ca_V 1.1a$ activation. Thus, membrane depolarization to about 0 mV causes the bulk movement of the $Ca_V 1.1a$ VSDs, which is sufficient to activate EC coupling. However additional depolarization is necessary to effect final transitions of VSD IV of $Ca_V 1.1a$ that remain below detectability in Q_{ON} but are necessary for opening of the channel pore. Interestingly, when exon 29 is included in VSD IV the activated state of the channel appears to be less stable, as also the channel open probability is dramatically reduced compared to the $Ca_V 1.1e$ splice variant lacking exon 29 (Tuluc *et al.* 2009, 2015).

How does the 19 amino acid sequence of exon 29 affect these dramatic changes of the gating properties in VSD IV? One possibility, consistent with its position at the extracellular face of the channel, would be that it serves as a protein–protein interaction site for an extracellular modulator. A probable candidate would be the $\alpha_2\delta$-1 subunit, which is located extracellularly and modulates current kinetics in the same direction as inclusion of exon 29. However, siRNA depletion of $\alpha_2\delta$-1 equally affected activation kinetics in $Ca_V 1.1$ channels with and without exon 29, demonstrating that this sequence is not necessary for the modulatory action of $\alpha_2\delta$-1 on $Ca_V 1.1$ gating properties (Tuluc *et al.* 2009). This notion is further supported by the recent cryo-EM structure of the $Ca_V 1.1$ complex, according to which VSD IV does not participate in the interaction with $\alpha_2\delta$-1 (Wu *et al.* 2015), as well as by a very recent voltage-clamp fluorometry study indicating that in $Ca_V 1.2$ $\alpha_2\delta$-1 differentially modulates VSDs I, II

and III, whereas VSD IV is not affected by the presence or absence of $\alpha_2\delta$-1 (Savalli *et al.* 2016).

Alternatively, intrinsic structural properties of the exon 29 sequence could alter the function of VSD IV. In fact *de novo* structure *Rosetta* modelling and secondary structure prediction tools suggested that inclusion of exon 29 not only alters the length of the linker between IVS3 and IVS4 but also endowed it with distinct structure (helix and/or β-sheet) and hydrophobicity (Tuluc *et al.* 2015). Our models further predicted that inclusion of the exon 29 sequence would result in major structural rearrangements of this IVS3–S4 linker during the transitions from the closed state to the activated state. It is likely that any such structural features and dynamic rearrangements will increase the necessary activation energy and thus require stronger depolarization for the activation process. Indeed the substitution of two lysines for asparagines, which abolished secondary structure and reduced the hydrophobicity of the IVS3–S4 linker, caused a substantial left-shift of the voltage dependence of activation of $Ca_V 1.1a$ (Tuluc *et al.* 2016). Thus, the structure and not merely the length of the IVS3–S4 linker is critical for its role in regulating the voltage sensitivity of VSD IV.

If the IVS3–S4 linker containing exon 29 by itself is able to right-shift the voltage sensitivity of a VSD, this function should not be limited to VSD IV. However, inserting the IVS3–S4 linker into the corresponding position of VSD I of $Ca_V 1.1e$ failed to reproduce the right-shifted voltage dependence of activation of $Ca_V 1.1a$ (Tuluc *et al.* 2016). Apparently exon 29 exerts its modulatory function in VSD IV but not in VSD I. Where is the difference? Sequence comparison of the two VSDs showed that in S4 the innermost positively charged amino acids (R4) are spaced differently (third position in IS4, fourth position in IVS4) and that IVS3 contains an additional negatively charged residue (aspartate 1196; D4) that is absent in IS3. Homology structure modelling of VSD IV with and without the exon 29 sequence in four different activation states predicted that D4 could act as additional counter-charge for the two outermost arginines (R1 and R2) during the late voltage sensor transition (Tuluc *et al.* 2015). Importantly the structure model further predicted striking differences in the distance and number of bonds between these residues between $Ca_V 1.1a$ and $Ca_V 1.1e$. In the intermediate state 2 and in the activated state the $Ca_V 1.1e$ splice variant lacking exon 29 had more and stronger (shorter distances) hydrogen bonds between D4 and R1 and R2 compared to $Ca_V 1.1a$. Considering the biophysical properties of the two splice variants the models suggested that these interactions of oppositely charged residues may facilitate the transition of the voltage sensor into the activated state and its subsequent stabilization in the activated state (Fig. 4).

This model was tested and confirmed using site directed mutagenesis and patch-clamp analysis of channels

reconstituted in dysgenic myotubes (Tuluc *et al.* 2015). Mutations in $Ca_V1.1e$ that abolished the charges of D4, R1, or R2 all resulted in right-shifted voltage dependence and reduced amplitude, comparable to the voltage sensitivity of $Ca_V1.1a$. The same mutations in the presence of exon 29 (in $Ca_V1.1a$) did not result in a further shift of the voltage dependence. Thus, VSD IV of $Ca_V1.1$ comprises a functionally critical intramolecular interaction between oppositely charged residues of the S3 and S4 transmembrane helices that has not been reported in previous studies of VSDs of other voltage-gated cation channels. This newly identified interaction is important for proper voltage sensitivity of the calcium channel and it makes this VSD amenable to modulation by alternative splicing in the IVS3–S4 linker. When the exon 29 sequence is included in the IVS3–S4 linker, S3 and S4 are displaced from each other and the important interactions between D4 and R1/R2 are weakened or lost, resulting in similar perturbations of channel voltage sensitivity and open probability as mutations of any one of the participating amino acids.

Moving exon 29 into VSD I does not reconstitute this modulatory mechanism because it does not contain D4 in IS3 (Tuluc *et al.* 2016). Surprisingly, also the transfer of the IVS3–S4 linker plus IVS3 (containing D4) was not sufficient to restore the mechanism in the first repeat. Conversely, moving IS3 and the IS3–S4 linker into VSD IV perturbed channel gating, even though this loop does not contain exon 29. Apparently the critical difference between the two VSDs resides in the sequence of the IVS4 helix, which requires the D4 countercharge in S3 for proper function.

Interestingly, the charged aspartate (D4) in IVS3 is highly conserved among the members of high voltage-activated calcium channels (Ca_V1, Ca_V2). Moreover, alternative splicing in the IVS3–S4 linker is a common feature among the CaV family members (Perez-Reyes *et al.* 1990; Lin *et al.* 1997, 1999; Bourinet *et al.* 1999; Liao *et al.* 2007), and in some cases it has been shown to affect voltage dependence of activation (Tang *et al.* 2004). Therefore it is possible that this mechanism, which is responsible for the dramatic differences in the gating properties of the two skeletal muscle $Ca_V1.1$ splice variants, actually might represent a more general mechanism for regulating the voltage sensitivity and open probability of voltage-gated calcium channels. However, voltage-clamp fluorometry study of $Ca_V1.2$ indicated that in this Ca_V isoform VSD IV does not contribute to channel gating (Pantazis *et al.* 2014). Future studies will need to examine this possibility.

## Structural determinants of slow activation kinetics of $Ca_V1.1$

Whereas exclusion of the exon 29 sequences in VSD IV normalizes voltage dependence of activation and the open probability of $Ca_V1.1$, it does not alter the slow activation kinetics (Tuluc *et al.* 2016). Previously work showed that transfer of IS3 and the IS3–S4 linker from $Ca_V1.1$ into $Ca_V1.2$ could confer slow activation kinetics but did not alter the voltage dependence of activation (Nakai *et al.* 1994). Together these observations indicate that the two properties are determined by similar sequence domains in two different VSDs. However, contrary to the voltage sensitivity mechanism, slow activation kinetics could not be conferred to fast activating $Ca_V1.1$ channel constructs by moving sequences from the first to the fourth repeat (Tuluc *et al.* 2016). Notably, the same sequences (IS3 and the IS3–S4 linker) which were sufficient to transfer slow activation from $Ca_V1.1$ to VSD I of $Ca_V1.2$, were not sufficient to transfer slow activation properties from VSD I to VSD IV within $Ca_V1.1$. This indicated that VSDs I of $Ca_V1.1$ and $Ca_V1.2$ resemble each other more closely than VSDs I and IV within $Ca_V1.1$. Based on the original domain swapping experiments it had been assumed that the specific slow activation mechanism resides in the IS3 and IS3–S4 linker sequences (Nakai *et al.* 1994). The recent findings that any changes in VSD I abolish slow activation, but that transfer of the entire sequence containing IS3, the IS3–S4 linker, and IS4 cannot confer slow activation properties to VSD IV demonstrate that regulation of activation kinetics depends on the integrity of the entire VSD I and probably also of the pore region (Tuluc *et al.* 2016). Thus, determining slow activation kinetics is a property of the first VSD and utilizes a different and more complex molecular mechanism than the focused mechanism controlling voltage sensitivity in VSD IV.

Modular organization of $Ca_V1.1$ calcium channels

Together these findings indicate that $Ca_V1.1$ is organized in a modular manner. The four VSDs are homologous but not identical in structure and function, enabling them to contribute differentially to channel gating. Specific gating properties seem to be regulated by distinct individual VSDs. We now know that VSD I determines activation kinetics and VSD IV regulates the voltage dependence of activation. Because the bulk of $Ca_V1.1a$ gating charges moves rapidly and at lower membrane potential than current activation (Tuluc *et al.* 2009), we assume that all four VSDs need to move to the activated state to initiate pore opening. For a given gating property, a single VSD can be limiting: the slowest VSD might determine the speed of activation, the least voltage-sensitive VSD the voltage dependence of activation, regardless to how fast and at which voltages the other VSDs are activated. A similar model was suggested 20 years ago to explain the slow activation kinetics of frog muscle fibres (Melzer *et al.* 1995). Riccardo Olcese's team have recently introduced the voltage-clamp fluorometry method to study $Ca_V1.2$ channel gating and thus provided direct experimental
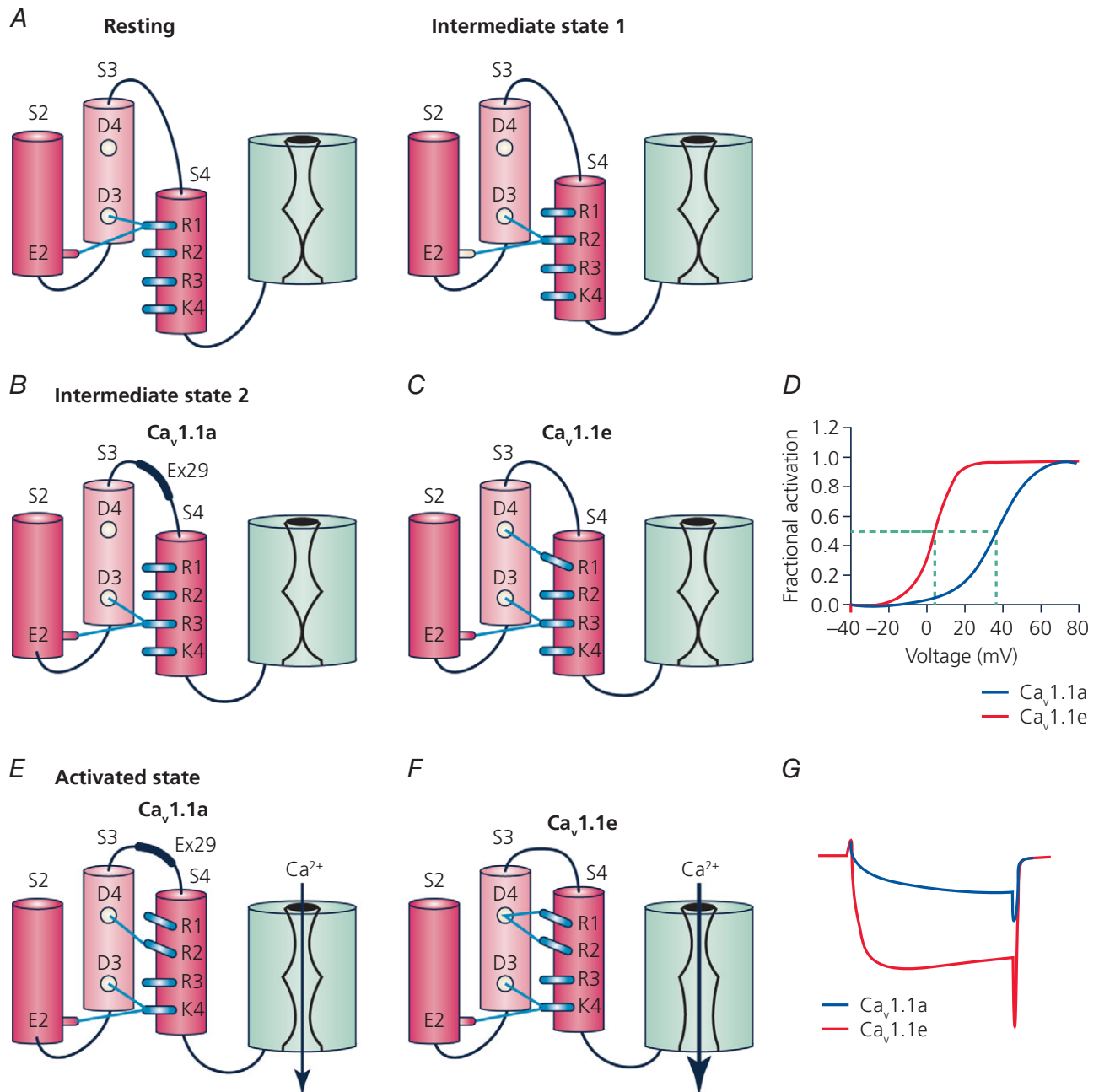
Figure 4. Model showing the molecular mechanism by which alternative splicing of exon 29 regulates the voltage dependence of activation and open probability of Ca$_V$1.1

A, in the resting and intermediate state 1, R1 and R2, respectively, interact with two countercharges (glutamate E2 in IVS2 and aspartate D3 in IVS3) forming the charge transfer centre. Consequently there are no interactions of the IVS4 arginines with D4 of IVS3 and also no modulation by insertion/exclusion of exon 29 in the IVS3–S4 linker. *B–D*, in the intermediate state 2, R3 occupies the charge transfer centre and only in the absence of exon 29 (Ca$_V$1.1e) does R1 establish an interaction with D4 that facilitates the transition of the voltage sensor into the active state and therefore shifts the voltage dependence of CaV1.1e to the left. *E* and *F*, in the activated state, D4 of Ca$_V$1.1e forms several hydrogen bonds with both R1 and R2, thus stabilizing the activated state and increasing the open probability. *B* and *E*, inclusion of exon 29 in the IVS3–S4 linker of the adult splice variant Ca$_V$1.1a reduces the number and strength of the hydrogen bonds between D4 and R1 and R2. Consequently channel gating is severely perturbed and currents curtailed in the adult splice variant of the skeletal muscle calcium channel.

evidence that different VSDs contribute differentially to channel gating (Pantazis *et al.* 2014). In Ca$_V$1.2 VSDs II and III critically determine voltage dependence and kinetics of channel opening. Interestingly, in Ca$_V$1.2 VSD I is of minor importance and VSD IV appears not to participate at all in channel opening. Evidently, Ca$_V$1.1 and Ca$_V$1.2 operate on the same principle that the four VSDs contribute differentially to channel gating, but differ in that they utilize distinct VSDs for this purpose.

This difference may be associated with the specific requirement of Ca$_V$1.1 to activate EC coupling. Differential use of VSDs for different gating properties and functions enables Ca$_V$1.1 to gate EC coupling and calcium conduction at different voltages and with different kinetics. Because activation of EC coupling occurs at substantially lower membrane potentials than current activation, and because activation of EC coupling is fast, we propose that VSDs I and IV are not critically involved in activation of EC coupling. This hypothesis is further supported by a malignant hyperthermia mutant of the innermost arginine of VSD I (R174W) which abolished LTCC in response to test pulses of up to +60 mV without perturbing EC coupling (Eltit *et al.* 2012). A primary role of VSDs II and III in EC coupling is consistent with the strategically favourable position of sequences which are critical for the functional interaction with RyR1 in the cytoplasmic loop connecting repeats II and III (Grabner *et al.* 1999). Whether both or only one of these two VSDs are required for activation of EC coupling is currently unknown.

Importance of curtailing calcium influx for skeletal muscle fibre type specification

So far we have reviewed the evidence indicating that curtailing LTCC in skeletal muscle might be important and we summarized our current knowledge of the molecular mechanisms which determine the poor voltage sensitivity and slow kinetics of Ca$_V$1.1 current activation. What remains to be discussed is the physiological importance of curtailing the calcium currents. Mouse models have been generated with mutations in the Ca$_V$1.1 channel pore that abolish the calcium conductance (Dayal *et al.* 2014; Lee *et al.* 2015). Analyses of mouse motor behaviour and skeletal muscle contraction revealed no differences between wild-type and non-conducting Ca$_V$1.1 mutants. Nevertheless, the mice of the Hamilton group revealed deficient refilling of SR calcium stores during sustained activity and an increased type IIb fibre content and increased fatigability (Lee *et al.* 2015), and they suffered from changed calcium-dependent fatty acid metabolism (Georgiou *et al.* 2015). This phenotype indicates that LTCCs are dispensable for EC coupling but may play a role in muscle differentiation and during prolonged activity of mammalian skeletal muscle.

Given this limited role of LTCC in skeletal muscle function, how important is it to curtail the currents? During normal development expression of the calcium-conducting Ca$_V$1.1e in the fetal stage is almost completely replaced by the virtually non-conducting adult Ca$_V$1.1a splice variant (Tuluc & Flucher, 2011; Sultana *et al.* 2016). The consequences of circumventing this alternative splicing event have recently been analysed in a genetic mouse model in which exon 29 was constitutively knocked out (Sultana *et al.* 2016). Overall the development and motor performance of these exon 29 knockout mice was normal. However they displayed reduced voluntary running and reduced grip force. This was confirmed in isolated muscles, where significantly reduced contractile force, an increased endurance and a shift of tetanic fusion to lower frequencies was observed. Because these effects of permanent exclusion of exon 29 resembled the contractile properties of slow muscle fibres, the fibre type composition has been analysed. Indeed, both fast extensor digitorum longus and slow soleus muscles of exon 29 knockout mice experienced a substantial shift towards slower muscle fibre types compared to wild-type controls. Consistent with the interpretation that increased LTCC during EC coupling caused a dysregulation of fibre type specification, this shift was accompanied by an up-regulation of the chief activity- and calcium-dependent regulators of fibre type composition, calcineurin and CaMKII, in exon 29 knockout mice.

As expected from an increased expression of slow oxidative muscle fibres, young exon 29 knockout muscle fibres displayed an increased activity of succinyl dehydrogenase (SDH). Surprisingly, however, in older animals SDH activity was reduced. Electron microscopic analysis of these muscle fibres revealed severe mitochondrial damage and a reduction in total mitochondrial volume that would explain the reduced SDH activity (Sultana *et al.* 2016). Calcium recording in isolated muscle fibres of exon 29 knockout mice demonstrated increased depolarization-induced calcium influx, an increased contribution of LTCC to refilling of SR calcium stores, and an increased incidence of calcium sparklets at rest. Evidently the increased depolarization-induced calcium influx was not reflected by a parallel increase in contractile force. In part this may be counteracted by the expected reduction of maximal force associated with the shift towards slower muscle fibre types. In fact the observed activation of the slow fibre pathway may be part of a compensatory response to the aberrantly increased calcium signals in muscles constitutively expressing the calcium-conducting Ca$_V$1.1e isoform. On the other hand the increased calcium influx during activity or the altered calcium homeostasis probably caused the progressive damage and loss of mitochondria. Together these findings indicate that curtailing calcium influx by developmentally regulated

splicing of $Ca_V1.1$ is important for the normal regulation of muscle fibre types and to prevent muscle fibres from mitochondrial damage. Apparently, curtailing LTCC in skeletal muscle is of minor importance for calcium's primary signalling function in EC coupling, but is of considerable importance for calcium's second signalling function in activity-dependent regulation of the fibre type composition.

$Ca_V1.1e$ calcium influx is linked to muscle disease

The observed detrimental effects of exclusion of exon 29 on mitochondria suggest a contribution of the resulting $Ca_V1.1e$ LTCC to muscle disease. It is well known that calcium overload can lead to mitochondrial damage in any excitable tissue. Moreover, mitochondrial damage is often the first manifestation of muscle damage in muscle diseases associated with dysregulated calcium homeostasis (Brookes *et al.* 2004; Boncompagni *et al.* 2009). Most importantly, muscle weakness in patients with myotonic dystrophy is accompanied by the aberrant expression of the calcium-conducting developmental $Ca_V1.1e$ splice variant in adult muscle (Tang *et al.* 2012; Santoro *et al.* 2014). Although aberrant expression of $Ca_V1.1e$ in adult mice by itself did not cause a dystrophic phenotype or severe muscle weakness, the observed mitochondrial damage might be a first sign of muscle damage. During the much longer life span in humans and in combination with other genetic defects in myotonic dystrophy it is likely that the additional calcium influx through $Ca_V1.1e$ will lead to muscle disease. If this can be confirmed, targeting $Ca_V1.1e$ LTCC with licensed calcium channel drugs may be a reasonable therapeutic strategy to ease the symptoms of myotonic dystrophy (Benedetti *et al.* 2015).

Conclusion

Several lines of evidence indicate that the developmental switch from a calcium conducting to an effectively non-conducting $Ca_V1.1$ splice variant is of physiological and pathological relevance for skeletal muscle. The existence of multiple strategies to abolish or reduce calcium conduction in skeletal muscle calcium channels highlights the importance of curtailing calcium currents. In $Ca_V1.1a$ unique molecular mechanisms in distinct VSDs determine slow activation kinetics, right-shifted voltage dependence of activation, and low open probability. The latter two gating properties can be regulated by alternative splicing of exon 29. When the splicing event is circumvented the continued expression of the calcium-conducting $Ca_V1.1e$ splice variant leads to a dysregulation of muscle fibre type specification. Furthermore, aberrant expression of $Ca_V1.1e$ causes mitochondrial damage in mice and correlates with the degree of muscle weakness in human myotonic dystrophy

patients. The simultaneous use of cytoplasmic calcium signals to control muscle contraction and regulate the specification of fibre type properties, as well as the potential danger to mitochondrial integrity emanating from a high calcium load necessitates a tight control of calcium. Evidently, in skeletal muscle this tightrope act is better achieved when activity-dependent influx of calcium through $Ca_V1.1$ channels has been curtailed.

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Additional information

Competing interests

The authors declare no competing interests.

Author contributions

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