Chapter 2
Structure-Function Relationship of the Voltage-Gated Calcium Channel $Ca_v1.1$ Complex

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Abstract Voltage-gated calcium (Ca_v) channels are miniature membrane transistors that convert membrane electrical signals to intracellular Ca^{2+} transients that trigger many physiological events. In mammals, there are ten subtypes of Ca_v channel, among which $Ca_v1.1$ is the first $Ca_v\alpha1$ to be cloned. $Ca_v1.1$ is specified for the excitation–contraction coupling of skeletal muscles, and has been a prototype in the structural investigations of Ca_v channels. This article summarized the recent advances in the structural elucidation of $Ca_v1.1$ and the mechanistic insights derived from the 3.6 Å structure obtained using single-particle, electron cryomicroscopy. The structure of the $Ca_v1.1$ complex established the framework for mechanistic understanding of excitation–contraction coupling and provides the template for molecular interpretations of the functions and disease mechanisms of Ca_v and Na_v channels.

Keywords Voltage-gated calcium channel · DHPR · $Ca_v1.1$ · Structure

Abbreviations

Ca_v	Voltage-gated calcium channel
DHPR	Dihydropyridine receptor
E-C coupling	Excitation-contraction coupling
Na_v	Voltage-gated sodium channel
RyR	Ryanodine receptor
TM	Transmembrane
VGIC	Voltage-gated ion channel
VSD	Voltage sensing domain

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2.1 Introduction

Voltage-gated calcium (Ca_v) channels are a large family of membrane proteins that are activated upon the change of membrane potentials. The Ca^{2+} ions permeated by the Ca_v channel act as second messengers that trigger a cascade of cellular events involved in a multitude of physiological processes such as muscle contraction, synaptic transmission, hormone secretion, gene expression and cell death (Fig. 2.1) [1, 2].

2.1.1 Classification of Voltage-Gated Calcium Channels

Ca_v channels exhibit tissue specificity. Ten subtypes of Ca_v channels have been identified in mammals through the voltage-clamp measurements of Ca^{2+} currents in distinct tissues and organisms (Table 2.1) [3, 4]. The ten subtypes can be divided into three subfamilies based on their sequence similarity. The Ca_v1 channels activate at high voltage and conduct large and long lasting ion currents, thus designated as the L-type calcium channels [5]. The Ca_v1 channels can be specifically inhibited by dihydropyridine and its derivatives. They are thereby also named as the dihydropyridine receptor (DHPR) [6, 7]. In contrast, the Ca_v3 members are designated as T-type calcium channel for activation at low voltage and conduct tiny single channel current, and the current is transient [4, 8]. The Ca_v2 channels can be further divided to the P/Q-type, the N-type, and the R-type based on their Ca^{2+} current properties and inhibition by specific toxins [4, 9–11].

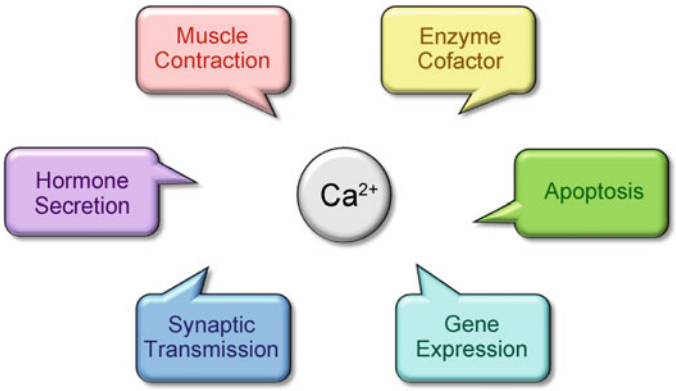

Fig. 2.1 The physiological functions of Ca^{2+} ions. Ca^{2+} ions play an important role in muscle contraction, apoptosis, gene expression, synaptic transmission, hormone secretion, and can also act as enzyme cofactor

Table 2.1 Classification of voltage-gated calcium channels

Ca^{2+} current type	Ca^{2+} channel	Specific blocker	Functions
L	$Ca_v1.1$	DHPs	E-C coupling Gene regulation
	$Ca_v1.2$		E-C coupling Hormone secretion Gene regulation
	$Ca_v1.3$		Hormone secretion Gene regulation
	$Ca_v1.4$		Visual transduction
N	$Ca_v2.1$	ω-CTx-GVIA	Neurotransmitter release Dendritic Ca^{2+} transients
P/Q	$Ca_v2.2$	ω-Agatoxin	Neurotransmitter release Dendritic Ca^{2+} transients
R	$Ca_v2.3$	SNX-482	Neurotransmitter release Dendritic Ca^{2+} transients
T	$Ca_v3.1$	None	Repetitive ring
	$Ca_v3.2$		Repetitive ring
	$Ca_v3.3$		Repetitive ring

2.1.2 Molecular Properties of Ca_v

Ca_v channels belong to the voltage-gated ion channel superfamily, which has a conserved core fold. Unlike their prokaryotic homologues, which are homotetramers, the ion conducting α1 subunit of eukaryotic Ca_v channels is a single peptide chain with four similar repeats linked by intracellular loops [12]. Each repeat contains six transmembrane helices named S1–S6, among which S1–S4 of each repeat form the voltage sensing domain (VSD) and S5–S6 of the four repeats constitute the ion conducing pore (Fig. 2.2) [14].

Ca_v1 and Ca_v2 channels also contain several auxiliary subunits that form a complex with the α1 subunit (Fig. 2.2). The auxiliary subunits include the extracellular α2δ subunit, the cytosolic β subunit and sometimes the transmembrane γ subunit [1]. The auxiliary subunits are not essential for the channel permeation, but they can regulate the membrane trafficking and channel properties of the α1 subunit. For example, the α2δ subunit can facilitate channel translocation to the cell surface, and the β subunit modulates the kinetic properties of the channel [15–17]. In skeletal muscle, the β subunit in $Ca_v1.1$ plays an essential role in the excitation-contraction coupling [18–20]. The γ subunit assists channel inactivation [21, 22].

Not only α subunit has different subtypes, each auxiliary subunit may have distinct subtypes and splicing isoforms. Four genes encoding the β subunit have been identified, and each can yield different isoforms by alternative splicing [16, 23]. The α2 and δ subunits result from proteolytic cleavage of a single gene product. Besides, four genes have been reported encoding the α2δ subunit [24]. Combinations of the distinct α1 subtypes with various auxiliary subunits give rise to the diversity of Ca_v channels.

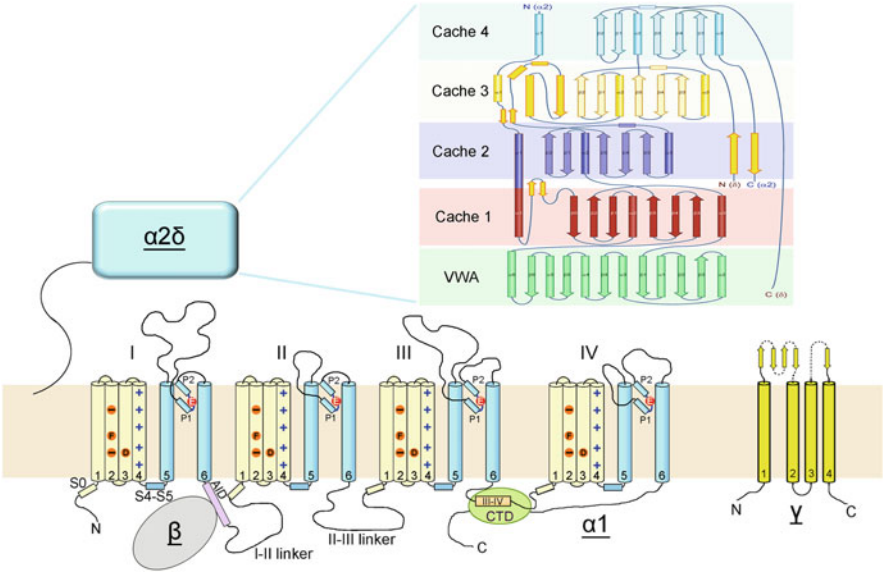

Fig. 2.2 Topology of Ca_v channels. Ca_v channels contain several subunits: the ion permeation subunit α1, the extracellular α2δ subunits, the cytosolic β subunit and sometimes the transmembrane γ subunit. The topology shown here is of $Ca_v1.1$. The upper right pannel is adapted from Wu et al. [13]

2.1.3 Channelopathies

Voltage-gated calcium channels play essential roles in various physiological processes, and their disorders will trigger serious diseases, such as hypokalemic periodic paralysis ($Ca_v1.1$ related), cardiac arrhythmia ($Ca_v1.2$ related), autism spectrum disorder ($Ca_v1.2$ related), stationary night blindness ($Ca_v1.4$), cerebellar ataxia ($Ca_v2.2$) [1, 25].

Ca_v channels represent important drug targets. The α1 subunits are directly targeted by various natural toxins and clinical drugs that treat hypertension, arrhythmia, and angina pectoris. The α2δ-1 subunit is targeted by the gabapentinoid drugs gabapentin and pregabalin [26, 27].

2.2 Structural Studies of Voltage-Gated Calcium Channels

Because of the physiological and pathological importance, the structural studies of Ca_v channels have been pursued for decades. However, the long sequence of the single peptide chain with various intracellular linkers makes the channels resistant to recombinant overexpression, nor to say crystallization. Besides, the existence of the

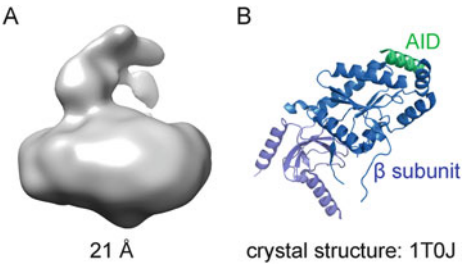

Fig. 2.3 Previous structural studies of Ca_v channels. (**a**) Low resolution cryo-EM map of $Ca_v1.1$. The nominal resolution is 21 Å [28]. (**b**) Crystal structure of the β subunit in complex with AID [29]

auxiliary subunits and various post-translational modifications such as glycosylation add further challenges to obtain a crystal structure of the Ca_v channel complex. Despite extensive efforts, the structural information of eukaryotic Ca_v channels have remained elusive. There were only low resolution cryo-EM maps reported (Fig. 2.3a) [28, 30–32]. High resolution crystal structures were obtained for the β1 subunit alone and in complex with the α1-interacting domain (AID) motif from the α1 subunit (Fig. 2.3b) [29, 33, 34].

Prokaryotic homologues of voltage-gated calcium or sodium channel are homotetramers, hence relatively easier than the eukaryotic ones for structural pursuit using X-ray crystallography. In the past several years, researchers have been focusing on the structural characterizations of the prokaryotic homologues of Ca_v or Na_v channel. A few crystal structures of bacterial Na_v channels were obtained, including Na_vAb [35], Na_vRh [36] and Na_vMs [37] since 2011. Moreover, the crystal structure of a Na_vAb variant that exhibits calcium selectivity, hence named Ca_vAb, was determined [38]. These structures represent imperative steps towards molecular understanding of the Na_v/Ca_v channels. However, there are still many questions remaining to be answered, including the most straightforward questions like how the four repeats of α1 subunit are organized spatially and how the auxiliary subunits interact with the α1 subunit. A high resolution structure of a eukaryotic Ca_v channel is required to address these questions.

The recent technological breakthrough in electron microscopy (EM), including the development of direct electron detector for image acquisition and new algorithms for data processing, provided an unprecedented opportunity to solve structures of challenging targets that were nearly insurmountable by X-ray crystallography. Structural biology by single-particle cryo-EM, used to be nick-named 'blob-ology', has been used to solve macromolecular structures at resolutions that can resolve side chains. Compared to X-ray crystallography, cryo-EM has two obvious advantages: (1) only several microliters of sample solutions are required, and (2) crystallization is spared. Cryo-EM has become a prevailing approach for structural biology of membrane proteins and macromolecular machineries since 2013 [39, 40]. The eukaryotic Ca_v channels have molecular weights beyond 300 kDa with the auxiliary subunits, representing suitable targets for cryo-EM.

$Ca_v1.1$ is the first $Ca_vα1$ to be cloned, and it has been a prototype in the functional, structural, and mechanistic investigations of Ca_v channels [12, 41]. There is only one isoform of β subunit, $β_{1a}$, in skeletal muscle [42], and the $Ca_v1.1$ complex is

exclusively expressed in skeletal muscle with relatively high abundance. It thereby may be easier to purify the $Ca_v1.1$ complex to homogeneity for cryo-EM imaging than for other Ca_v channels from other tissues. Several purification methods for $Ca_v1.1$ from rabbit skeletal muscle have been reported in literature [43–45]. Recently, a revised strategy for purification of $Ca_v1.1$ from rabbit skeletal muscle membrane was developed, using GST-fused β_{1a} subunit to compete with the endogenous β_{1a} subunit to pull down the whole complex [46]. This strategy is simple and more specific. The protein obtained using this protocol is of high purity and well behaved, representing an ideal target for single particle cryo-EM analysis.

In 2015, the cryo-EM structure of rabbit $Ca_v1.1$ was obtained at 4.2 Å resolution [46]. Most of the secondary structure elements of the protein complex were revealed in this structure. All the subunits were identified and the arrangement of the four repeats in α1 subunit has been revealed. In the following year, the resolution of the $Ca_v1.1$ structure was further improved to 3.6 Å resolution, which allowed assignment of most side chains [13]. This is the first near atomic structure of a eukaryotic Ca_v channels. The high resolution structure provides important insights into the understanding of the structure-function relationship of Ca_v and the related Na_v channels.

2.3 Structural Analysis of $Ca_v1.1$

2.3.1 The Overall Architecture of $Ca_v1.1$

The structure of $Ca_v1.1$ is constituted by the ion-permeating core subunit α1, the extracellular α2δ subunit, the intracellular β_{1a} subunit, and the transmembrane γ subunit. The overall structure is approximately 170 Å in height and 100 Å in the longest dimension of the width (Fig. 2.4a).

2.3.2 Structure of the α1 Subunit

The α1 subunit in eukaryotic Ca_v channels is a single peptide chain that folds into four similar repeats. Therefore, an evident and interesting question arose: how are the four homologous repeats arranged? Clockwise, counterclockwise, or intertwined?

Careful examination of the 4.2 Å cryo-EM reconstruction suggested that the four repeats are arranged in a clockwise manner in the extracellular view, which was then unambiguously confirmed by the 3.6 Å cryo-EM map (Fig. 2.4b). This repeat organization principle may be conserved in all eukaryotic Ca_v or Na_v channels.

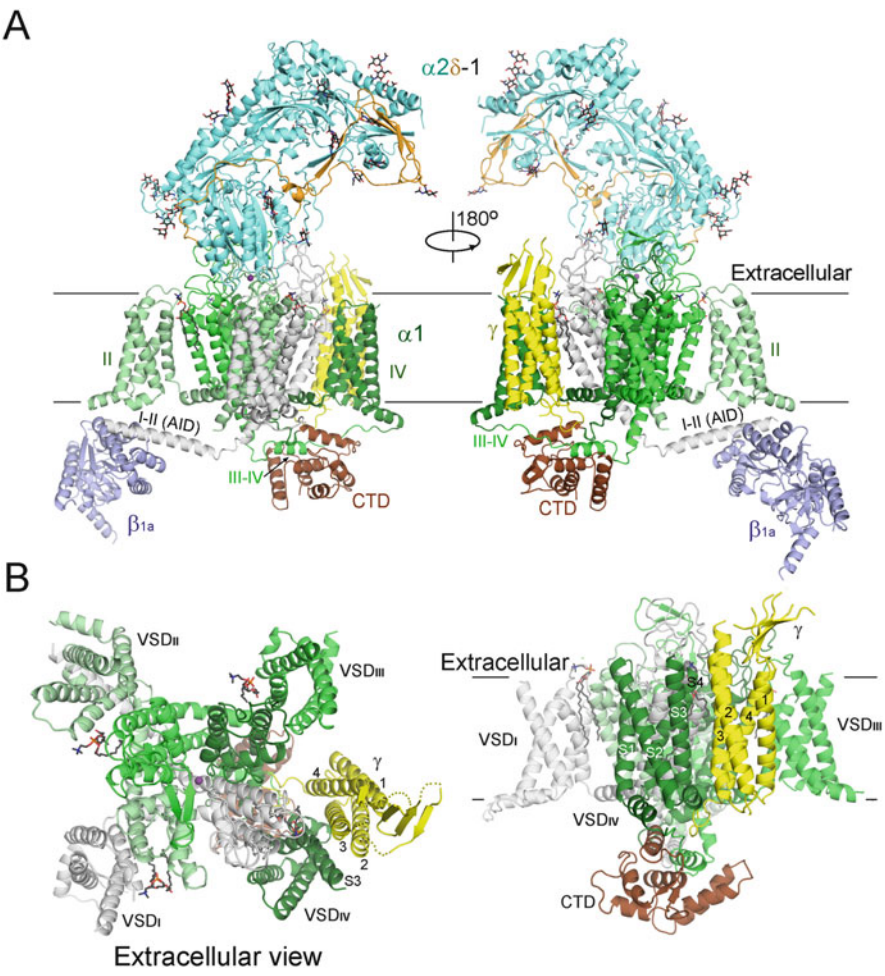

Fig. 2.4 Structure of the $Ca_v1.1$ channel complex from rabbit. (**a**) Overall structure of $Ca_v1.1$. The structure is color-coded for different subunits. The four repeats of the α1 subunit are colored from white to dark green and CTD is colored brown. (**b**) Spatial arrangement of α1 subunit. The four repeats are arranged clockwisely in the extracellular view. Adapted from Wu et al. [13]

2.3.3 The VSDs of $Ca_v1.1$

The VSDs are responsible for voltage sensing. Among the four transmembrane segments S1–S4 in each VSD, S4 contains repetitively aligned basic residues Arg or Lys that occur every three residues. They are named the "gating charges". The four VSDs of $Ca_v1.1$ are similar but non-identical with each other. The S3 segments of prokaryotic homologues Na_vAb and Na_vRh are largely unfolded on the extracellular side, whereas the S3 segments of $Ca_v1.1$ are full transmembrane helices.

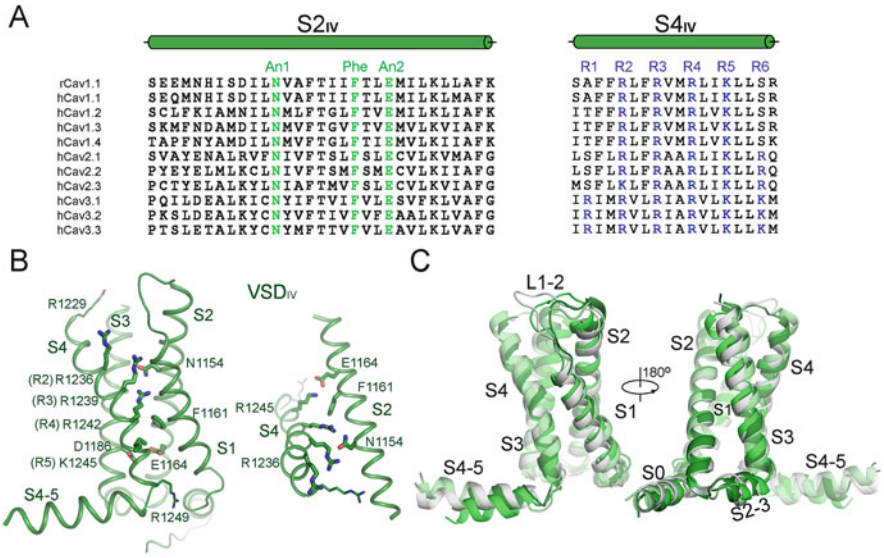

Fig. 2.5 VSD_{IV} of $Ca_v1.1$. (**a**) Sequence alignment of $S4_{IV}$ helix. The conserved R/K highlighted blue. (**b**) Structure of the VSD_{IV} of $Ca_v1.1$. (**c**) Structural superimposition of the four VSDs of $Ca_v1.1$. Adapted from Wu et al. [13]

According to the sequence alignment of $Ca_v1.1$ and ten human Ca_v channels, there are up to six gating charge residues on each S4 segment, defined as R1–R6 (Fig. 2.5a). For the rabbit $Ca_v1.1$, there are R1–R5 on $S4_I$, R2–R6 on $S4_{II}$, R1–R5 on $S4_{III}$, and R2–R5 on $S4_{IV}$.

In each VSD, all the gating charges are aligned on one side of the 3_{10} helix of S4. The R5 residues and $R6_{II}$ are below, whereas the R1–R4 residues are above the conserved occluding Phe in the charge transfer centre, representing the depolarized or 'up' conformation of VSDs (Fig. 2.5b). Considering the closed pore and the 'up' VSDs, the structure of $Ca_v1.1$ shown here may represent a potentially inactivated state. The R1–R4 residues are above the conserved occluding Phe in the charge transfer center, representing the depolarized or up conformation of VSDs. This is consistent with the sample conditions of 0 mV membrane potential (depolarized state).

2.3.4 The Selectivity Filter

The selectivity filter (SF) is responsible for ion selection. In $Ca_v1.1$, the SF is constituted by an outer site comprising the side chains of four essential Glu residues and an inner site formed by the carbonyl oxygen of the two preceding residues.

The SF of different voltage gated ion channels exhibits distinct chemical and structural features. The selectivity filter of K_V channels is constituted by the carbonyl oxygen of the residues. But in Ca_v and Na_v channels, the side chains of the outer site

residues are important for selectivity. In Na_v channels, the key residues are Asp/Glu/Lys/Ala (DEKA).

The SF of $rCa_v1.1$ is constituted by the side groups of Glu292/614/1014/1323 and the two preceding residues in each repeat that contribute the carbonyl oxygen (C=O). A consecutive stretch of density stands along the selectivity filter vestibule that can be deconvoluted to a round disc in the centre of the four Glu residues and a sphere surrounded by the eight C=O groups. Two Ca^{2+} ions are tentatively assigned to the middle disc and the inner sphere. The two maps (3.6 Å and 4.2 Å) were from two different batches of protein which was purified in 10 mM and 0.5 mM Ca^{2+}, respectively. The shape and position of the density in the selectivity filter vestibule in the two maps are different (Fig. 2.6a). The heights of the two Ca^{2+} ions in the 3.6 Å

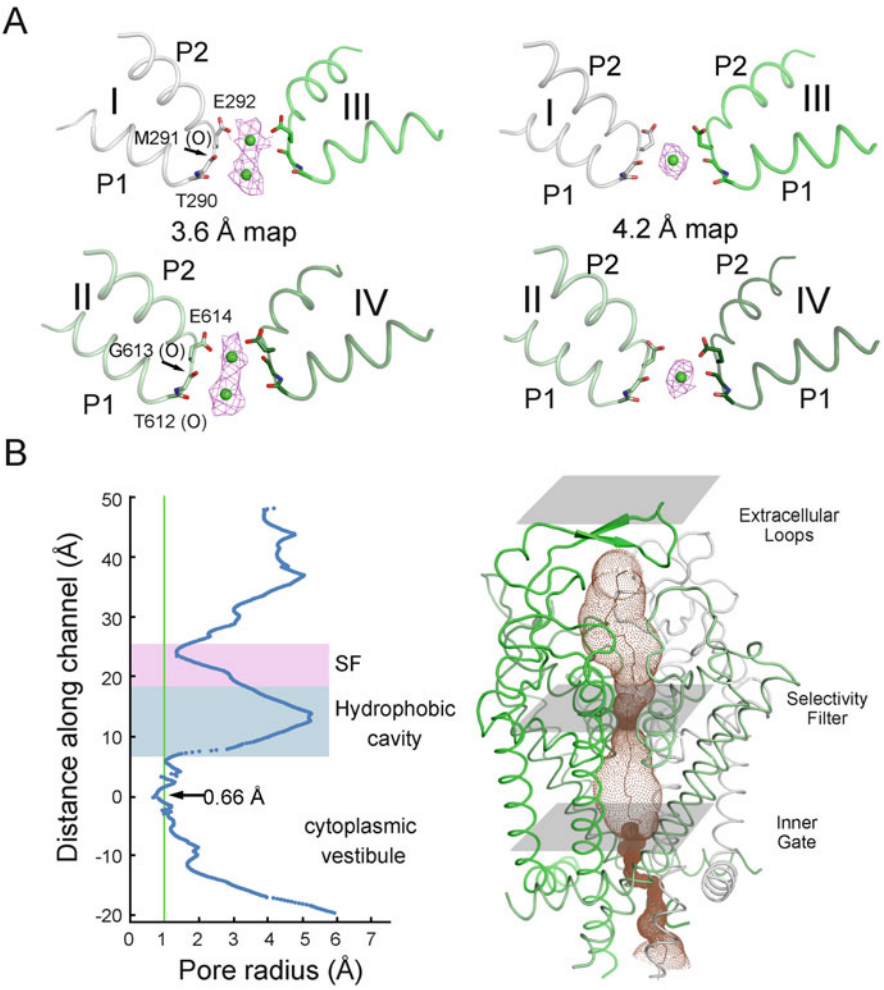

Fig. 2.6 Selectivity filter. (**a**) Structures of the selectivity filter obtained from two different maps. (**b**) The permeation path of the pore domain. Adapted from Wu et al. [13]

map are similar to those in Ca_{vAb} [38], except that the inner one is slightly off the central axis and closer to repeats I and II.

Above the entrance to the Ca^{2+} permeation path, the extended extracellular loops, which are stabilized by many disulfide bonds, form a windowed dome. The window is enriched in negatively charged residues, which may help attract cations. Below the SF is a hydrophobic cavity with fenestrations. The radius in the inner gate is less than 1 Å too narrow to permeate Ca^{2+} ions (Fig. 2.6b). Therefore the structure is in a closed conformation. Considering the closed pore and the 'up' VSDs, the reported structure of $Ca_v1.1$ may represent a potentially inactivated state.

2.3.5 The Auxiliary Subunits

In the structure of the rabbit $Ca_v1.1$ channel complex, four tandem cache domains and one VWA domain are identified in the α2δ subunit. Although these domains are organized separately in space, they involve intertwined sequences. Interestingly, although the δ subunit and α2 subunit are separated in the primary sequence, they co-fold with each other. The δ subunit contributes three β-strands to the fourth Cache domain. Cys1074 in the δ subunit forms a disulfide bond with Cys406 in the VWA domain. The extended conformation of δ is stabilized through multiple intra and inter-subunit disulfide bonds. In total, four disulfide bonds were observed between the α2- and δ-subunits and two within the δ-subunit.

There was a debate on whether the δ subunit is a transmembrane protein or a GPI (glycosylphosphatidylinositol)-anchored protein. In the 3.6 Å resolution EM map, there is no extra density for a TM helix. The density for δ subunit ended after Cys1074, which happened to be the predicted site for GPI modification. Besides, in the related MS result, no peptide can be detected after Cys1074. The structure appeared to support that δ subunit is a membrane anchored protein.

In the $Ca_v1.1$ complex structure, four transmembrane helices (named TM1–4), an extracellular β sheet and the cytosolic amino (N)- and C-terminal loops are resolved for the γ subunit. The transmembrane interface between α1 and γ is mediated by TM2 and TM3 of γ and the S3 and S4 segments in α1-VSD_{IV}. All of the residues constituting the interface are hydrophobic residues, which are unlikely to provide the specificity between γ and VSD_{IV}. On the intracellular side, the C-terminus of γ subunit contains some polar residues that are hydrogen-bonded with the III–IV linker in the α1 subunit, which may provide the interaction specificity with VSD_{IV} but not for other VSDs. The direct contact between γ and VSD_{IV} may affect the conformational changes of the latter during voltage dependent activation or inactivation, thereby providing the molecular basis for the antagonistic modulation of the γ subunit.

Before the determination of the structure of $Ca_v1.1$ complex, the crystal structure of β has been reported. The different cryo-EM maps of $Ca_v1.1$ revealed distinct conformations of the intracellular domains. The AID motif is sandwiched between α1-VSD_{II} and β. Comparison of the different EM 3D reconstructions reveals shifts of

the C terminus of $S6_I$ and the ensuing I–II helix. Meanwhile, the β-subunit undergoes a pronounced displacement between the two reconstructions.

2.3.6 *Structural Mapping of Disease-Associated Mutations*

Ca_v channels and the closely related Na_v channels play a major role in a multitude of physiological and pathological processes. Hundreds of mutations have been identified in these channels. The structure presented here represents the first atomic model of a single-chain eukaryotic Ca_v or Na_v channel. Structural mapping of the disease-associated mutations will greatly foster mechanistic understanding of the related disorders and provide the opportunity for novel drug development targeting these channels (Fig. 2.7).

2.4 Voltage-Gated Calcium Channels in Excitation-Contraction Coupling

Excitation-contraction coupling (E-C coupling) of muscles is an important and fundamental physiological process. Multiple proteins are involved in this signal transduction cascade, among which the ryanodine receptor RyR and Ca_v channels are two key components [47].

RyRs are high-conductance calcium release channels located on the sarco/endoplasmic (SR/ER) membrane. They are responsible for the rapid release of Ca^{2+} from intracellular stores during E-C coupling [48–50]. As the largest known ion channel, the homotetrameric RyR has a molecular mass of more than 2.2 MDa with each protomer containing about 5000 residues [51, 52]. In mammals, there are three isoforms: RyR1 and RyR2 are primarily expressed in skeletal and cardiac muscles, respectively, and RyR3 was originally found in the brain [53–56]. Recently, the near atomic 3D structures of RyR1 and RyR2 in multiple conformations have been reported [57–60]. Details of RyRs are discussed in a later chapter.

Ca_v channels, which are located in the transverse tubule (or T-tubule) are activated upon plasma membrane depolarization, and then induce the activation of the downstream RyRs. The sudden increase of the cytoplasmic Ca^{2+} triggers a cascade of cellular events that eventually result in muscle contraction. Ca^{2+} ions are then pumped back to the SR by the calcium ATPase SERCA, leading to muscle relaxation [47].

$Ca_v1.1$ (in skeletal muscle) and $Ca_v1.2$ (in cardiac muscle) sense the changes of the membrane potential of the plasma membrane and activate RyR1 and RyR2, respectively, but via different mechanisms (Fig. 2.8). In skeletal muscle, $Ca_v1.1$ undergoes conformational changes in response to membrane depolarization, which then activate RyR1 through physical association. It is called the "mechanical

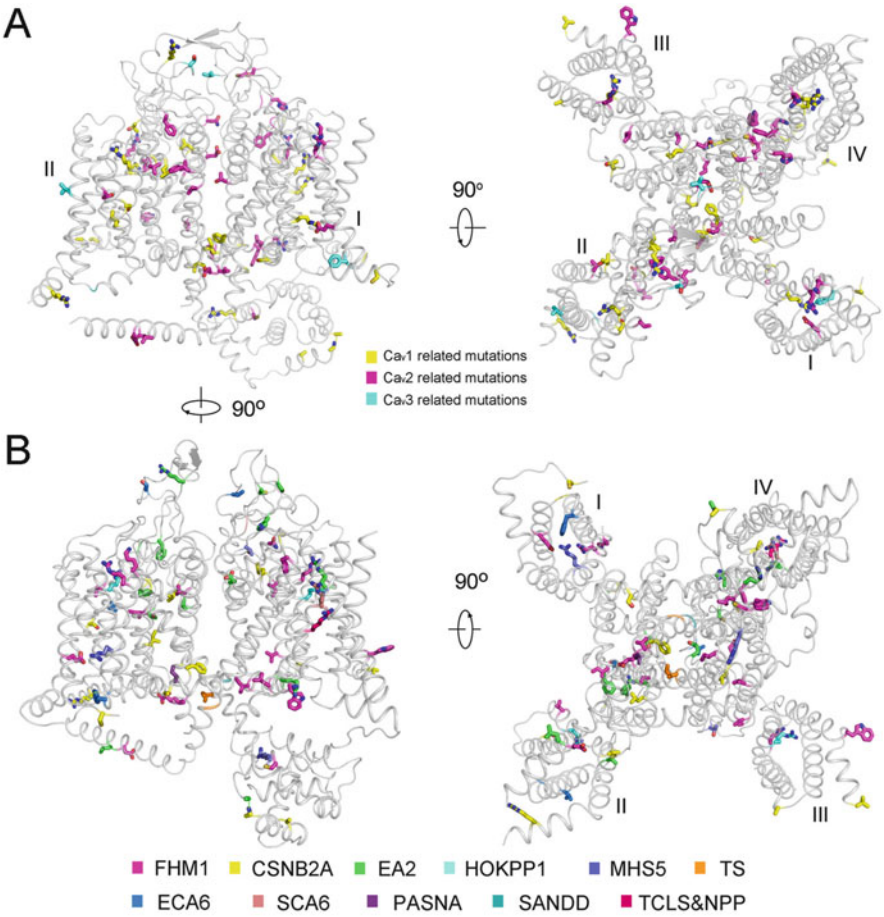

Fig. 2.7 Structural mapping of disease-associated mutations identified in the human Ca_v channels. The disease-related mutations identified in six human Ca_v channels are mapped to the structure of the r$Ca_v1.1\alpha1$ and color coded for different subtypes of Ca_v channels (**a**) or disorders (**b**). HOKPP1: periodic paralysis hypokalemic 1; MHS5: malignant hyperthermia 5; TCLS&NPP: Transient compartment-like syndrome and normokalaemic periodic paralysis; TS: Timothy syndrome; BRGDA3: Brugada syndrome 3; SANDD: sinoatrial node dysfunction and deafness; PASNA: primary aldosteronism, seizures, and neurologic abnormalities; NIDDM: non-insulin-dependent diabetes mellitus; CSNB2A: night blindness, congenital stationary, 2A; AIED: Aaland island eye disease; SCA6: spinocerebellar ataxia 6; FHM1: migraine, familial hemiplegic, 1; EA2: episodic ataxia 2; ECA6: epilepsy, childhood absence 6

coupling" mechanism [61]. In cardiac muscle, it is the Ca^{2+} influx mediated by $Ca_v1.2$ that activates RyR2, a mechanism known as the "calcium induced calcium release" (CICR) [62].

To understand the activation mechanism of RyR1 by $Ca_v1.1$, the accurate interaction between $Ca_v1.1$ and RyR1 is needed. Although the near atomic resolution

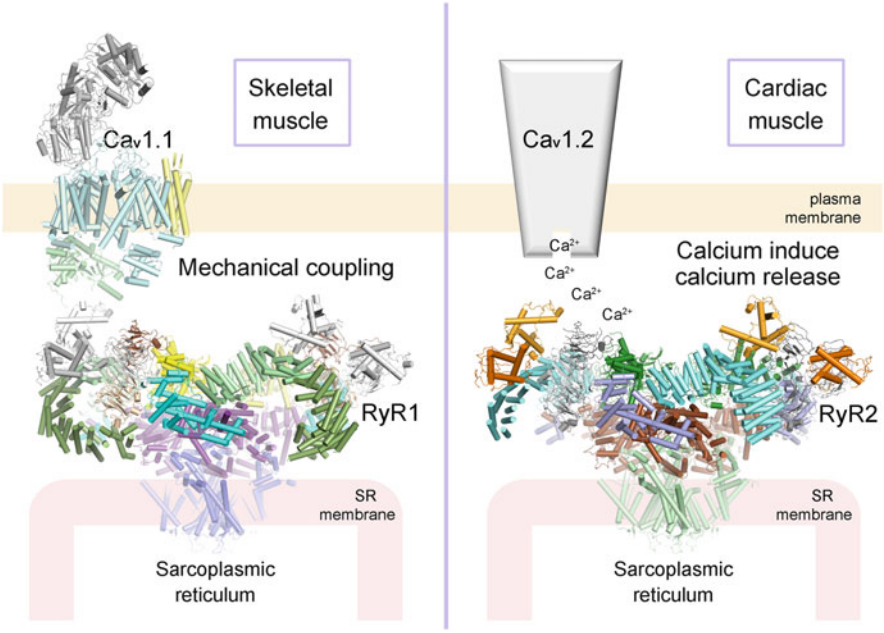

Fig. 2.8 Working models for activation of RyR1/2 by $Ca_v1/2$, respectively. In the skeletal muscle, RyR1 is activated by the membrane depolarization-induced conformational changes of $Ca_v1.1$ through physical interactions. It is called the "mechanical coupling" mechanism. In cardiac muscle, $Ca_v1.2$ permeates Ca^{2+} ions into the cytosol, leading to the activation of RyR2, a mechanism known as the "calcium induced calcium release"

structures of $Ca_v1.1$ and RyR1 have been solved, there is no detailed structural information on the $Ca_v1.1$ and RyR1 supercomplex. According to the structural analysis, a speculative mechanism is proposed [57]. As reported, RyR1 is activated through direct physical contacts with the $Ca_v1.1$ [61, 63–65]. Multiple areas, such as the SPRY3 domain of RyR1 and β-subunit of $Ca_v1.1$, are involved in their coupling [66]. The conformational changes of the VSDs of $Ca_v1.1\alpha1$ may induce shifts of the β-subunit and other cytoplasmic segments of $Ca_v1.1$, subsequently triggering the motion of the adjacent RyR1 cytoplasmic domains. Note that the SPRY3 domain of RyR1 is in direct contact with the N-terminal domain (NTD) within the same protomer. Potential shifts of the SPRY3 domain may be translated to the conformational changes of NTD, and subsequently the Handle domain and the Central domain. So the structural shifts triggered by $Ca_v1.1$ at the periphery of the RyR1 cytoplasmic region are propagated along the superhelical assemblies of the cytoplasmic domains to the Central domain, eventually leading to the opening of the intracellular gate. Nonetheless, the speculative mechanism still awaits experimental evidence.

2.5 Perspective

Voltage-gated calcium channels play essential roles in a multitude of physiological processes. The structure of $Ca_v1.1$ complex provides clues to the gating mechanism and selectivity, which establishes a foundation for mechanistic understanding of E-C coupling and provides a three-dimensional template for molecular interpretations of the functions and disease mechanisms of Ca_v and Na_v channels.

In the past few decades, most high resolution protein structures were obtained from X-ray crystallography. Until a few years ago, we witnessed the breakthrough of cryo-EM. The resolution was improved to near atomic resolution (beyond 4 Å), a "resolution revolution". A number of important protein or protein complex structures were solved. However, regardless of single-particle cryo-EM or X-ray crystallography, purification of the protein is required. Therefore, some proteins or protein complexes that are resistant to in vitro purification cannot yield high resolution structure. To visualize the E-C coupling ultrastructure formed by RyR1 and $Ca_v1.1$, we may eventually have to employ another technology, electron tomography (ET), which can image the samples in situ. The rapid technological development of ET may allow structural resolution the RyR1-$Ca_v1.1$ supercomplex to molecular details in the near future.

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