L-type calcium channel targeting and local signalling in cardiac myocytes

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Abstract

In the heart, Ca2⁺ influx via Cav1.2 L-type calcium channels (LTCCs) is a multi-functional signal that triggers muscle contraction, controls action potential duration, and regulates gene expression. The use of LTCC Ca2⁺ as a multi-dimensional signalling molecule in the heart is complicated by several aspects of cardiac physiology. Cytosolic Ca2⁺ continuously cycles between ~100 nM and ~1 µM with each heartbeat due to Ca2⁺ linked signalling from LTCCs to ryanodine receptors. This rapid cycling raises the question as to how cardiac myocytes distinguish the Ca2⁺ fluxes originating through L-type channels that are dedicated to contraction from Ca2⁺ fluxes originating from other L-type channels that are used for non-contraction-related signalling. In general, disparate Ca2⁺ sources in cardiac myocytes such as current through differently localized LTCCs as well as from IP₃ receptors can signal selectively to Ca2⁺-dependent effectors in local microdomains that can be impervious to the cytoplasmic Ca2⁺ transients that drive contraction. A particular challenge for diversified signalling via cardiac LTCCs is that they are voltage-gated and, therefore, open and presumably flood their microdomains with Ca2⁺ with each action potential. Thus spatial localization of Cav1.2 channels to different types of microdomains of the ventricular cardiomyocyte membrane as well as the existence of particular macromolecular complexes in each Cav1.2 microdomain are important to effect different types of Cav1.2 signalling. In this review we examine aspects of Cav1.2 structure, targeting and signalling in two specialized membrane microdomains—transverse tubules and caveolae.

Keywords

L-type calcium channel • T-tubule • Caveolae • Channel trafficking • Calcium signalling

This article is part of the Spotlight Issue on: T-tubules and ryanodine receptor microdomain signalling in cardiac hypertrophy and failure.

1. Cardiac L-type channel-structure function

High-voltage-activated calcium (Cav) channels convert electrical signals into Ca2⁺ influx to trigger biological responses in excitable cells. Cav channels are multi-subunit protein complexes comprised of a pore-forming α₁ subunit assembled with auxiliary (β, δ, and γ) subunits, and calmodulin (Figure 1). There are seven different types of Cav channel α₁ subunit (Cav1.1—Cav1.4 and Cav2.1—Cav2.3) encoded by different genes.¹ Under normal conditions, adult ventricular myocytes express only the Cav1.2 α₁C subunit.

1.1 The pore-forming α₁C subunit

The LTCC α₁C subunit contains four homologous domains (DI—DIV) each with six membrane-spanning segments (S1—S6) connected by intracellular loops and bracketed by cytosolic N- and C-termini. The S1—S4 segments from each domain comprise the voltage sensor, whereas the four S5—S6 segments form the channel pore and contain the selectivity filter.² The α₁C subunit undergoes extensive alternative splicing, and transcript scanning of human heart cDNA libraries indicates the existence of multiple α₁C splice variants.² The functional significance of α₁C alternative splicing in the heart is unknown, although experiments in heterologous cells indicate some alternatively spliced Cav1.2 variants display moderate changes in channel gating and/or trafficking.³,⁴ Although the α₁C mRNA predicts a protein of 240 kDa protein, western blots of heart tissue typically show an additional prominent band at 190 kDa. This is attributed to post-translational cleavage of α₁C in the C-terminus creating two fragments of 190 and 50 kDa, respectively.⁴ In heterologous cells, the cleaved α₁C C-terminus non-covalently associates with the main body of the channel and inhibits channel activity.⁵ The α₁C C-terminus is important for channel trafficking to the cell surface,⁶ and is a critical
CaV gating properties including: increasing channel open probability (\(P_o\)) and imparting unique profiles of voltage-dependent inactivation.9,10

In heterologous cells, these impart unique inactivation and single-channel-gating properties.18,19 In contrast to mouse, all four \(\alpha_2\) subunits interact with the extracellular domain I–II intracellular loop of pore-forming \(\alpha_{1C}\) subunits to the plasma membrane.9,10 For \(\alpha_{1C}\), the mechanism involves \(\beta\)-subunit binding to \(\alpha_{1C}\) I–II loop and causing rearrangement of \(\alpha_{1C}\) intracellular loops in a manner that shifts a balance of power between export signals on the I–II loop and retention signals elsewhere.5 In addition, \(\alpha_{1C}\) prevent targeting of \(\alpha_{3\beta_1}\) subunits to the proteasome leading to increased surface expression.15,16 Beyond enhancing trafficking, \(\alpha_{1C}\) also regulate multiple channel-gating properties including: increasing channel open probability (\(P_o\)), causing a hyperpolarizing shift in the voltage-dependence of activation, and imparting unique profiles of voltage-dependent inactivation.9,10

There appears to be species differences in the complement of \(\beta\) subunit isoforms expressed in adult mammalian heart. In adult mouse heart, only \(\beta_2\) contains a conserved core comprised of src homology 3 (SH3) and guanylate-kinase like (GK) domains and three variable unstructured regions—N-terminus, C-terminus, and a HOOK domain that separates SH3 and GK. An \(\alpha_1\)-binding pocket (ABP) in the \(\alpha_{2}\) GK domain binds with high (nM) affinity to a conserved 18-residue \(\alpha_1\) interaction domain (AID) located in the domain I–II intracellular loop of pore-forming \(\alpha_{1}\) subunits.14 In heterologous cells, \(\alpha_{1C}\) dramatically increase the trafficking of \(\alpha_{1C}\) subunits to the plasma membrane.9,10 For \(\alpha_{1C}\), the mechanism involves \(\beta\)-subunit binding to \(\alpha_{1C}\) I–II loop and causing rearrangement of \(\alpha_{1C}\) intracellular loops in a manner that shifts a balance of power between export signals on the I–II loop and retention signals elsewhere.5 In addition, \(\alpha_{1C}\) prevent targeting of \(\alpha_{3\beta_1}\) subunits to the proteasome leading to increased surface expression.15,16 Beyond enhancing trafficking, \(\alpha_{1C}\) also regulate multiple channel-gating properties including: increasing channel open probability (\(P_o\)), causing a hyperpolarizing shift in the voltage-dependence of activation, and imparting unique profiles of voltage-dependent inactivation.9,10

There are four distinct \(\alpha_{2\delta}\) subunit isoforms (\(\alpha_2\delta-1\–\alpha_2\delta-4\)). These proteins are the product of a single gene that is post-translationally cleaved into separate \(\alpha_2\) and \(\delta\) peptides held together by disulphide bonds.25,26 The large \(\alpha_2\) protein is entirely extracellular and tethered to the plasma membrane via the \(\delta\) subunit which has a short hydrophobic region that traverses the membrane in the manner of a type 1 transmembrane protein. It has also been shown that some \(\alpha_2\delta\) subunits can anchor to the plasma membrane using a glycosylphosphatidylinositol (GPI) anchor.26 The \(\alpha_2\) proteins contain a von Willebrand A (VWA) domain, a protein–protein interaction motif found in many proteins including integrin receptors and extracellular matrix proteins involved in cell adhesion.27 The \(\alpha_2\delta\) VWA domains contain a metal ion-dependent adhesion site (MIDAS).25–27 The \(\alpha_2\) proteins also contain a Cave domain, a signalling motif held in common with some prokaryotic chemotaxis receptors.25,26 In general, \(\alpha_2\delta\) subunits increase the amplitude of LTCC currents by approximately two- to three-fold in heterologous systems, and modulate current kinetics and voltage-dependence.29,30 The mRNA for \(\alpha_2\delta-1\) and \(\alpha_2\delta-2\) has been detected in heart, \(\alpha_2\delta-3\) is exclusively expressed in rat atria, and \(\alpha_2\delta-4\) is not present in the heart. \(\alpha_2\delta-1\) knockout mice display a significantly reduced \(I_{Ca,L}\) (45% decrease), slowed inactivation, and depolarizing shifts in the voltage-dependence of activation and inactivation.31 In neurons, \(\alpha_2\delta\) subunits interact with the extracellular...
matrix protein thrombospondin and promote synaptogenesis. It is unknown whether α2δ subunits have similar functionally relevant interactions with extracellular matrix proteins in the heart.

1.4 γ subunit

There are eight distinct γ isoforms (γ1–γ8). Topologically, γ subunits are membrane proteins with a predicted four membrane-spanning segments and intracellular N- and C-termini. The cDNA of four γ subunit isoforms (γ4, γ6, γ7, and γ8) has been detected in human heart, although only γ6 expression has been confirmed at the protein level in rat heart. In heterologous expression studies all these four γ subunits co-immunoprecipitate with CaV1.2 α1C subunit. Functionally, they produce distinctive effects on CaV1.2 current amplitude and voltage-dependence of gating in a manner that is dependent on the β-subunit isoform present within the channel complex. Nevertheless, the functional impact of γ subunit expression and diversity on CaV1.2 channel targeting and signalling in heart cells is unknown.

1.5 Calmodulin

LTCCs undergo negative and positive feedback modulation by Ca2+-ions termed CaV1.2-dependent inactivation (CDI) and CaV1.2-dependent facilitation (CDF), respectively. The ubiquitous CaV1.2-sensing protein, calmodulin (CaM), is the Ca2+-sensor for both CaV1.2 CDI and CDF. At resting intracellular Ca2+, CaM is associated with CaV1.2 channels. The proximal CaV1.2 α1C C-terminus contains critical structural elements (EF hand, pre-IQ, and IQ regions) necessary for apoCaM and Ca2+-CaM binding and transduction of CDI and CDF. Crystal structures of Ca2+-CaM bound to either the α1C IQ peptide, or pre-IQ–IQ region have been described. Beyond, endowing Ca2+-dependent regulation, CaM binding to the α1C C-terminus is also important for targeting CaV1.2 to the cell surface. The physiological importance of CaM regulation of LTCC in the heart has been demonstrated in two ways. First, over-expressing a mutated CaM (CaM1234) that no longer binds Ca2+ in adult cardiac myocytes eliminates LTCC CDI and results in ultra-long action potentials. Secondly, mice expressing a mutation (IQ to EQ) that disrupts CaM binding to α1C displayed a lower LTCC current, developed cardiomyopathy, and died prematurely.

2. L-type channel targeting in T-tubules

2.1 Channel lifespan as an estimate of dynamic trafficking

Cardiac ion channels occur at specific membrane sub-domains and in specific densities. Both localization and density remain remarkably consistent over the cardiac lifespan. Given that there is little or extremely slow (well less than 10% per year) turnover of cardiomyocytes, a natural conclusion would be that the ion channels within each cardiomyocyte are equally stable. However, studies that measure channel turnover reveal otherwise. Connexin43, the most well-studied cardiac channel protein, has a half-life of 1–3 h. In non cardiac systems, potassium channels have a turnover rate of about 2 h. L-type calcium channel half-life is not as well explored in the heart, but in cell lines and neurons Cav1.2 protein has a half-life of about 3 h. Our personal recordings of Cx43 and L-type calcium channel membrane turnover in cardiomyocytes, which were used to design pharmacological interventions that affect their trafficking, concur with the multiple hour timeframes reported in the literature. It is remarkable to consider that over the course of any day, most channels in our hearts are newly formed and ‘older’ channels degraded. Channel half-lives on the order of hours also indicate that trafficking to and from the membrane is highly dynamic and must be well regulated.

In the last two decades it has been increasingly clear that channel trafficking to the plasma membrane relies on cytoskeleton-based transport. Cytoskeletal components are easily able to accommodate rapid transport. For instance, kinesin-1-based transport along microtubules occurs at a rate of about 1 μm per second, which is theoretically fast enough to cover the 100 μm longitudinal axis of a ventricular cardiomyocyte in <2 min. Cytoskeletal components themselves are not static. Individual microtubule fibres are dynamic and can grow as well as suffer catastrophe all within several minutes. Thus the cytoskeleton delivery apparatus can well accommodate both ongoing steady-state trafficking and a sudden change in trafficking needs such as during adrenergic-mediated cardiac high output states or during acute cardiac injury.

We recently found that for Connexin43-based gap junction channels, more than 80% of the cytoplasmic channels are stationary and associated with actin fibres. Given the high turnover rate of channel protein, it is possible that there is a ready-made and constantly refreshed reservoir of channel that occurs below the plasma membrane and can be delivered to the membrane on a very short notice. Given the rapid turnover of all cardiac ion channels, intracellular reservoirs could contain ready-made redundant channel whose purpose is to maintain or increase density of channel in the plasma membrane. These reservoirs could buffer channel density in conditions of stress or insult.

The possibility exists (and is not well tested) that while overall channel protein turnover rate is high, functional channels that have been inserted in the plasma membrane may be less stable or, if anchored by scaffolding proteins, may be more stable than their cytoplasmic counterparts. For instance, pulse-chase experiments identified that membrane-bound Cav1.2 has a half-life as short as 3 h, whereas total cellular Cav1.2 half-life could be as much as 25 h. Thus the plasma membrane population of channel can turn over more quickly that total channel protein turnover. Accurate measurement of channel life cycles involves a combination of pulse-chase experiments with biochemical separation between plasma membrane-bound and general cytoplasmic populations, combined with detailed live cell imaging with tags that can be used to mark channels at certain times such as photoconversion of fluorescent colours. We encourage such studies in the future to understand the timing of channel localization in each phase of its life-cycle. Such studies would require primary cardiomyocytes and, for L-type calcium channel studies, further segregation of channel life cycles involves a combination of pulse-chase experiments with biochemical separation between plasma membrane-bound and general cytoplasmic populations, combined with detailed live cell imaging with tags that can be used to mark channels at certain times such as photoconversion of fluorescent colours. We encourage such studies in the future to understand the timing of channel localization in each phase of its life-cycle. Such studies would require primary cardiomyocytes and, for L-type calcium channel studies, further segregation would be necessary between the T-tubule membrane component and non-T-tubule plasma membrane.

2.2 Forward transport to T-tubules

Post-transcription and translation, the proteins that form cardiac ion channels such as Cav1.2 for the L-type calcium current are completed in the Golgi apparatus where they are also inserted in membrane vesicles for delivery to the plasma membrane (Figure 2). The cytoskeleton transports the vesicles to the plasma membrane, with most studies focusing on microtubules as the fibres responsible for forward...
A critical aspect of forward transport in cardiomyocytes is localization to membrane sub-domains. It is entirely possible that channels may be delivered to random regions of the plasma membrane, only to later laterally diffuse to their appropriate sub-domain. However, the temporal and stochastic inefficiency of non-specific channel insertion, together with unexplained mechanisms of subsequent lateral localization other than chance interaction with a sub-domain-specific anchor protein, suggests that specificity of delivery from the Golgi to the surface membrane may also occur. We previously found the Cx43 gap junctions are localized to cell–cell-bordered regions by a combination of the plus-end-tracking proteins EB1 and p150 (Glued) at the microtubule ends, and the adherens junction membrane complex, which captures the EB1-tipped microtubules, to allow for Cx43 hemichannels to be offloaded onto adherens junction-containing membrane. This directed targeting paradigm of ion channel delivery also holds for LTCCs, as we discuss below.

T-tubule invaginations of ventricular cardiomyocyte plasma membrane occur with a species-dependent spatial period of several micrometres and are enriched with L-type calcium channels. This local enrichment is necessary for calcium-induced calcium release with nearby ryanodine receptors, which is important for beat-to-beat excitation–contraction (EC) coupling in the heart. An understanding of LTCC trafficking to T-tubules is beginning to emerge. As discussed earlier in this review, the β subunit is generally necessary for surface expression. Moreover, the α2δ subunit synergizes with the β subunit to promote surface channel expression. These auxiliary subunits enhance surface expression by promoting channel export from the ER and can also stabilize the channel on its journey to the membrane, and limit internalization. It is possible that subunits contribute to localization to particular plasma membrane-specific sub-regions such as T-tubules, but data for such a phenomenon are presently lacking.

Several years ago we found that Cav1.2-based channels adhere to the directed targeting paradigm. In particular, the membrane scaffolding protein BIN1 which lines T-tubules provides the membrane anchor that attaches dynamic microtubules, allowing delivery of LTCCs directly to T-tubule membrane. Using HL-1 cells that express Cav1.2 but not T-tubules, and non-muscle cell lines that do not either express Cav1.2 or naturally form T-tubules, we found that exogenous BIN1 caused the formation of deep invaginations in the cell membrane enriched with endogenous or overexpressed Cav1.2, suggesting that BIN1-containing membrane without other typical cardiac muscle proteins is sufficient to recruit LTCCs. As we discuss below.

Unresolved issues in the directed targeting paradigm is where in the pathway to the membrane is specificity of delivery obtained and whether the minimal subset of channel, microtubule plus-end-tracking protein, and membrane anchor are sufficient to get each channel to its respective sub-domain. There is considerable evidence that specificity of delivery is obtained early in the Golgi apparatus, in effect membrane-bound vesicles receive their ‘postal address’ early in the

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**Figure 2** Forward trafficking of cardiac ion channels. Post-transcription and translation, cardiac ion channels are packaged into vesicles and transported by the microtubule cytoskeleton to specific sub-domains on the plasma membrane. Specificity of final delivery of connexon 43 channels to gap junctions is due, in part, to the connexon protein, the microtubule plus-end-tracking proteins EB1 and p150(Glued) and the adherens junction complex serving as membrane anchor. Specificity of final delivery of Cav1.2 channels to T-tubules is due, in part, to Cav1.2 protein, and BIN1 serving as membrane anchor. Microtubule plus-end-tracking proteins have not yet been determined for Cav1.2 to T-tubule trafficking. It has also been identified that the actin cytoskeleton slows down forward delivery and is associated with the majority of cytoplasmic Connexin 43 containing vesicles, possibly serving as sorting and reservoirs related rest stops on the way to the plasma membrane. Presumably actin fibres function in a similar capacity for Cav1.2 delivery.
transport pathway. Alternatively, vesicles may obtain specificity en route to the plasma membrane. Actin-based rest stops on the trafficking highway may be opportunities for either different microtubules or additional accessory-guiding proteins to be utilized to bring each channel to its appropriate sub-domain. Biochemical-based inquiries will aid exploration of trafficking-related binding partners of Cav1.2 such as calmodulin, GPCRs, and AKAP79 as well as other proteins that make up the LTCC. Furthermore, detailed live cell imaging can allow the pathway of the LTCC to be investigated as it exits the Golgi and makes its way to T-tubule membrane.

### 2.3 T-tubule membrane as destination

An intriguing and certainly controversial possibility about T-tubule membrane is that it undergoes dynamic remodelling. In mice and rats, T-tubule networks are deeper and more extensive than in rabbit and humans. In general, the extent of a T-tubule network in ventricular myocytes is proportional to the host heart rate due to the need for faster and uniform EC coupling at higher rates. However, heart and myocyte size, as well as chamber and myocyte cell type, all impact the expected extent of each T-tubule network. It remains an active area of interest to explore both the genetic determinates of T-tubule development and changes in a T-tubule network due to environmental and metabolic factors. Furthermore, it is known that the heart is able to contract without T-tubules. In mice and rats, T-tubules develop only in the second to fourth weeks of life. Therefore, embryonic and neonatal hearts in these animals have organized sarcomeres and generate a blood pressure, but without T-tubules. Similarly, T-tubule networks have not been reported for avian, reptile, and amphibian hearts.

Not only do embryonic and neonatal hearts not need T-tubules, but T-tubules are also lost over time in disease and culture conditions. Isolated cardiomyocytes are viable yet rapidly loose their T-tubules over the first 3 days of culture. Interestingly, actin stabilization limits cell culture-associated T-tubule loss. It has also been reported in animals and humans that both ischaemic and non-ischaemic heart failure (HF) is associated with T-tubule remodelling with less-deep and less-dense T-tubule networks in ventricular cardiomyocytes. The mechanisms of T-tubule loss in HF are not well understood.

The lack of T-tubules in embryonic and neonatal hearts, and the reduction of T-tubules in culture and disease indicate that T-tubules are very important but not fixed and vital structures of the cardiomyocyte and that without appropriate signalling and environmental conditions, T-tubules will recede. T-tubules may be present to optimize cardiac function, but are not essential to cardiomyocyte survival. Thus T-tubules can be dynamic structures that are continuously generated and degraded. Stress may inhibit de novo tubule membrane formation while maintaining or accelerating degradation. In a recent report, we found that calcium channels are intracellular localized in human HF, and that transcription of their membrane anchor, BIN1, is reduced. The most straightforward conclusion from this finding is that, without its membrane anchor, calcium channels are no longer delivered to T-tubules. However, BIN1 is also part of the membrane sculpting BAR domain family. It is an untested possibility that BIN1 reduction not only reduces trafficking of LTCCs to T-tubules, but decreased BIN1 is a contributing factor to disease-related reduction of T-tubule membrane. Many cell types release small (100–1000 nm) vesicles of plasma membrane known as microparticles. BIN1-containing microparticles are present in blood and plasma-derived BIN1 levels are reduced in HF, correlating with the clinical assessment of cardiac status and predicting ventricular arrhythmia. These findings suggest that remodelled T-tubule membrane is not just internalized, but also externalized and released into blood.

The existence T-tubule membrane turnover adds additional complexity to understanding LTCC trafficking of T-tubules. In a homeostatic equilibrium, not only would LTCC forward trafficking be needed to compensate for the rate of LTCC internalization, but also for the rate of T-tubule membrane loss. We hasten to add that despite the provocative data that suggest continuous T-tubule remodelling, such a phenomenon remains to be proven.

### 2.4 LTCC internalization from T-tubules

General internalization of LTCCs is poorly understood with a severe paucity of studies conducted in cardiomyocytes. Evidence exists that the beta subunit can enhance dynamin-dependent internalization in oocytes, and that neurons may undergo depolarization and calcium-dependent internalization. The reader is referred to several excellent reviews on LTCC internalization in neurons and other cells. We could not identify published primary data on LTCC internalization from cardiomyocyte T-tubules.

### 3. L-type channel targeting and signalling in caveolae

#### 3.1 Caveolae

Caveolae are a type of lipid raft and appear as small 50- to 100-nm diameter flask-shaped invaginations of the plasma membrane that are enriched in cholesterol and sphingolipids. They are defined by the presence of 18–22 kDa caveolin proteins, of which there are three types (caveolin 1–3). Muscle cells predominantly contain caveolin-3. Caveolins are hairpin-shaped proteins characterized by: a central membrane-associated region; cytoplasmic N- and C-termini; an oligomerization domain that permits their self-association; and a scaffolding domain that binds signalling proteins. In addition to caveolins, another class of cytosolic proteins, the cavin, is necessary for caveolae formation. Many signalling proteins are found to be concentrated in caveolae in different cell types, leading to the notion that these structures act as important organisational centres for cellular signalling. Typically, proteins target to caveolae using a hydrophobic caveolin-binding motif (ΦXXXΦXXXΦ and ΦXΦXXXΦ, where Φ is an aromatic residue) that binds the caveolin scaffolding domain.

#### 3.2 CaV1.2 targeting to caveolae

Electron microscopic studies of cardiac myocytes identify a high density of caveolae in the surface sarcolemma, and at a lower density in T-tubules where they are excluded from dyadic junctions. Several lines of evidence indicate that a subset of LTCCs in ventricular myocytes reside in caveolae. First, immunogold-labelled CaV1.2 has directly been observed in caveolae by electron microscopy. Secondly, cell fractionation and western blotting experiments indicate that a portion of cardiac CaV1.2 is present in detergent-resistant membranes that contain caveolin-3. Thirdly, immunoprecipitation of CaV1.2 in heart cells pulls down caveolin-3 and vice versa. Fourthly, quantitative co-immunofluorescence experiments demonstrate a subset of CaV1.2 co-localized with caveolin-3. A caveat for these last two is that a subset of caveolin-3 on the cardiac myocyte surface, such
as those present in T-tubules, is probably not associated with caveo-
lae. Estimates of the fraction of CaV1.2 present in caveolae range from
~25% of CaV1.2 in adult feline ventricular myocytes to ~50% in mice. The molecular determinants and mechanisms responsible
for CaV1.2 trafficking to caveolae in the heart are unknown, but pre-
sumably involve direct interactions between caveolin-3 and specific
subunit/s within the LTCC complex. Studies aimed at identifying
the specific molecular interactions responsible for CaV1.2 targeting to
caveolae in the heart are required to determine whether caveola-
targeted CaV1.2 channels are molecularly distinct from those targeted to
T-tubules.

### 3.3 Signalling function of caveolae CaV1.2 channels

There are conflicting data as to whether caveolea-localized CaV1.2 channels participate in EC coupling in adult ventricular myocytes. Geometrically, 50-nm diameter caveolae cannot be accommodated at dyadic junctions where CaV1.2 channels and ryanodine receptors approach within 12–20 nm of each other, a physical constraint confirmed by electron microscopy. Hence, it might be expected that caveolae LTCCs would not contribute appreciably to the CaV1.2-induced Ca2+-release that underlies ventricular EC coupling. However, acutely eliminating caveolae in adult rat ventricular myocytes with methyl-β-cyclodextrin resulted in diminished Ca2+- transients and cell shortening accomplished by a decrease in the gain of EC coupling. Immunofluorescence experiments indicate that caveolin-3 co-localizes with a fraction of extradyadic RYRs. One possibility is that CaV1.2 localized in such non-dyadic RYR-neighbouring caveolae contributes to or modulates EC coupling in adult ventricular myocytes. An alternative view is that caveolea-localized CaV1.2 channels do not participate in EC coupling but instead act locally to control other Ca2+-dependent signalling processes in the heart cells. For example, it has been hypothesized that caveolea-localized CaV1.2 channels selectively signal to the nucleus via the transcription factor NFAT (nuclear factor of activated T cells). To test this idea, House et al. recently developed an approach to selectively inhibit caveolae-localized LTCCs that takes advantage of RGK (Rad, Rem, Rem2, and Gem/Kir) proteins, a family of Ras-like GTPases that potently inhibit CaV1. channels. Rem autonomously targets to the plasma membrane using a polybasic C-terminus extension, and this localization is important for CaV1 channel inhibition. The House group replaced the Rem C-terminus with a caveolin-targeting motif, creating Rem263-cav. When expressed in adult feline ventricular myocytes, Rem263-cav is targeted to caveolae and prevents activity-dependent GFP-NFAT translocation to the nucleus without compromising contractility or Ca2+-transient amplitude.

It is not straightforward to envision how caveolae LTCCs can be used to activate NFAT signalling in the heart in a manner that is tightly regulated and specific. This is because LTCCs, being voltage-gated ion channels, open with each cardiac action potential and flood their microdomain with a high local [Ca2+], estimated to be in the ~100 μM range. Therefore, there must be mechanisms in place to ensure that caveolae LTCCs do not non-specifically activate NFAT signalling during normal physiological cardiac action potentials. One hypothetical possibility is that caveolea-localized CaV1.2 channels are held in an inactive low open probability state until acted upon by a second signal (such as phosphorylation initiated by a hypertrophic hormone). Such a mechanism would be analogous to the inactivation of endothelial nitric-oxide synthase (eNOS) when it is targeted to caveolae. However, there is no evidence for this type of regulation of CaV1.2 in the heart since acute ablation of caveolae in adult rat ventricular myocytes had no impact on LTCC current amplitude. Nevertheless, direct measurements of local caveolae Ca2+ signals is necessary to either convincingly rule in or rule out the possibility that caveolae LTCCs in the heart are normally held in an inactive state. Alternatively, specificity in the LTCC to NFAT signalling pathway may be conferred by the Ca2+-decoding properties of Ca2+-sensitive effector proteins present in the caveolae LTCC microdomain.

### 3.4 Ca2+-sensing effectors: CaM, calcineurin, and CaM kinase II

There are two functionally distinct pools of CaM in the LTCC microdomain whose properties have been recently reviewed. First, there is CaM that is associated with the CaV1.2 C-terminus as a virtual subunit and is the sensor for CDI. Secondly, there is a local pool of signalling CaM that when calcified can be used to locally activate Ca2+-CaM-dependent effector proteins such as calcineurin and Ca2+-CaM-dependent protein kinase II (CaMKII). The total CaM concentration ([CaM]) in myocytes is ~6 μM, of which only 50–75 nM is free CaM. Mori et al. used CaV1.2 fused to single CaM molecules as a biosensor to estimate a local [CaM] of 2.5 mM in the LTCC microdomain in HEK 293 cells, several orders of magnitude above the measured total cellular free [CaM] of 50 nM. The precise mechanism underlying such local CaM enrichment is unknown, but could involve apoCaM bound to CaM buffer proteins. Whether CaM is similarly enriched in the microdomain of LTCCs in cardiomyocytes has yet to be directly confirmed. CaM consists of two lobes (N- and C-lobes) joined together by a central α helix, with each lobe containing two Ca2+-binding EF hand motifs. The two lobes differ in their affinity for Ca2+ (N-lobe Kd ~12 μM, C-lobe Kd ~1 μM) and the kinetics of Ca2+ dissociation (~1000 s-1 for N-lobe, ~10 s-1 for C-lobe). Furthermore, the affinity and kinetics of Ca2+-binding can dramatically be altered by CaM binding to target proteins. The relatively low affinity of CaM for Ca2+ makes it selectively responsive to microdomains of high [Ca2+] occurring at the mouth of LTCCs and other Ca2+-sous in myocytes (e.g. RyRs and IP3Rs) rather than the bulk cytosolic 1 μM-amplitude Ca2+-transients that drive contraction. The fast dissociation kinetics of Ca2+ from the N-lobe ensures that once calcified, CaM travels a distance of ~100 nm before losing Ca2+ bound to the N-lobe, effectively restricting the range of action of calcified CaM to the microdomain of the activating Ca2+-source.

Calcineurin, a serine-threonine phosphatase, is a heterodimer comprised of catalytic (CnA) and regulatory (CnB) subunits. CnA (~60 kDa) consists of an N-terminal catalytic domain, a helical CnB-binding region, a CnM-binding region, and a C-terminus autoinhibitory peptide that binds the catalytic domain to keep the enzyme in an inactive state. CnB (~19 kDa) contains four EF hands, and under basal (low Ca2+)-conditions binds to CnA in a manner that renders the CnM-binding region unavailable for CnM association. CnA-dependent activation of calcineurin takes place in several steps. First, Ca2+ binds to CnB causing a conformational change that makes the CnM-binding region available. In this state, the enzyme is partially active (10% of full activity). Secondly, Ca2+-CaM binds the exposed CnM-binding region of CnA, causing a
conformational change that relieves the action of the autoinhibitory peptide on the catalytic domain, resulting in full enzymatic activity.

The link between calcineurin and NFAT activation was first described in activated T lymphocytes, but is now recognized as a widespread signalling paradigm in many systems, including in the heart. Under basal conditions, NFAT is phosphorylated and trapped in the cytosol. Activated calcineurin dephosphorylates NFAT, allowing its translocation into the nucleus where it initiates transcription of NFAT-responsive genes. Chronic activation of the calcineurin/NFAT signalling pathway causes pathological cardiac hypertrophy in the heart.104,105 Three distinct sarcolemmal sources of Ca2+ have been linked to activation of calcineurin in the heart—LTCCs, T-type Ca2+-channels, and TRPC channels.24,106–108 Experiments in neonatal rat ventricular myocytes suggest that LTCCs may be most tightly coupled to calcineurin—NFAT signalling.108

In the heart, calcineurin is part of a macromolecular complex with CaV1.2 channels.90,109 (Figure 3). Two distinct binding sites have been identified for calcineurin targeting to the CaV1.2 macromolecular complex. First, calcineurin binds to the A kinase-anchoring protein, AKAP 5 (also known as AKAP 79 and AKAP 150).90 Secondly, calcineurin binds directly to the CaV1.2 N- and C-terminus.109 The role of calcineurin binding to the CaV1.2 C-terminus in the heart is unclear, because in AKAP 5 knockout mice calcineurin is no longer pulled down with the CaV1.2 macromolecular complex.90 AKAP 5 is also necessary for calcineurin association with caveolin-3 in the heart.90 This has important implications for the hypothesis that caveolae-localized, rather than dyadic LTCCs selectively activate calcineurin—NFAT signalling.93 (Figure 3). It is unclear whether dyadic LTCCs are associated with calcineurin. From a design perspective, there may be an advantage to not having calcineurin associated with dyadic LTCCs, since modelling suggests that calcineurin at the dyadic cleft would be constitutively active due to the large local [Ca2+] generated by LTCCs and RyRs, and the high affinity of calcineurin for Ca2+-CaM.101 Overall, fundamental questions remain regarding how caveolae-localized LTCCs activate calcineurin/NFAT signalling in the heart.

CaMKII is a multi-functional serine/threonine protein kinase with numerous biological functions in many cell types including the heart.110 The CaMKII holo-enzyme is a multimer of 12 monomeric subunits, each of which has three distinct domains: an N-terminal catalytic domain that mediates kinase activity, a central regulatory domain that exerts basal autoinhibitory control of the kinase domain, and an association domain that mediates subunit assembly.110,111 Ca2+-CaM activates CaMKII by binding the regulatory domain and relieving autoinhibition. Sustained elevations in Ca2+-CaM result in autophosphorylation of CaMKII that leads constitutive, Ca2+-CaM-independent enzymatic activity.111,112 There is overwhelming evidence that autonomous CaMKII activity is elevated and plays a prominent role in cardiac pathogenesis in animal models of HF,113,114 and in failing human heart.115,116 CaV1.2 channels in the heart display an activity-dependent facilitation of current amplitude that is mediated through activated CaMKII,117,118 and is an important mechanism for dynamically regulating cardiac contractility. However, when excessive, CaMKII-mediated enhancement of ICa,L contributes to a prolonged APD, increased incidence of early and delayed after-depolarizations (EADs and DADs), and a propensity for cardiac arrhythmias.119 CaMKII associates with and phosphorylates both the pore-forming α1C subunit and the auxiliary β subunit.121 Evidence has been provided supporting the importance of phosphorylation of both α1C and β1,121 in CaMKII-mediated facilitation of ICa,L. It is unknown whether caveolae- and T-tubule-localized LTCCs differentially associate with CaMKII.

4. Dysregulation of L-type channel targeting and signalling in cardiac disease

Mutations of LTCC proteins are associated with multiple diseases of both electrical (arrhythmia) and mechanical (HF) phenotype. Gain-of-function mutations in CaV1.2, which cause near-complete loss of voltage-dependent channel inactivation and calcium overload in multiple tissues, are linked to Timothy syndrome123,124, which is notable for a long QT interval, ventricular arrhythmia, and also structural heart defects. Interestingly, the disease-causing mutations, G406R and G402S, were found within exons 8 and 8a, which are alternatively spliced in a mutually exclusive fashion. In patients with hypertrophic HF, aberrant splicing of the mutually exclusive exons 31 and 32 was detected such that re-expression of the foetal exon contributed to disease progression.124

Loss-of-function mutations in CaV1.2 occur in patients with Brugada syndrome which is characterized by a short QT interval and sudden cardiac death.125 When missense mutations at G490R and A39V were co-expressed with other LTCC subunits in CHO cells, a clear reduction of ICa,L was observed. Confocal microscopy studies revealed that channel trafficking was unaffected. Thus, it was hypothesized that the G490R mutation, which is located in a linker region, interferes with β subunit binding to inhibit current density. Another study revealed that a V2041I mutation in the C-terminus also reduces ICa,L amplitude by decreasing channel conductance and altering current inactivation.125

Abnormal EC coupling, resulting from deregulation at the onset of Ca2+ influx through the T-tubule network, is increasingly implicated in HF progression and sudden cardiac death.126,127 Measurements of whole-cell LTCC current density in cardiomyocytes from failing human hearts or animal models of HF either find a decrease128–131 or no change in ICa,L.122–124 Interestingly, in a canine
tachycardia-induced model of HF Cav1.2 maximal gating charge ($Q_{\text{max}}$) was decreased by ~60% even though whole-cell $I_{\text{Ca,L}}$ was unchanged. Because $Q_{\text{max}}$ provides a measure of the number of channels with moveable voltage sensors in the sarcolemma, this result implied that there were less surface Cav1.2 channels in this model of HF, but that the channels present were more active. Indeed, single-channel experiments found a higher open probability for LTCCs from failing compared with non-failing human ventricles. However, while we recently confirmed that the total cellular Cav1.2 content is unchanged in HF, we also found that the channels are internalized. Testing the forward trafficking machinery of Cav1.2 in failing hearts, we found that human HF involves a reduction in BIN1 at both the protein and mRNA message level, implying that the reduction is transcriptional. Subsequent studies in adult cardiomyocytes confirmed that decreased BIN1 reduces forward trafficking of Cav1.2, also diminishing intracellular calcium transients (in isolated cells and zebrafish hearts) and contractility (of zebrafish hearts). Recent rat studies confirmed that BIN1 is decreased in failing hearts and recovers with successful treatment. It is therefore possible that reduced contractility of progressively failing heart can be traced, in part, to decreased translocation of the LTCC scaffold BIN1.

5. Perspectives

Cardiac Cav1.2 channels exist in multiple discrete sub-domains with the plasma membrane, including in different locations within each cardiac T-tubule and in caveolae. The channel itself, like all cardiac ion channels, is short lived with a half-life that is likely on the order of hours. Therefore, the channel's trafficking to specific sub-domains must be highly regulated and dynamic. The channel is heavily dependent on channel subunits, cardiac cytoskeleton, and scaffolding proteins for proper membrane localization. Existing data indicate that Cav$\alpha$β subunits increase forward trafficking and prevent degradation, and extracellular subunits αβ increase LTCC current. The γ subunit affects current density but is less well studied. Cav1.2 delivery to T-tubules is microtubule dependent and facilitated by the BIN1-scaffolding protein that occurs at T-tubule membrane. Data exist on other cardiac ion channels for the role of actin as rest stops on the way to the plasma membrane, and cytoskeleton-based specificity of delivery is an active area of investigation. The regulatory protein calmodulin affects both gating and channel trafficking, and distinctive properties of Cav$\alpha$β-calmodulin association with calcineurin vs. CaM Kinase II may provide local signaling specificity to Cav1.2 channels. In failing cardiomyocytes, trafficking of channels to the plasma membranes is impaired, and the channels themselves undergo gating changes that increase open probability possibly as a compensatory result. In the future, detailed biochemical and imaging studies in cardiomyocytes and interpreted in the context of the LTCC life cycle (forward trafficking, existence on the membrane, and internalization) will help identify key regulators of this important channel.

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