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Different Subcellular Populations of L-type Ca^{2+} Channels Exhibit Unique Regulation and Functional Roles in Cardiomyocytes

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Abstract

Influx of Ca^{2+} through L-type Ca^{2+} channels (LTCCs) contributes to numerous cellular processes in cardiomyocytes including excitation-contraction (EC) coupling, membrane excitability, and transcriptional regulation. Distinct subpopulations of LTCCs have been identified in cardiac myocytes, including those at dyadic junctions and within different plasma membrane microdomains such as lipid rafts and caveolae. These subpopulations of LTCCs exhibit regionally distinct functional properties and regulation, affording precise spatiotemporal modulation of L-type Ca^{2+} current ($I_{Ca,L}$). Different subcellular LTCC populations demonstrate variable rates of Ca^{2+}-dependent inactivation and sometimes coupled gating of neighboring channels, which can lead to focal, persistent $I_{Ca,L}$. In addition, the assembly of spatially defined macromolecular signaling complexes permits compartmentalized regulation of $I_{Ca,L}$ by a variety of neurohormonal pathways. For example, β-adrenergic receptor subtypes signal to different LTCC subpopulations, with β_2-adrenergic activation leading to enhanced $I_{Ca,L}$ through caveolar LTCCs and β_1-adrenergic stimulation modulating LTCCs outside of caveolae. Disruptions in the normal subcellular targeting of LTCCs and associated signaling proteins may contribute to the pathophysiology of a variety of cardiac diseases including heart failure and certain arrhythmias. Further identifying the characteristic functional properties and array of regulatory molecules associated with specific LTCC subpopulations will provide a mechanistic framework to understand how LTCCs contribute to diverse cellular processes in normal and diseased myocardium.

Keywords

Cardiomyocyte; Calcium channel; Subcellular localization; Microdomain; Calcium signaling

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1. Introduction

In the heart, voltage-dependent L-type Ca^{2+} channels (LTCCs) are essential to numerous cellular processes including excitability, excitation-contraction (EC) coupling, hormone secretion, and regulation of gene expression. Participation in such diverse functions demands that the influx of Ca^{2+} through L-type channels (L-type Ca^{2+} current, $I_{Ca,L}$) is tightly controlled and compartmentalized within the cardiac myocyte. It has long been recognized that discrete clusters of LTCCs exist along the sarcolemma, and studies in recent years have greatly extended our understanding of how specific subcellular localization impacts channel function and regulation by a variety of neurohormonal and second messenger pathways [1-6].

A number of important LTCC subpopulations have been identified in cardiomyocytes that associate with unique macromolecular signaling complexes and scaffolding proteins, which enables spatiotemporal modulation of $I_{Ca,L}$. These include channels that are localized to dyadic junctions as well as extradyadic channels that reside in biochemically distinct regions of surface membrane known as membrane microdomains. Plasma membrane microdomains, including lipid rafts and caveolae, exhibit unique lipid composition and protein components and coordinate numerous cellular functions including various signal transduction pathways and protein recycling [7-9]. Numerous signaling molecules have been localized to caveolae including components of the β_2-adrenergic receptor/adenylyl cyclase/protein kinase A (PKA) cascade [5,6]. This review will highlight the evolving understanding of distinct subcellular populations of LTCCs in cardiomyocytes and their differing regulation and contributions to Ca^{2+} signaling in the heart.

2. LTCCs in the heart

2.1. Molecular composition of cardiac LTCCs

LTCCs are multimeric complexes consisting of a pore forming α_1 subunit and auxiliary β, $\alpha_2\delta$, and α subunits [10]. The α_1 subunit serves as the main functional component of the channel complex and consists of four homologous domains (I-IV) each containing six transmembrane segments (S1-S6). $Ca_v1.2$ (α_{1C}, encoded by the *CACNA1C* gene) is the predominant α_1 subunit in ventricular myocardium, whereas both $Ca_v1.2$ and $Ca_v1.3$ (α_{1D}, encoded by *CACNA1D*) are expressed in atrial tissue as well as nodal cells, where $I_{Ca,L}$ contributes to automaticity [11-15]. Extensive alternative splicing of $Ca_v1.2$ has been reported, and these splice variants play unique roles in cardiovascular physiology, pharmacology, and disease [16,17]. One important example is alternative splicing of $Ca_v1.2$ within transmembrane segment IS6, which impacts sensitivity to the dihydropyridine class of LTCC blockers. Differential expression of these splice variants lead to higher or lower sensitivity in smooth and cardiac muscle, respectively [18,19].

Ca^{2+} channel auxiliary subunits further add to the functional diversity of LTCCs. The cytosolic β subunits promote trafficking of the channel complex to the plasma membrane and modulate gating properties of the channel [20-22]. The β subunits are encoded by four distinct genes (*CACNB1-4*), each of which undergoes alternative splicing to generate a total of 18 or more unique β subunit isoforms in human myocardium [23]. The $\alpha_2\delta$ subunits arise from a common precursor protein that is post-translationally cleaved and relinked via a disulfide bridge. The extracellular α_2 peptide is heavily glycosylated and the δ peptide contains a single transmembrane domain [24]. Of the four $\alpha_2\delta$ subunits (encoded by *CACNA2D1-4*), $\alpha_2\delta$-1-3 are expressed in atrial tissue whereas $\alpha_2\delta$-1 and $\alpha_2\delta$-2 are present in ventricular myocardium [25,26]. The $\alpha_2\delta$ subunits modify both channel gating properties and surface membrane expression of the L-type channel complex [20,27]. Ca^{2+} channel α subunits, of which eight exist (encoded by *CACNG1-8*), were originally demonstrated to

associate with voltage-dependent Ca^{2+} channels in skeletal muscle and brain [28,29]. However, recent evidence suggests several α subunits including α4, α6, α7, and α8 are present in cardiac muscle and associate with the cardiac $Ca_v1.2$ channel complex, altering both activation and inactivation properties of the channel [30].

2.2. Distinct LTCC subpopulations in cardiac myocytes

2.2.1. Dyads—A critical subpopulation of LTCCs is that which participates in EC coupling. A number of studies applying immunoconfocal and electron microscopy techniques have demonstrated that a subset of LTCCs form dyadic complexes with Ca^{2+}-release channels (ryanodine receptors) on apposing junctional sarcoplasmic reticulum (SR) [1,2,31,32]. Upon membrane depolarization, activation of these LTCCs leads to an influx of Ca^{2+} into the dyadic cleft space, which triggers the opening of ryanodine receptors and subsequent release of SR Ca^{2+} stores. This Ca^{2+}-induced Ca^{2+} release (CICR) mechanism underlies the rise in free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) that activates myofilament proteins leading to muscle contraction [33].

Studies have estimated that approximately 75% of LTCCs reside at dyad junctions in cardiac myocytes [34]. In mammalian ventricular cardiomyocytes, dyadic couplings occur predominantly within the transverse (T)-tubule network, which represents a complex system of interconnected membrane structures continuous with the extracellular space [35]. T-tubules extend deep into the myocyte at each myofibrillar Z-line, bringing the plasma membrane in close proximity with junctional SR through the width of the cell, thereby allowing synchronous Ca^{2+} release throughout the cell with each depolarization. However, as many as 25% of dyads are found at the surface sarcolemma in ventricular myocytes [35,36]. Studies in which the T-tubule system is isolated from the surface membrane using osmotic shock led to an 75-80% decrease in $I_{Ca,L}$ without significantly altering SR Ca^{2+} load; therefore, it is hypothesized that although the majority of LTCCs reside in the T-tubular membrane, surface membrane LTCCs may play a particularly important role in SR Ca^{2+} loading [37].

Dyadic LTCCs are the target of numerous neurohormonal signaling pathways, most prominently the β-adrenergic/PKA signaling pathway, which increases contractility partially through enhancing $I_{Ca,L}$ [38]. Whereas extradyadic LTCCs do not contribute directly to EC coupling, these channels likely play roles in several other cellular processes. These extradyadic channels are not found randomly throughout the sarcolemma, but associate with important signaling molecules in biochemically-distinct membrane microdomains [3,4,6,39].

2.2.2. Lipid rafts—The plasma membrane is a heterogeneous mixture of lipids, cholesterol, and proteins that can form localized regions that compartmentalize cellular processes. The tight packing of sphingolipids and cholesterol forms liquid-ordered microdomains popularly termed “lipid rafts”, which serve a number of important cellular functions including signal transduction and membrane trafficking [7,8]. Although the small (10-200 nm), heterogeneous, and dynamic nature of lipid rafts has made their visualization and characterization challenging, one common feature is that these microdomains resist solubilization with non-ionic detergents such as Triton X-100 at 4°C and can be found in low density fractions on density gradients [8,40,41]. A number of studies utilizing this biochemical methodology have demonstrated the presence of $Ca_v1.2$ in low density fractions from ventricular myocardium [5,42,43], strongly suggesting LTCCs localize to lipid raft domains.

2.2.3. Caveolae—Caveolae represent a subset of lipid rafts that are morphologically distinct structures appearing in electron micrographs as flask-shaped invaginations of

membrane that are 50-100 nm in diameter. Caveolae are rich in cholesterol and are associated with the integral membrane protein caveolin [44]. Three genes (*CAV1-3*) encode six known caveolin subtypes (caveolin-1α and −1β; caveolin-2α, −2β, and −2α; and caveolin-3) that have varying tissue distribution [45]. Caveolin (Cav)-1 and Cav-2 are expressed in most cell types, whereas expression of Cav-3 is restricted to cardiac, skeletal, and smooth muscle types, as well as some types of neurons [45-47].

The lack of morphologically distinct caveolae in skeletal muscle from Cav-3 knockout mice highlights the importance of caveolin in caveolae formation [48]. Cholesterol is an additional component necessary for caveolar assembly and maintenance, because depletion of membrane cholesterol dramatically reduces caveolae number [49]. More recently, the cytosolic protein Polymerase I and Transcript Release Factor (PTRF), also known as Cavin, was also demonstrated to be required for caveolar biogenesis [50].

Several methods have been used to demonstrate the presence of LTCCs within caveolae in cardiomyocytes, exploiting the presence of Cav-3 as a marker to help differentiate the localization of proteins to caveolae versus non-caveolar lipid rafts. First, co-immunoprecipitation from ventricular myocytes using anti-Cav-3 or anti-$Ca_V1.2$ showed that the two proteins are associated [5,51]. Immunofluorescence studies of isolated ventricular myocytes using confocal microscopy also demonstrated extensive colocalization between Cav-3 and $Ca_V1.2$ [5,43]. Perhaps most strikingly, immunogold electron microscopy showed colabeling of Cav-3 and $Ca_V1.2$ in close association within morphologically distinct caveolae (Fig. 1A,B) [5,51].

2.2.4. Caveolin-3 scaffolds—The presence of caveolins in cell types lacking morphologically distinct caveolae such as neurons has sparked interest in understanding the physiological role of caveolins independent of caveolae. It has been suggested that extra-caveolar caveolin forms scaffolds that regulate protein trafficking and signal transduction in some cell types [52]. Cav-3 scaffolds may be particularly important in cardiac myocytes, which have relatively few caveolae compared to other cell types such as endothelial cells and smooth muscle myocytes, yet express Cav-3 at high levels [52,53]. In adult rat cardiomyocytes, a significant amount of Cav-3 is found in heavy (non-buoyant) fractions, and immunoprecipition studies showed that Cav-3 associates with a number of signaling proteins including G-protein coupled receptors, adenylyl cyclase, and heterotrimeric G proteins even in heavy fractions [54]. These findings suggest Cav-3 may regulate signal transduction in sarcolemmal regions outside of caveolae in cardiac myocytes. However, no data yet directly demonstrate localization of LTCCs to noncaveolar Cav-3 scaffolds and additional approaches such as immunogold electron microscopy are needed to carefully investigate this possibility.

2.2.5. Nucleus—The carboxyl (C)-terminus of $Ca_V1.2$ undergoes proteolytic cleavage and serves as an autoinhibitory domain by reassociating with the truncated $Ca_V1.2$ subunit at the surface membrane [55-57]. Recently, the $Ca_V1.2$ C-terminus was also localized to the nucleus in cardiac myocytes, where it autoregulates transcription [58]. Western blotting of nuclear fractions showed expression of the cleaved $Ca_V1.2$ C-terminus in nuclear fractions, and GFP-tagged $Ca_V1.2$ C-terminus showed nuclear localization [58]. Interestingly, expression of various β subunits fused to GFP in ventricular myocytes also revealed significant localization of β_4 in the nucleus; however, the significance of this is currently unclear [59].

2.2.6. Other subcellular compartments—Additional specialized compartments may exist that could prove important for LTCC modulation. For example, a recent study using electron tomography identified electron-dense bridges between dyadic clefts and the

mitochondrial outer membrane in mouse ventricular myocardium, and $I_{Ca,L}$ has been demonstrated to alter mitochondrial function either directly or through association with actin filaments [60-62]. This raises the possibility of a functional coupling between dyadic LTCCs and mitochondria. Similarly, a close spatial relationship exists between the T-tubule membrane, dyadic cleft, and the nuclear envelope [63]. However, the detailed identity and function of these different subcellular populations of LTCCs are largely unknown at this time.

Furthermore, the subcellular localization of LTCCs may be more complex than currently appreciated. There is a dynamic range in the size of dyadic junctional spaces, for instance, which could differentially impact LTCC function at individual dyads [60]. Likewise, subcompartmentalization of membrane microdomains into smaller nanodomains may be necessary to more precisely define their function and impact on LTCCs. For example, it is currently unknown whether subpopulations of LTCCs coupled to different signaling pathways coexist within the same caveola. Addressing these possibilities will require refinement of available biochemical and microscopy techniques.

3. Subcellular localization impacts LTCC function

With each heart beat, $I_{Ca,L}$ activates ryanodine receptors to release SR Ca^{2+} stores, leading to a transient rise in global $[Ca^{2+}]_i$ from ~100 nM during diastole to ~1 μM during systole, which activates myofilament proteins, producing contraction. How then, is specificity of Ca^{2+} signaling achieved within the cardiomyocyte? It is increasingly recognized that the localization of LTCCs to structurally or biochemically distinct subcellular regions affords functional and physical compartmentalization. Within these spatially defined areas, local $[Ca^{2+}]$ may differ significantly from bulk cytosolic $[Ca^{2+}]_i$, which modulates LTCC function and differentially regulates a number of Ca^{2+}-dependent cellular processes [64,65].

3.1. Local effects on Ca^{2+}-dependent inactivation of LTCCs

Upon opening, LTCCs undergo rapid voltage-dependent and Ca^{2+}-dependent inactivation that limits the amount of Ca^{2+} entry during each action potential. The mechanism of Ca^{2+}-dependent inactivation (CDI) involves tethering of the Ca^{2+} sensor protein calmodulin (CaM) to the carboxyl tail of Ca 1.2 [66,67]. Ca^{2+}v ions near the mouth of the channel bind to CaM, which subsequently changes conformation to bind a nearby IQ motif, accelerating channel inactivation. The degree of CDI observed depends on local $[Ca^{2+}]$ and thus may differ among channels localized to various subcellular environments [68].

At the dyadic cleft, a spatially restricted area estimated between 4.39×10^5 nm^3 and 1.5×10^6 nm^3 in size, $I_{Ca,L}$ and subsequent SR Ca^{2+} release combine to dramatically increase local $[Ca^{2+}]$ from approximately 100 nM during diastole to 100-600 μM during systole [60,69]. Elevated $[Ca^{2+}]$ in the dyadic space during CICR greatly enhances CDI of LTCCs, which impacts action potential duration in ventricular myocytes (Fig. 2) [70-75]. This was demonstrated in rat ventricular myocytes by depleting SR Ca^{2+} stores with thapsigargin to abolish CICR, which slowed CDI, resulting in a 40-70% increase in $I_{Ca,L}$ during the action potential waveform and prolonging action potential duration [72].

Interestingly, LTCCs outside of T-tubules have been shown to exhibit slower inactivation kinetics, indicating surface membrane channels are less sensitive to SR Ca^{2+} release [74,76]. It is not clear whether subpopulations of surface membrane LTCCs localized to membrane microdomains such as lipid rafts and caveolae have a characteristic ‘signature’ of CDI. However, experiments using fluorescence-based Ca^{2+} sensors have indicated that subcaveolar $[Ca^{2+}]$ is higher than levels associated with the surrounding plasma membrane in endothelial cells [77]. Given the known clustering of LTCCs in lipid raft and caveolar

microdomains in cardiomyocytes, it is possible that locally elevated subcaveolar [Ca^{2+}] may contribute to unique CDI among caveolar LTCCs relative to channels residing outside of caveolae [5,43,51].

There is evidence that the conformational change of the CaM-bound C-terminus of $Ca_v1.2$ during CDI directly impacts Ca^{2+}-dependent cellular processes such as excitation-transcription coupling and SR Ca^{2+} release [78]. For instance, activation of cAMP-responsive element-binding protein (CREB)-mediated gene transcription upon depolarization is dependent on a freely moving $Ca_v1.2$ C-terminus as well as the binding of CaM. This was demonstrated through experiments in which the $Ca_v1.2$ C-terminus was either immobilized to the plasma membrane by fusing it to a pleckstrin homology domain or made insensitive to CaM, both of which diminished CDI and abolished CREB activation despite an influx of Ca^{2+} and corresponding rise in $[Ca^{2+}]_i$ [79]. These findings suggest at least one mechanism of excitation-transcription coupling is attributed more to the movement of CaM-bound $Ca_v1.2$ C-terminus than to $I_{Ca,L}$ or changes in $[Ca^{2+}]_i$ [78, 79]. Thus, overall differences in the degree and kinetics of CDI among subpopulations of LTCCs provide a mechanism to differentially regulate Ca^{2+} signaling processes within distinct subcellular domains of cardiac myocytes.

3.2. Coupled gating of LTCCs

Ca^{2+} influx through single LTCCs or small clusters of channels can be measured with high spatiotemporal resolution using total internal reflection fluorescence (TIRF) microscopy [80,81]. Whereas single L-type channels normally exhibit random, infrequent openings that permit small rises in submembrane [Ca^{2+}] termed "Ca^{2+} sparklets", some channels associate in highly active clusters that generate localized regions of significantly elevated [Ca^{2+}] [82,83]. These "persistent Ca^{2+} sparklets" arise from the coupled opening of as many as six adjacent LTCCs [83]. Coupled gating of neighboring LTCCs associated with persistent Ca^{2+} sparklets is enhanced by the scaffold protein A-kinase anchoring protein (AKAP) 5 (also known as AKAP 79/150) as well as protein kinase C alpha (PKCα) [83]. Thus, subpopulations of LTCCs associating with AKAP5 and PKCα may serve as specialized Ca^{2+}-signaling domains that are coupled to discrete cellular functions requiring locally high [Ca^{2+}] [83,84]. Whether LTCCs giving rise to persistent Ca^{2+} sparklets localize to specific subcellular compartments remains unclear; however, AKAP5 has been shown to associate with Cav-3, indicating caveolae or Cav-3 scaffolds may serve an important role in modulating persistent Ca^{2+} sparklet activity [85]. Future work is needed to define the precise subcellular localization and physiological role of persistent Ca^{2+} sparklets in cardiac myocytes.

3.3. Membrane microdomains and excitation-transcription coupling

LTCCs provide a critical link between cellular excitability and gene regulation. Considering transcriptional regulation is on a time course of minutes to hours, it has remained puzzling how cardiac myocytes regulate Ca^{2+}-dependent gene regulation given the rhythmic cycling of cytosolic $[Ca^{2+}]_i$ with each heart beat. This has led some to propose that specific membrane microdomain compartments are responsible for Ca^{2+}-dependent signal transduction to the nucleus [86,87].

CaM plays a significant role in coupling $I_{Ca,L}$ to transcriptional regulation in cardiac myocytes, particularly in the activation of hypertrophic signaling [88,89]. Ca^{2+}-CaM activates a number of transcriptional pathways, and the two most prominent in cardiac muscle involve either Ca^{2+}-CaM-dependent protein kinases (CaMK) or the phosphatase calcineurin (protein phosphatase 2B, PP2B) [88]. The importance of $I_{Ca,L}$ in stimulating hypertrophic remodeling was recently demonstrated in transgenic mice with cardiac-specific

overexpression of the LTCC β_{2a} subunit to increase $I_{Ca,L}$, which develop hypertrophy that is inhibited using blockers of LTCCs, CaMK, or calcineurin [90].

Relatively little is currently known regarding whether specific LTCC populations are responsible for signaling to the nucleus through the CaM-CaMK pathway. Interestingly, CaMKIIα has been localized to lipid rafts in neurons, and disruption of lipid rafts using methyl-β-cyclodextrin reduced phosphorylation of CaMKII substrates [91,92]. However, several studies have shown that overexpression of the nuclear localized CaMKIV and CaMKII-δ_B isoforms stimulate cardiomyocyte hypertrophy whereas the nonnuclear CaMKIIα does not seem to play a significant role in hypertrophic remodeling [93,94]. There is evidence that $I_{Ca,L}$ triggers translocation of CaM from the cytosol to the nucleus, where it activates nuclear CaMKIV [95]. In cardiomyocytes, CaMKIV induces a hypertrophic response through activation of the transcription factor MEF2 [94].

Activation of the Ca^{2+}-CaM-calcineurin pathway leads to dephosphorylation of members of the nuclear factor of activated T cells (NFAT) transcription factor family within the cytosol, which then translocate to the nucleus to regulate genes involved in cardiac hypertrophy [96]. In arterial smooth muscle cells and neurons, a macromolecular signaling complex between $Ca_v1.2$, AKAP5, and calcineurin couples $I_{Ca,L}$ with NFAT-mediated gene transcription [97,98]. Recently, calcineurin has been demonstrated to associate with $Ca_v1.2$, AKAP5, and Cav-3 in ventricular myocytes [85]. This raises the possibility that LTCCs within caveolae or associated with Cav-3 scaffolds specifically couple to calcineurin-NFAT-mediated gene regulation (Fig. 2); however, little experimental evidence currently exists to directly support this hypothesis. It is worth noting that Cav-3 has a known role in cardiac hypertrophy, as knockout of Cav-3 leads to hypertrophic cardiomyopathy in mice [99].

3.4. Membrane microdomains and excitation-secretion coupling

LTCCs provide a critical influx of Ca^{2+} necessary for secretion of hormones in some cell types [100,101]. $Ca_v1.2$ associates with several soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins involved in exocytosis [102,103]. In pancreatic β-cells, it has been shown that the coupling of LTCCs to SNARE proteins permits locally elevated [Ca^{2+}] near exocytotic machinery to trigger insulin secretion [102].

In atrial myocytes, LTCCs regulate secretion of atrial natriuretic peptide (ANP), a hormone with diuretic, natriuretic, and vasodilatory properties [104]. However, the precise role of $I_{Ca,L}$ remains unclear. Some studies have shown that $I_{Ca,L}$ stimulates ANP release, but other studies have suggested that $I_{Ca,L}$ inhibits ANP secretion [105-112]. ANP release is also largely controlled by mechanical stretch, and $I_{Ca,L}$ is coupled to the regulation of stretch-induced ANP secretion, suggesting there may be different roles for Ca^{2+} in basal versus stimulated release [112-114].

Little is known regarding the subcellular localization of ANP release machinery in atrial myocytes. However, several aspects of caveolae make this microdomain an attractive candidate for coordinating secretion of ANP. First, ANP has been localized to caveolae in atrial myocytes using electron microscopy [115]. Second, the localization of SNARE proteins to cholesterol-rich microdomains has been reported in various cell types and methyl-β-cyclodextrin treatment reduces exocytosis of secretory vesicles [116-118]. Finally, caveolae contain a subpopulation of LTCCs and are essential for translating mechanical stretch to activation of downstream signaling pathways, both of which impact the rate of ANP release [5,119]. Thus, it is possible that ANP release from atrial myocytes occurs in specialized membrane microdomains such as caveolae where LTCCs have preferred access to secretory machinery.

3.5. Modulation of $I_{Ca,L}$ by membrane cholesterol and lipids

Relative to the bulk plasma membrane, lipid rafts and caveolae are enriched in sphingolipids, cholesterol, and phosphoinositides, which have been shown to modulate the functional properties of a number of ion channels [39,120]. Experimental approaches to investigate the role of cholesterol or lipids on ion channel function are often complicated because these interventions also target a large number of signaling molecules that regulate ion channel activity. However, there is some evidence that cholesterol directly modulates $I_{Ca,L}$. Exposure of smooth muscle cells to cholesterol-enriched liposomes led to a gradual increase in $I_{Ca,L}$ and a positive shift in the voltage dependence of inactivation that was speculated to result from changes in membrane physical properties [121]. Importantly, lipid-altering strategies such as statin drugs or dietary supplementation with n-3 polyunsaturated fatty acids have shown promise as anti-arrhythmic therapy. These interventions are believed to exert effects via several mechanisms including modulation of ion channel expression and function [122-124]. The antiarrhythmic action of polyunsaturated fatty acids such as eicosapentaenoic acid has been partially attributed to a decrease in $I_{Ca,L}$ [122,125]. Likewise, simvastatin has been shown to suppress $I_{Ca,L}$ in mouse ventricular myocytes [126]. Studies have also shown that statins and diets rich in n-3 polyunsaturated fatty acids alter lipid raft and caveolar composition [127-130]. Future work is needed to investigate whether these lipid-altering therapies specifically result in changes in the localization, function, or regulation of LTCCs within membrane microdomains.

4. Unique regulation of LTCC subpopulations

Neurohormonal regulation of LTCCs is central to the ability of the heart to adapt to changing physiological needs by altering heart rate and contractility [131,132]. Membrane microdomains such as lipid rafts and caveolae are particularly well-suited for regulation of $I_{Ca,L}$ because of the targeting of proteins involved in a variety of signaling cascades to these domains. Co-localization of signaling molecules with LTCCs enables highly localized and specific regulation of the channels.

4.1. Localization of LTCCs to a caveolar macromolecular signaling complex is required for β_2-adrenergic regulation

The sympathetic nervous system drives the fight-or-flight response through activation of β-adrenergic receptors, and one of the essential downstream targets of β-adrenergic regulation is the LTCC [38]. Three subtypes of β-adrenergic receptors exist ($\beta_{1\text{-}3}$) in myocardium and notable differences exist in the manner by which each receptor affects downstream pathways. For example, chronic activation of β_1-adrenergic receptors results in cardiomyocyte apoptosis, whereas β_2-adrenergic receptor stimulation is cardioprotective [133]. Furthermore, β_1 receptor activation causes a global cellular response that includes PKA-mediated phosphorylation of multiple proteins involved in EC coupling including LTCCs and the SR protein phospholamban, yet β_2 receptors signal locally to increase $I_{Ca,L}$ without affecting other PKA-dependent processes such as phospholamban phosphorylation [134,135].

A number of recent studies have helped explain these divergent physiological responses observed with β_1 versus β_2-adrenergic receptor activation. A subpopulation of LTCCs was identified as part of a caveolar macromolecular complex with the β_2-adrenergic receptor, adenylyl cyclase, $G\alpha_s$, $G\alpha_i$, and protein phosphatase 2A (PP2A) in ventricular myocytes [5]. Disruption of caveolae in neonatal mouse ventricular myocytes with either methyl-β-cyclodextrin or short interfering RNA (siRNA)-mediated knockdown of Cav-3 abolished β_2-adrenergic stimulation of $I_{Ca,L}$ (Fig. 1C,D) without effecting β_1-adrenergic enhancement of $I_{Ca,L}$[5]. Similar studies utilizing methyl-β-cyclodextrin or intracellular application of Cav-3

antibody in adult rat ventricular myocytes showed disruption of normal β_2 regulation of $I_{Ca,L}$ [42]. These results demonstrate specific coupling of LTCCs with β_2-adrenergic receptors within caveolae or in a complex scaffolded by Cav-3 and suggest that channels coupled to β_1-adrenergic signaling molecules localize outside of cholesterol-rich membrane microdomains. Interestingly, $I_{Ca,L}$-mediated cardiomyocyte apoptosis resulting from β_1-adrenergic activation involves CaMKII, which has not been reported in caveolae [136]. Thus, the specific coupling of caveolar LTCCs with β_2-adrenergic receptors may stimulate pro-survival signaling (Fig. 2) [86,137].

Activation of $G\alpha_s$-coupled receptors activates adenylyl cyclase, resulting in production of cAMP. Monitoring subcellular localization of cAMP pools using fluorescence resonance energy transfer (FRET)-based biosensors in ventricular myocytes has demonstrated that stimulation of β-adrenergic receptors using isoproterenol leads to cAMP accumulation within cytosolic and caveolar compartments, whereas activation of another $G\alpha_s$-coupled receptor, the E-type prostaglandin receptor 4, results only in cytoplasmic cAMP production [138]. Thus, restricted production of cAMP within the caveolar compartment following β_2-adrenergic receptor activation may explain the localized signaling between β_2 receptors and LTCCs. This idea is consistent with studies in which disruption of caveolae with methyl-β-cyclodextrin led to more diffuse cAMP regulation after β_2-adrenergic activation that included PKA phosphorylation of phospholamban [139]. Spatially restricted cAMP generation associated with β_2 adrenergic activation may also involve coupling of β_2 receptors with $G\alpha_i$ or localized expression of phosphodiesterase 4D isoforms [140,141].

4.2. AKAP5 essential for β-adrenergic stimulation of $[Ca^{2+}]i$ transient

Whereas selective activation of β_2-adrenergic receptors within caveolae does not have far-reaching regulatory effects on phospholamban or contractile proteins, β_1-adrenergic stimulation leads to global signaling events that increase contractility and speed relaxation. Recently, a macromolecular signaling complex involving the scaffolding protein AKAP5 and Cav-3 was demonstrated to be essential for β-adrenergic regulation of EC coupling proteins including LTCCs, ryanodine receptors and phospholamban [85]. Knockout of AKAP5 in mice abolished isoproterenol (a nonselective β-adrenergic receptor agonist) stimulation of $[Ca^{2+}]_i$ transients, as well as phosphorylation of ryanodine receptor and phospholamban. Intriguingly, enhancement of whole cell $I_{Ca,L}$ by isoproterenol remained intact in AKAP5 knockout mice. Further work demonstrated that in wild type mice, $Ca_v1.2$ channels associated with Cav-3 were preferentially phosphorylated at Serine 1928 (S1928) after treatment with isoproterenol whereas the fraction of $Ca_v1.2$ not associating with Cav-3 did not undergo significant phosphorylation. In AKAP5 knockout mice, however, the converse was observed as isoproterenol predominantly led to phosphorylation of non-Cav-3-associated $Ca_v1.2$ channels. Thus, loss of AKAP5 leads to β-adrenergic stimulation of $I_{Ca,L}$ acting on a different subpopulation of LTCCs that does not communicate directly with ryanodine receptors. However, the physiological relevance of S1928 phosphorylation is debated as recent studies have demonstrated that S1928 is not necessary for the PKA-mediated increase in $I_{Ca,L}$ [131,142,143]. Nevertheless, the authors provided important evidence to propose a model in which AKAP5 serves as a scaffold to assemble proteins involved in EC coupling with a β-adrenergic signaling complex within T-tubules, and interestingly this macromolecular signaling complex included Cav-3 (Fig. 2).

Questions remain regarding the precise subcellar localization of this AKAP5/$Ca_v1.2$/Cav-3 complex. In ventricular myocytes, caveolae are found in both surface and T-tubular sarcolemma but are absent from dyadic junctions [53,144]. A number of studies have demonstrated Cav-3 expression within the T-tubule system in ventricular myocytes, where Cav-3 has some degree of colocalization with $Ca_v1.2$ and ryanodine receptor [54,145]. Considering caveolar structures are excluded from dyads, the observation that Cav-3

colocalizes with both $Ca_v1.2$ and ryanodine receptor within the cell interior may suggest the presence of Cav-3 scaffolds at or very close to dyadic junctions. A recent study using a triple immunolabeling strategy revealed that a small but significant percentage (~3-5%) of Cav-3 colocalizes with both $Ca_v1.2$ and ryanodine receptor in atrial myocytes, suggesting Cav-3 is expressed at or adjacent to select dyads; however, atrial myocytes lack the highly developed T-tubule system seen in adult ventricular myocytes, which may lead to differences in dyad architecture between the two cell types [146,147]. Another study using confocal microscopy has reported little to no Cav-3 localization at dyad junctions in rat ventricular myocytes [148]. Because perturbation of cholesterol rich microdomains reduces $[Ca^{2+}]_i$ transient amplitude and knockout of Cav-3 in mice leads to reduced myocardial contractility measured by fractional shortening, it seems possible that Cav-3 is expressed sufficiently close to dyadic junctions to play an important role in regulating EC coupling (Fig. 2) or alternatively influx of Ca^{2+} through distinct caveolar LTCCs contributes significantly to loading SR stores [42,99]. Additional strategies such as immunogold electron microscopy may yield insight into the localization of Cav-3 relative to dyadic $Ca_v1.2$ channels.

5. Targeting LTCCs to subcellular compartments

Considering spatially defined subpopulations of LTCCs regulate discrete cellular functions, understanding the mechanisms responsible for appropriate channel targeting to various subcellular compartments in cardiac myocytes is important, yet these pathways are largely undefined. Recent advances in our understanding of LTCC trafficking in cardiomyocytes will be briefly discussed.

5.1. Influence of Ca^{2+} channel auxiliary subunits on subcellular localization of LTCCs

The LTCC β subunits may play a significant role in defining subcellular L-type Ca^{2+} channel populations. Early evidence that β subunits can directly impact subcellular Ca^{2+} channel targeting came from studies in polarized epithelial cells, in which $Ca_v2.1$ subunits were trafficked to the basolateral membrane by β_{1a} and β_4 subunits but exhibited apical membrane localization when coexpressed with β_{2a} [149]. Disparities in the localization of various β subunits have been also observed in cardiomyocytes. Western blots of canine ventricular membrane fractions and immunoconfocal microscopy of isolated ventricular myocytes demonstrated that β_{1b}, β_2, and β_3 isoforms localize predominantly to T-tubule membranes while expression of β_{1a} and β_4 was detected more strongly at the surface sarcolemma [23]. Furthermore, adenovirus-mediated expression of various β subunits fused to GFP showed marked differences in subcellular localization among β subunit isoforms in cultured adult rat ventricular myocytes [59]. There is mounting evidence that caveolar localization of $Ca_v1.2$ channels requires an association between β_2 subunits and Cav-3, suggesting β subunits play an active role in targeting L-type Ca^{2+} channel complexes in cardiac myocytes [150].

A role for $\alpha_2\delta$ subunits in subcellular targeting of LTCCs has also been proposed. The $\alpha_2\delta$ subunits undergo posttranslational modification to add glycosylphosphatidyl inositol (GPI) anchors, which are known to localize proteins to highly ordered microdomains such as lipid rafts and caveolae [151-152]. Another possibility is that trafficking of cardiac LTCCs to caveolae may involve Cav-3. Cav-1 has been demonstrated to direct trafficking of the voltage-dependent K^+ channel $K_v1.5$ to cholesterol-rich rafts [153]. The skeletal muscle $Ca_v1.1$ LTCC subunit has been reported to interact directly with Cav-3; however, this has not been demonstrated with $Ca_v1.2$ [154]. Future work will be necessary to carefully address whether other auxiliary subunits are important for targeting LTCC complexes to specific membrane microdomains such as caveolae.

5.2. BIN1 targets channels to T-tubules in cardiac myocytes

Localization of LTCCs to dyadic junctions within T-tubule structures is essential for EC coupling, and mislocalization of channels caused by remodeling in heart failure is thought to contribute to impaired contractility. T-tubule biogenesis involves the membrane scaffolding protein BIN1, and recent studies have implicated BIN1 in targeting LTCCs to T-tubules [155,156]. BIN1 localized to T-tubules in human and mouse ventricular myocytes in that BIN1 tethers microtubule structures to coordinate anterograde trafficking of $Ca_v1.2$ channels to T-tubule structures [156]. Importantly, knockdown of BIN1 reduced plasmalemmal expression of $Ca_v1.2$ channels and diminished $[Ca^{2+}]_i$ transients, suggesting fewer dyadic LTCCs [156].

5.3. Internalization and degradation of LTCCs

Although BIN1 plays an important role in anterograde trafficking of LTCCs to the plasma membrane, other mechanisms likely regulate channel retrieval and intracellular trafficking. There is some evidence for regulated internalization of LTCC complexes in cardiomyocytes; exposure of ventricular myocytes to isoproterenol for several minutes led to β-arrestin-1-mediated internalization of $Ca_v1.2$ into clathrin-coated vesicles [157]. However, it was unclear whether a specific subpopulation of LTCCs was targeted for internalization (i.e. bulk plasma membrane vs. caveolar); however, caveolar endocytosis is thought to be largely clathrin-independent, which suggests internalized channels are extracaveolar [158]. Furthermore, the destination of these internalized channels was not defined. Evidence exists for endocytic recycling of some cardiac ion channels to the surface membrane, and this process is normally regulated by the small GTPase Rab11 [159-161]. However, Rab11b has been demonstrated to target surface membrane LTCCs for degradation in cardiomyocytes [162]. Thus, recycling of cardiac LTCCs to the plasma membrane may occur via an alternative pathway, or internalized $Ca_v1.2$ channels may be preferentially degraded rather than recycled. In neurons, L-type $Ca_v1.2$ and $Ca_v1.3$ channels internalized in the presence of glutamate are targeted to the lysosome, which is thought to protect against excitotoxicity [163,164].

6. Altered microdomains disrupt LTCC function in cardiac disease

Dysregulation of LTCCs contributes to the pathophysiology of numerous heart diseases including heart failure, atrial fibrillation, and long and short QT syndromes [165-170]. Several reports have suggested that the geometry and protein composition of subcellular compartments associated with LTCC activity are altered in some cardiac diseases [171-174]. These changes could directly impact the function and regulation of LTCCs and contribute to defects in Ca^{2+} signaling, dysregulated EC coupling, and electrical instability [175].

6.1 Mutations in $Ca_v1.2$ and LTCC auxiliary subunits

Mutations in the genes encoding $Ca_v1.2$ and LTCC auxiliary subunits including β_{2b} and $\alpha_2\delta$-1 have been reported in patients with inherited arrhythmia disorders [165,169,176]. A number of mutations in $Ca_v1.2$, β_2, and $\alpha_2\delta$-1 lead to a loss-of-function phenotype characterized by dramatically reduced $I_{Ca,L}$. Although in most cases the reduction in $I_{Ca,L}$ was attributed to changes in biophysical properties of the channel, one mutation in $Ca_v1.2$ (A39V) associated with short QT syndrome and sudden death disrupted LTCC trafficking [165,176]. It is unclear whether any of these reported loss-of-function mutations alter the specific subcellular localization of LTCCs. However given the potential role of auxiliary β and $\alpha_2\delta$ subunits in the subcellular targeting of LTCCs (see Section 5.1), it is possible these phenotypes could result from the mislocalization of critical LTCC subpopulations.

Timothy syndrome (TS) is a multisystem disorder characterized by ventricular arrhythmias and autism resulting from gain-of-function mutations (G406R, G402S) in $Ca_v1.2$ that interfere with voltage-dependent channel inactivation [169]. Interestingly, TS mutant G406R $Ca_v1.2$ channels have recently been demonstrated to exhibit increased propensity for coupled gating and persistent Ca^{2+} sparklets [83]. TS $Ca_v1.2$ channels in transgenic ventricular myocytes exhibited prominent localization at the surface sarcolemma and intercalated discs relative to T-tubules, suggesting these mutant channels represent a distinct subcellular population [167]. TS $Ca_v1.2$ channels appear to be abnormally coupled to AKAP5, undergoing frequent and prolonged openings that are eliminated by knockout of AKAP5 [167]. AKAP5 ablation also protects TS transgenic mice from arrhythmias, indicating persistent Ca^{2+} sparklet activity plays a significant role in the TS phenotype. Precisely where TS $Ca_v1.2$ channels target remains unclear, but caveolae represent an intriguing candidate due to the known association between AKAP5 and Cav-3 [85].

6.2. CAV3 mutations associated with arrhythmia and cardiomyopathy

Congenital long QT syndrome (LQTS) is a potentially lethal disorder associated with delayed cardiac repolarization, prolonged QT interval, and ventricular arrhythmias and arises due to mutations in a number of ion channels and scaffolding proteins [178]. Recently, mutations in the *CAV3* gene were identified in a subset of LQTS patients (designated LQT9). Initial studies showed that the *CAV3* mutations resulted in increased late Na^+ current through $Na_v1.5$ channels, which could prolong action potential duration [179]. Because LTCCs and important regulatory molecules such as β_2-adrenergic receptors associate with Cav-3, alterations in $I_{Ca,L}$ may also contribute to the pathophysiology of LQT9. However, additional information regarding the electrophysiological effects of LQTS *CAV3* mutations is not available. Other mutations in *CAV3* have been linked to familial hypertrophic cardiomyopathy in one family and dilated cardiomyopathy with AV conduction defects in another [180,181]. The molecular mechanisms leading to these phenotypes are not well understood.

In addition to *CAV3*, mutations in the gene encoding the caveolar protein PTRF/Cavin have also been linked to LQTS, sinus bradycardia, and supraventricular and ventricular tachycardias [182]. Further work will be required to understand the detailed mechanisms underlying cardiac arrhythmias and cardiomyopathies induced by mutations in caveolar proteins.

6.2. Remodeling in failing heart

Heart failure is characterized by weakened myocardial contractile force, partially due to abnormal EC coupling resulting in reduced SR Ca^{2+} release [171,183,184]. A number of studies have demonstrated extensive remodeling of the T-tubular system in failing heart, which likely contributes to inefficient EC coupling [167,185-187]. T-tubule structural remodeling in heart failure is associated with a significant (~50%) reduction in the density of LTCCs, and $Ca_v1.2$ channels within T-tubules may be specifically decreased compared to channels in the surface membrane [167,188]. It has also been suggested that T-tubule remodeling may alter the geometry of the dyadic cleft microdomain [171,174,189]. Alternatively, changes in T-tubular architecture and composition could potentially disrupt macromolecular signaling complexes and lead to dysregulation of LTCCs. Blunted β-adrenergic regulation of $I_{Ca,L}$ has been observed in animal heart failure models and in human heart failure [167,190]. Changes in the relative density, localization, and coupling of β-adrenergic receptor subtypes have been observed [190-193]. For example, in a canine tachycardia-induced heart failure model, enhanced β_2-adrenergic signaling through $G\alpha_i$ strongly blunted the increase in $I_{Ca,L}$ by β_1-adrenergic stimulation [190].

6.4. Atrial fibrillation

Chronic atrial fibrillation involves important structural and electrical changes including significant downregulation of $I_{Ca,L}$ that results in shortened action potential duration and reduced atrial contractility [194,195]. Some studies have attributed the decrease in $I_{Ca,L}$ to a reduction in transcription of $Ca_v1.2$ or various LTCC auxiliary subunits genes resulting in reduced mRNA and protein levels, whereas other reports have suggested impaired posttranslational modifications such as dephosphorylation of $Ca_v1.2$, altered channel trafficking, or enhanced degradation [196-200]. A decrease in T-tubule density was observed in atrial myocytes from a sheep model of atrial fibrillation, which may underlie dysynchronous CICR and reduced contractility [201]. However, it remains unclear whether specific populations of LTCCs are altered in atrial fibrillation.

7. Conclusions and future directions

In cardiac myocytes, LTCCs are localized to multiple distinct subcellular compartments that impact their function and regulation (Fig. 2). The significance of dyadic LTCCs in EC coupling has long been recognized, but other subpopulations, such as those localized to caveolae, are increasingly implicated in a variety of cellular functions and signaling pathways. Many cardiac diseases involve changes in subcellular architecture and organization, thus altered subcellular localization of LTCCs with associated changes in channel function properties can produce aberrant electrophysiology with resulting arrhythmias as well as dysregulation of various Ca^{2+}-dependent cellular processes. Many gaps in our knowledge remain. For example, technical limitations still preclude detailed single channel studies of critical populations of LTCCs localized to T-tubules and thus out of reach from the patch pipette or TIRF microscope. Future studies will undoubtedly reveal additional details regarding how LTCCs and associated Ca^{2+}-signaling molecules are assembled and targeted to defined subcellular compartments. Furthermore, a better understanding of the various subcellular populations of LTCCs may enable new therapeutic approaches for prevalent forms of heart disease such as heart failure and atrial fibrillation.

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Abbreviations

LTCC	L-type Ca^{2+} channel
EC	excitation-contraction
$I_{Ca,L}$	L-type Ca^{2+} current
SR	sarcoplasmic reticulum
T-tubules	transverse tubules
CICR	Ca^{2+}-induced Ca^{2+} release
$[Ca^{2+}]_i$	free intracellular Ca^{2+} concentration
PKA	protein kinase A
Cav	caveolin
PTRF	Polymerase I and Transcript Release Factor
C-terminus	carboxyl terminus

CDI	Ca^{2+}-dependent inactivation
CaM	calmodulin
CREB	cAMP-responsive element-binding protein
TIRF	total internal reflection fluorescence
AKAP	A-kinase anchoring protein
PKCα	protein kinase C alpha
CaMK	Ca^{2+}-CaM-dependent kinase
PP2B	protein phosphatase 2B
NFAT	nuclear factor of activated T cells
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
ANP	atrial natriuretic peptide
PP2A	protein phosphatase 2A
FRET	fluorescence resonance energy transfer
GPI	glycosylphosphatidyl inositol
TS	Timothy syndrome
LQTS	congenital long QT syndrome

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Highlights

- L-type Ca^{2+} channels regulate diverse cellular processes in the heart.
- Different L-type Ca^{2+} channel subpopulations exist in cardiomyocytes.
- Function and regulation of L-type Ca^{2+} channels depend on subcellular localization.
- Altered localization of L-type Ca^{2+} channels plays a role in heart disease.

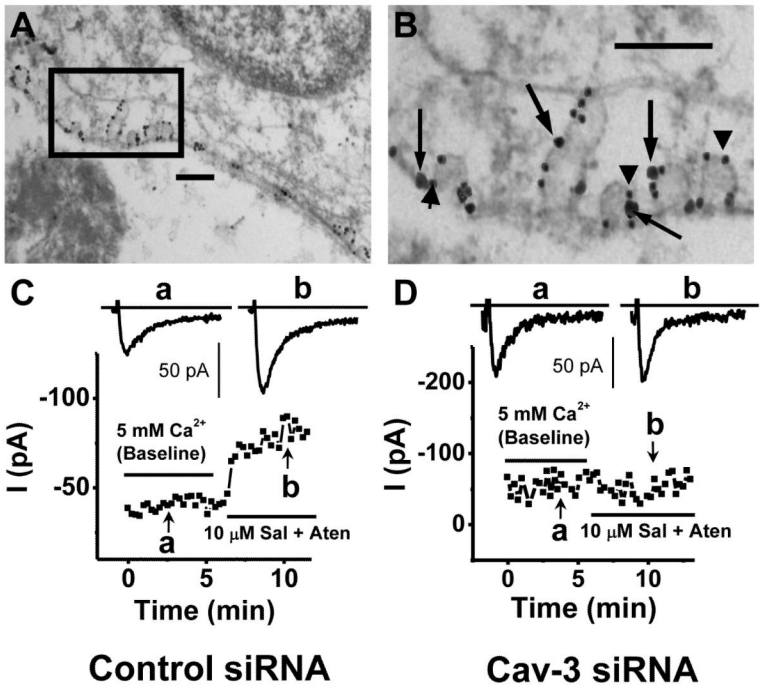

Figure 1. Cardiomyocyte L-type Ca^{2+} channels localized to caveolae are regulated by β_2-adrenergic receptor activation

(A,B) Immunogold electron micrographs of mouse neonatal ventricular myocytes demonstrate co-localization of the $Ca_v1.2$ subunit of L-type Ca^{2+} channel (large particle, arrows) with Cav-3 (small particle, arrowheads) within caveolae. Scale bars represent 200 nm. (C) β_2-adrenergic receptor activation with 10 μM salbutemol (Sal) plus 10 μM atenolol (Aten) increases peak $I_{Ca,L}$ in neonatal mouse ventricular myocytes under control conditions. (D) β_2-adrenergic stimulation of $I_{Ca,L}$ is lost in cells in which caveolae are disrupted using Cav-3 siRNA. Copyright (2006) National Acadamy of Sciences, USA [5].

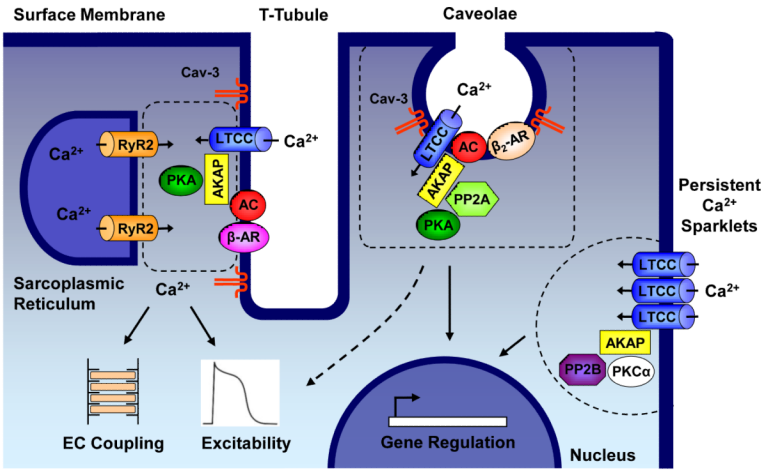

Figure 2. Schematic of L-type Ca^{2+} channel subpopulations and associated macromolecular signaling complexes that contribute to diverse processes within the cardiac myocyte

Within T-tubules, some L-type Ca^{2+} channels (LTCCs) form dyadic junctions with ryanodine receptors (RyR2) on the apposing sarcoplasmic reticulum (SR). A small influx of Ca^{2+} through LTCCs triggers release of SR Ca^{2+} stores through RyR2, leading to the intracellular $[Ca^{2+}]_i$ transient, which is essential for activation of myofilament proteins leading to contraction. β-adrenergic receptor (β-AR) stimulation of $[Ca^{2+}]_i$ transients involves a signaling complex consisting of LTCCs, β-AR, adenylyl cyclase (AC), protein kinase A (PKA), caveolin-3 (Cav-3), and A-kinase anchoring proteins (AKAPs) [85]. Multiple AKAPs are important for regulating LTCC function in the heart including AKAP5 and AKAP15 [85,131]. Additional LTCC subpopulations in the surface sarcolemma are implicated in signaling to the nucleus to regulate the transcription of genes involved in cell survival or cardiac hypertrophy and may also contribute to membrane excitability. Within caveolae, LTCCs associate in a macromolecular protein complex with β_2-AR, AC, PKA, protein phosphatase 2A (PP2A), and Cav-3. Caveolar LTCCs are locally stimulated by β_2-AR activation [5]. Coupled gating of closely neighboring LTCCs results in persistent Ca^{2+} sparklets, which are enhanced by AKAPs and protein kinase C alpha (PKCα) and may serve as a local source of Ca^{2+} to stimulate calcineurin (PP2B) signaling to the nucleus [83,97].