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Caveolae, Ion Channels and Cardiac Arrhythmias

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Abstract

Caveolae are specialized membrane microdomains enriched in cholesterol and sphingolipids which are present in multiple cell types including cardiomyocytes. Along with the essential scaffolding protein caveolin-3, a number of different ion channels and transporters have been localized to caveolae in the heart including L-type Ca^{2+} channels ($Ca_v1.2$), Na^+ channels ($Na_v1.5$), pacemaker channels (HCN4), Na^+/Ca^{2+} exchnager (NCX1) and others. Closely associated with these channels are specific macromolecular signaling complexes that provide highly localized regulation of the channels. Mutations in the caveolin-3 gene (*CAV3*) have been linked with the congenital long QT syndrome (LQT9), and mutations in caveolar-localized ion channels may contribute to other inherited arrhythmias. Changes in the caveolar microdomain in acquired heart disease may also lead to dysregulation and dysfunction of ion channels, altering the risk of arrhythmias in conditions such as heart failure. This review highlights the existing evidence identifying and characterizing ion channels localized to caveolae in cardiomyocytes and their role in arrhythmogenesis.

1. Introduction

The cardiac action potential is generated by the highly orchestrated activity of dozens of ion channel proteins as well as membrane transporters and exchangers. These transmembrane proteins govern the flux of ions across the sarcolemma of cardiomyocytes generating the ionic currents responsible for excitation. Abnormalities in the function or regulation of the ion channel proteins, whether acquired or inherited, underlie many different forms of arrhythmias. A critical factor for understanding the function and regulation of ion channels is their subcellular localization. The purpose of this review is to examine the contribution of ion channels localized to caveolae in normal cardiac physiology and in the genesis of arrhythmias.

2. Membrane microdomains

The cell surface membrane is a heterogeneous mixture of proteins, cholesterol, and lipids including glycero-, phospho- and sphingolipids. The sphingolipids and cholesterol laterally associate with one another within the plasma membrane and form liquid-*ordered* microdomains, popularly known as lipid rafts (Brown and Petersen, 1998; Jiao et al., 2008; Simons and Ikonen, 1997). These cholesterol and sphingolipid-enriched membrane microdomains are believed to coordinate multiple cellular processes including second

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messenger signaling and recycling of proteins to the membrane. In addition, lipid rafts have been implicated in the modulation of multiple different types of ion channel proteins. Various types of lipid rafts have been proposed based on different protein markers, morphological features, and their relative cholesterol to sphingolipid content (Echarri et al., 2007; Hanzal-Bayer and Hancock, 2007). A subset of lipid rafts present in cardiac muscle are caveolae which are morphologically distinct structures that will be the focus of this review.

2.1 Caveolae and caveolins

Caveolae were first identified in 1953 by Palade using electron microscopy to examine the endothelial cells of rat capillaries (Palade, 1953). Caveolae were named based on their morphological appearance on electron micrographs as 'little caves.' Typically, caveolae exhibit an invaginated flask-shaped structure of 50–100 nm in diameter which is contiguous with the surface plasmalemma; however, the shape of caveolae can also be modulated by the physiological state of the cells (Bruns and Palade, 1968a; Bruns and Palade, 1968b; Palade, 1961; Palade and Bruns, 1968; Simionescu et al., 1975). Caveolae are found in the plasma membrane in most cell types including the muscle cells, but not all cell types such as neurons which lack caveolae (Gabella, 1976; Mobley and Eisenberg, 1975; Napolitano, 1963). Different cell types possess different densities of caveolae in their plasma membrane. For example, approximately 50% of the surface plasmalemma of the adipocyte consists of caveolae (Thorn et al., 2003), while only 5% of fibroblast plasma membrane is made up of caveolae (Guillot et al., 1990). A major defining and essential feature of caveolae is the presence of caveolin proteins (Murata et al., 1995; Rothberg et al., 1992), which are scaffolding proteins that interact with cholesterol. Cholesterol is a major component of caveolae (Simionescu, 1983), and cholesterol depletion from caveolae makes them flat shaped and can reduce or eliminate caveolae from the cell (Rothberg et al., 1992). It is suggested that a critical amount of cholesterol is required for the formation and maintenance of caveolae (Chang et al., 1992); however, the exact relationship between the caveolins and cellular cholesterol is not yet clear. The oligomerization of the caveolins and interaction with cholesterol help generate the invaginated shape of these entities.

Three caveolin-encoding genes (*CAV1*, *CAV2*, and *CAV3*) are translated to express six known subtypes of the protein [caveolin-1α and -1β, caveolin-2α, -2β, -2γ, and caveolin-3] (Parton and Simons, 2007; Razani et al., 2002). Caveolins (18 – 20 kDa proteins) possess a unique hairpin structure with cytoplasmic N- and C-termini. Caveolin-1 (Cav-1) is expressed in most cell types including adipocytes, endothelial, epithelial, fibroblast and smooth muscle cells (Rothberg et al., 1992; Smart et al., 1999). Caveolin-3 (Cav-3) is specifically expressed in cardiac muscle as well as skeletal and smooth muscle (Song et al., 1996). Cavs-1 and -3 are expressed independently of each other and are required for the formation of caveolae, and caveolin-2 (Cav-2) associates with Cav-1 or -3 but is not involved in caveolae formation (Das et al., 1999; Li et al., 1998; Mora et al., 1999; Scherer et al., 1997).

2.2 Caveolae in cardiomyocytes

In the heart, caveolae are abundantly present in ventricular, atrial and the nodal cells. Cav-3 has been identified as an essential for formation of caveolae in cardiomyocytes based on knock-out mice studies or knock-down studies (Balijepalli et al., 2006; Galbiati et al., 2001). Expression of the other caveolin genes is variably reported in the cardiomyocytes depending on the stage of development and potential disease conditions. Cav-3 expression has been reported to be low at birth, but expression increases postnatally to reach maximum levels by day 5 followed by a decrease to expression levels seen in the adult cardiomyocytes (Ratajczak et al., 2005). Expression of Cav-3 parallels the rate of cardiac growth, which increases just after birth and attains a maximum value on postnatal day 4 (Ostadal et al., 1999). A recent report also indicates coexpression of Cav-2 and -3 in neonatal cardiac myocytes (Rybin et al.,

2003) Adult cardiac myocytes predominantly express Cav-3 as homo-oligomers (Kawabe et al., 2001; Scherer et al., 1997; Tang et al., 1996). Recent studies have suggested Cav-1 is also expressed in adult cardiomyocytes (Patel et al., 2007; Robenek et al., 2008), but we failed to detect Cav-1 in adult mouse ventricular myocytes using immunogold electron microscopy (Fig. 1). Although as shown in Fig. 1, Cav-1 is robustly expressed in endothelial cells and likely other cell types in the myocardium, we could not detect evidence for expression in mouse cardiomyocytes. Studies have likewise failed to detect Cav-2 expression in rat atrial or ventricular myocytes using immunolabeling techniques (Eldstrom et al., 2006), and similarly Cav-2 was not detected in mouse ventricular myocytes in contrast to its presence neighboring endothelial and endocardial cells (Woodman et al., 2002). Similarly, others have failed to detect Cav-1 and Cav-2 in lysates made from rat ventricular lysates (Yarbrough et al., 2002).

A relationship of caveolae to the transverse-tubular system in cardiomyocytes has been suggested but not clearly defined. An early study hypothesized that the formation of transverse-axial tubular system appears to be derived from the repetitive generation of caveolae, which form "beaded tubules" (Forbes et al., 1984). The Cav-3 knockout mice develop a progressive cardiomyopathy characterized by myocyte hypertrophy; however, the cardiac T-tubule system has not been specifically examined (Woodman et al., 2002). On the other hand, in skeletal muscle Cav-3 transiently associates with T-tubules during development and may be involved in the early biogenesis of the T-tubule system (Carozzi et al., 2000; Lee et al., 2002; Parton et al., 1997). Additionally the Cav-3 null mice show loss of caveolae and abnormalities in the organization of the T-tubule system in the skeletal muscle tissue (Galbiati et al., 2001). Further investigation is needed to clearly establish the role of caveolins in the development and maintenance of the T-tubule system in myocardium and changes in this relationship in disease.

3. Caveolar cardiac ion channels

The subcellular localization of ion channels to caveolae allows the integration of these channels into specific macromolecular signalling complexes in a distinct lipid microenvironment. Thus, the localization of these channels provides for their precise regulation and helps define their specific contributions to cellular physiology. Several ion channels and exchangers have been localized to caveolae, and other channels have been associated with non-caveolar lipid rafts in different cells types, including cardiomyocytes. We will describe available data describing caveolar ion channels and their functional properties.

3.1 Pacemaker Channels

The hyperpolarization-activated, cyclic nucleotide-gated (HCN) family of ion channels are responsible for generating the pacemaker current (funny current, I_f) which contributes to phase 4 depolarization in nodal cells and hence automaticity. The HCN4 channel is the predominant isoform expressed in nodal cells of the heart and is directly activated by cAMP (DiFrancesco and Tortora, 1991; Marionneau et al., 2005; Wainger et al., 2001). A number of laboratories including ours have provided evidence that HCN4 channels are localized to caveolae based on the presence of HCN4 in low density membrane fractions along with Cav-3 as well as the specific interaction of HCN4 with Cav-3 (Barbuti et al., 2004; Scherer et al., 1997; Ye et al., 2008). Disruption of caveolae by reducing membrane cholesterol using methyl-β-cyclodextrin (MβCD) alters the gating of HCN4 channels shifting the voltage dependence of activation of the channels by approximately 10 mV in the positive direction. In addition, the β_2-adrenergic receptor modulation of the HCN4 channel is lost when caveolae are disrupted using the cholesterol chelating drug MβCD consistent with a co-localization of β_2-adrenergic receptors and HCN4 channels in caveolae (Barbuti et al., 2007). Thus, the function and regulation of HCN4 channels in pacemaker cells in the heart are impacted by caveolar localization.

3.2 L-type Ca^{2+} channels

L-type Ca^{2+} channels are present in all types of cardiomyocytes and provide the critical influx of Ca^{2+} that triggers excitation-contraction coupling. Likewise, the influx of Ca^{2+} through these channels may impact other Ca^{2+}-dependent signaling pathways including gene regulation. Inward current through L-type Ca^{2+} channels contributes to the plateau phase of the ventricular action potential and also is the major contributor to the upstroke of the action potential in nodal cells. L-type Ca channels are multi-subunit complexes composed of a pore forming α_1 subunit and auxiliary subunits. Multiple genes encode α_1 subunits, and in the heart *CACNA1C* and *CACNA1D* genes are expressed. The *CACNA1C* endcoded $Ca_v1.2$ channel being the predominant isoform in working myocardium.

The first suggestion that caveolae may be important in Ca^{2+} signaling in cardiomyocytes came from a study in which depletion of caveolae by cholesterol chelation using MβCD resulted in a dose-dependent decrease in the frequency, amplitude and size of Ca^{2+} sparks (Lohn et al., 2000). These Ca^{2+} sparks reflect the primary Ca^{2+} release events in excitation-contraction coupling and require the highly localized crosstalk between sarcolemmal L-type Ca^{2+} channels and sarcoplasmic reticulum-localized ryanodine receptors. Because MβCD treatment did not directly impact the density or gating properties of L-type Ca^{2+} currents, the authors concluded that the localization of L-type Ca^{2+} channels to caveolae was essential for appropriate coupling to ryanodine receptors in the neonatal rat ventricular cardiomyocytes studied (Lohn et al., 2000). However, this study did not directly demonstrate that L-type Ca^{2+} channels are present in caveolae, and an alternative interpretation of the results could be that caveolae are essentially modulating sarcoplasmic reticulum function and localization independent of effects on L-type Ca^{2+} channels.

More recently, it has been demonstrated using a variety of techniques, including immunogold electron microscopy, that a subpopulation of $Ca_v1.2$ L-type Ca^{2+} channels are localized to caveolae in ventricular cardiomyocytes (Balijepalli et al., 2006; Shibata et al., 2006). Figure 1B shows an immunogold labeled electron micrograph of adult mouse ventricular myocardium demonstrating a colocalization of immunogold labeled Cav-3 and $Ca_v1.2$ along in surface sarcolemmal caveolae. In addition, immunoprecipitation studies have demonstrated that Cav-3 associates specifically with $Ca_v1.2$ channels and an associated macromolecular signaling complex composed of various molecules in the β_2-adrenergic/AC/PKA signaling pathway. Although disruption of caveolae in neonatal mouse ventricular myocytes using either MβCD or siRNA did not alter the ionic currents carried by L-type Ca^{2+} channels, it did disrupt β_2-adrenergic regulation of the channels (Balijepalli et al., 2006). Furthermore, studies focused on the regulation of L-type Ca^{2+} channels have provided supportive evidence that channels are co-localized with critical regulatory molecules in caveolae as will be discussed in detail later (Barouch et al., 2002; Wang et al., 2005; Warrier et al., 2007). Thus, there is strong evidence that a subpopulation of L-type Ca^{2+} channels are present in caveolae which is essential for their precise regulation, but questions remain as to the specific role of these channels in cardiac physiology and disease.

3.3 K^+ channels

Multiple different types of K^+ channels are expressed in cardiomyocytes each with distinct subunit composition and gating properties which uniquely contribute to cardiac excitability. Cardiac K^+ channels are divided into three main classes based on their subunit composition and functional properties: 1) voltage gated (K_v) responsible for I_{to}, I_{Kur}, I_{Kr}, and I_{Ks}; 2) inward rectifiers (K_{ir}) responsible for I_{K1}, I_{KACh}, and I_{KATP}, and 3) background K^+ currents (TASK-1, TWIK1/2). There is significant variability in the expression of K^+ channels in myocytes from different chambers of the heart as well as transmurally across the wall of the myocardium. In general the K^+ channels are key components for repolarization of the myocardium during the

action potential and contribute to the unique shape of the cardiac AP in different myocytes cell types. Specific subcellular localization and targeting to lipid raft domains has been investigated for a subset of these cardiac K^+ channels. Some K^+ channels have been localized to noncaveolar lipid rafts (K_v11.1 and K_{ir}2.1) while other K^+ channels (K_v1.5 and K_{ir}6.2) have been localized specifically to caveolae.

The voltage gated K_v11.1 (hERG) channel is expressed in all types of cardiomyocytes throughout the heart and is responsible for the rapid component of the delayed rectifier current, I_{Kr}. Membrane fractionation studies using canine ventricular myocytes have demonstrated that K_v11.1 channels are present in cholesterol and sphingolipid-enriched membrane fractions, but these channels are not associated with Cav-3 based on a lack of co-immunoprecipitation (Balijepalli et al., 2007). Thus, K_v11.1 channels are present in lipid raft domains in myocytes but not specifically present in caveolae. Nevertheless, alterations of sarcolemmal cholesterol levels using MβCD clearly modulate the gating of K_v11.1 channels suggesting that the channels are sensitive to the lipid composition of the membrane microdomain where they are present in cardiomyocytes (Balijepalli et al., 2007).

Inward rectifier K^+ channels are encoded by the K_{ir}2.x family of genes and the I_{K1} maintains the resting membrane potential and contributes to terminal repolarization. There is evidence that the membrane cholesterol content alters plasma membrane expression and the K_{ir}2.1 current densities; however, these channels are not localized to caveolar lipid rafts based on a lack of interaction with Cav-3 (McEwen et al., 2008; Romanenko et al., 2002).

3.3.1 K_v1.5—The K_v1.5 K^+ channel is expressed predominantly in the atrial myocardium and is responsible for the ultrarapid component of the delayed rectifier K^+ current, I_{Kur}. The function of this channel is regulated by multiple different proteins including auxiliary $K_v\beta$ subunits, protein kinases such as protein kinase A and MAGUKs such as SAP97 (Godreau et al., 2003). Studies in mouse L cell fibroblasts transfected with K_v1.5 first suggested that the K_v1.5 channel can preferentially localize to caveolae (Martens et al., 2001). Subsequent studies provided evidence that in cardiac muscle, K_v1.5 was present in caveolae based on membrane fractionation and immunogold electron microscopy of membrane sheets from rat ventricular myocytes (Shibata et al., 2006). Another study reported that Cav-3 and SAP97, a MAGUK protein, interact and recruit K_v1.5 into caveolae to form a tripartite complex that selectively regulates the expression of currents encoded by a glycosylation-deficient K_v1.5 mutant channel (Folco et al., 2004). More recently McEwen et al. (McEwen et al., 2008) demonstrated K_v1.5 associates with Cav-3, and this association is proposed to recruit K_v1.5 channels from non-lipid raft regions of the membrane to caveolae and in so doing modifying their gating. Furthermore, the association with Cav-3 has been suggested to involve the amino terminus of K_v1.5 based on two independent studies (Folco et al., 2004; McEwen et al., 2008).

Despite a significant body of literature from primarily heterologous expression systems demonstrating an association of K_v1.5 channels and Cav-3 in caveolae, there is controversy regarding the localization of these K_v1.5 channels to caveolae in cardiac muscle. Careful studies have provided evidence that K_v1.5 channels are not preferentially localized to caveolae in cardiomyocytes utilizing a variety of biochemical and molecular imaging techniques. A study using canine and rat atrial and ventricular myocardium argued that K_v1.5 is not localized to lipid rafts and does not co-immunoprecipitate with Cav-3 nor co-localize with Cav-3 either with confocal microscopy or immunogold electron microscopy (Eldstrom et al., 2006). Another study using rat atrial myocytes provided evidence that K_v1.5 channels can be localized to lipid rafts with their gating modulated by membrane cholesterol levels, but the channels did not significantly co-localize with Cav-3 (Abi-Char et al., 2007). In myocytes, K_v1.5 appeared to be localized in the greatest concentration to intercalated disc regions of the myocytes independent of caveolae as well as some cell surface localization (Abi-Char et al., 2007;

Eldstrom et al., 2006) Nevertheless, questions remain regarding the localization of the $K_v1.5$ channel to caveolae in the cardiomyocytes given issues related to antibody specificity, accessibility of epitopes with different fixation procedures (Bush et al., 2006), and potentially the dynamic nature of $K_v1.5$ localization.

3.3.2 Kv7.1—The *KCNQ1* gene encodes the pore forming subunit of the K^+ channel $K_v7.1$ or K_vLQT1, which is responsible for the slow component of the delayed rectifier current, I_{Ks}. Membrane fractionation studies of cardiac muscle have indicated that $K_v7.1$ localizes to low-density membrane fractions together with Cav-3. This suggests that these channels are localized to lipid rafts and possibly caveolae (Balijepalli et al., 2007; Nakamura et al., 2007). However, further studies are needed to support the localization of $K_v7.1$ channels to caveolae such as co-immunoprecipitation with Cav-3.

3.3.3 K_{ATP}—In the adult heart, the ATP-sensitive K^+ channels, K_{ATP} consist of $K_{ir}6.2$ and the sulfonylurea receptor 2 (SUR2) subunits. $I_{K,ATP}$, responds to the metabolic state of the cell and plays a role in shortening of the action potential during ischemia (Zingman et al., 2007)(. Additionally, these channels are integral to the protective response of the myocardium in ischemic preconditioning (Patel et al., 2005). Data from studies of vascular smooth muscle have shown that the related $K_{ir}6.1$ vascular K(ATP) channel is regulated by caveolae and associates with Cav-3 as well as Cav-1 (Jiao et al., 2008; Sampson et al., 2004). Limited data are available describing the localization of K_{ATP} channels in the cardiac sarcolemma, but a preliminary study has provided evidence that $K_{ir}6.2$/SUR2A channels associate with Cav-3 in rat cardiomyocytes (Garg et al., 2008).

3.4 Sodium Channels

The voltage-gated Na^+ channel ($Na_v1.5$ encoded by *SCN5A*) is responsible for the initial upstroke of the action potential in atrial and ventricular myocytes and contributes to propagation of electrical impulses in the heart. The importance of this channel in cardiac excitability has been illustrated by the reports of various mutations in *SCN5A* that are linked to cardiac arrhythmia syndromes such as conduction block, congenital long QT syndrome, Brugada syndrome and sudden cardiac death (Lehnart et al., 2007). The cardiac $Na_v1.5$ channel was one of the first ion channels to be detected in caveolae in the heart (Yarbrough et al., 2002). The localization of a pool of $Na_v1.5$ channels to caveolar membranes is particularly important for the βAR regulation of the channels. βAR stimulation of the cardiac $Na_v1.5$ channels occurs by both PKA-dependent phosphorylation of $Na_v1.5$ channels which typically results in increases in both current amplitude and the rate of current decay (Frohnwieser et al., 1997; Matsuda et al., 1992), and by direct $G_{\alpha s}$ modulation of the channel increasing current density (Lu et al., 1999). Based $G_{\alpha s}$-mediated increase in I_{Na} in ventricular myocytes results from the presentation of a pool of caveolar $Na_v1.5$ channels to the surface membrane (Shibata et al., 2006; Yarbrough et al., 2002). This recruitment of $Na_v1.5$ channels and caveolae to the continuity of the sarcolemma significantly increases the amplitude of I_{Na} which reaches steady state in less than five minutes (Shibata et al., 2006). The increase in I_{Na} is readily reversible with removal of βAR stimulation. Further studies have suggested that a functional interaction between $G_{\alpha s}$ and Cav-3 is responsible for the PKA-independent enhancement of I_{Na} (Palygin et al., 2008).

3.5 Na^+ / Ca^{2+} exchanger

The cardiac Na^+/Ca^{2+} exchanger (NCX) is an important Ca^{2+} signaling protein involved primarily in extrusion cytosolic Ca^{2+}, and NCX1 is the predominant isoform expressed in heart. NCX1 regulates cardiac excitation-contraction coupling by its major role in the decay phase of the intracellular Ca^{2+} transient and by modulating SR Ca^{2+} load and release (Bers et al., 1989; Goldhaber et al., 1999; Leblanc and Hume, 1990; Litwin et al., 1998; Vites and

Wasserstrom, 1996). The NCX is an electrogenic exchanger with a stoichiometry of 3 Na^+:1 Ca^{2+}, and thus NCX1 contributes to the ionic currents responsible for the action potential and under pathological conditions to the genesis of delay after depolarizations and resulting arrhythmias (Fozzard, 1992). An initial study reported that NCX1 has several caveolin binding motifs and that the NCX1 protein associates with Cav-3 (Bossuyt et al., 2002). Another study has provided evidence that annexin A5 directly interacts with NCX1 as part of multimolecular complexes including Cav-3 localized to caveolae in human ventricular myocytes (Camors et al., 2006). However, another study was unable to detect co-immunoprecipitation of NCX1 and Cav-3 from rat ventricular myocardium, nor was significant co-localization of NCX1 and Cav-3 detected in immunolabeled rat ventricular myocytes (Cavalli et al., 2007). Further studies are needed to clarify the localization of NCX1 to caveolae and the functional implication of this localization. Given that NCX protein density and function are known to be upregulated in pathological conditions such as cardiac hypertrophy and heart failure, defining the role of NCX1 and its potential caveolar localization will be important for understanding the pathophysiological processes including arrhythmias such as those associated with heart failure.

4. Regulation of caveolar ion channels

Caveolar localization of ion channels can regulate their functional properties in at least three distinct ways. First, the unique lipid environment of caveolae rich in sphingolipids and cholesterol can directly impact the biophysical properties of some ion channels. Second, caveolae provide localized macromolecular complexes that enable various signalling pathways to specifically regulate ion channels in a highly localized fashion. Third, caveolae can regulate the availability of ion channels by controlling their trafficking and thus access to the extracellular environment.

4.1 Lipid microenvironment regulation

Caveolae have a distinct lipid composition including cholesterol, sphingolipids (sphingomyelin and glycosphingolipids), and phosphatidylinositols (Liu et al., 1997), and this unique caveolar lipid microenvironment can significantly influence the functional properties of ion channels. In addition, the lipid microenvironment is dynamic and lipid-based signaling molecules can also directly impact channel function.

4.1.1 Ceramide regulation of K^+ channels—Depending on the cell type, caveolae may contain up to 95% of total cell sphingomyelin with the key enzyme, sphingomyelinase, responsible for sphingomyelin regulation also localized to caveolae microdomains (Bilderback et al., 1997; Liu and Anderson, 1995). Sphingomyelinase hydrolyzes sphingomyelin to form ceramide, a second messenger that stimulates multiple signaling cascades (Kolesnick and Kronke, 1998; Levade and Jaffrezou, 1999). A recent finding demonstrated that sphingomyelinase-D treatment resulted in activation of the $K_v2.1$ channels at negative potentials at which the channels usually remain deactivated (Ramu et al., 2006). However, such regulation has not yet been clearly described for caveolar localized channels.

4.1.2 Polyunsaturated fatty acids regulation of Na^+ and Ca^{2+} channels—Another class of important lipids relevant to cardiac arrhythmias are the polyunsaturated fatty acids (PUFAs) that can regulate the properties of the ion channels. It is known that PUFAs are components of caveolae and can profoundly alter the biochemical makeup of cell membrane lipid rafts/caveolae microdomains (Darios and Davletov, 2006; Ma et al., 2004; Seo et al., 2006). In cardiac myocytes, PUFAs have caused a reduction in cellular excitability and shortening of the action potential by altering the gating properties of Na^+ channels and reducion in I_{Na} (Kang et al., 1995; Xiao et al., 1995). Similarly, the PUFAs directly modulate $I_{Ca,L}$, and reduce the Ca^{2+}-sparks and Ca^{2+} transients and Ca^{2+} -activated membrane current (Xiao et al.,

1997). Thus, given these effects on cardiac ion channels, PUFAs have been suggested to exhibit antiarrhythmic properties (Xiao et al., 2005).

4.1.3 PIP2 regulation of channels—Another family of lipids, the phosphoinositides, reside within the intracellular leaflet of the plasmamembrane, interact with membrane proteins and have prominent roles in signaling (McLaughlin and Murray, 2005). Phosphatidylinositol 4,5-bisphosphate (PIP2) is a negatively charged membrane phospholipid present in caveolae and capable of regulating multiple different ion channels and transporters. In addition, PIP2 is the precursor for the important second messengers inositol 1,4,5-trisphosphate (IP_3) and sn-1,2-diacylglycerol (DAG) which are involved in multiple signaling cascades ranging from receptor activation, activation of protein kinases, ion channel/exchanger activity to changes in intracellular Ca^{2+} levels (Woodcock and Matkovich, 2005). Activation of phospholipase C (PLC) leads to the rapid breakdown of PIP2 to IP3 and DAG, and all of these lipid second messengers have the ability to directly or indirectly influence cardiomyocyte electrical activity and can contribute to cardiac arrhythmogenesis (Woodcock et al., 2008).

PIP2 levels in the surface membrane regulate the function of a variety of different ion channels and transporters (Suh and Hille, 2005). The first characterized transmembrane proteins directly regulated by PIP2 were the Na/Ca exchanger and the K_{ATP} channel ($K_{ir}6.2$/SUR2) (Fan and Makielski, 1997; Hilgemann and Ball, 1996), and both of these proteins are likely present in cardiac caveolae. Subsequent studies have demonstrated that multiple member of the K_{ir} family of channels including $K_{ir}2$, $K_{ir}3$, and $K_{ir}6$ are regulated by PIP2 levels. In general, increased levels of PIP2 activate K_{ir} channels and decreased levels lead to lower levels of activity (Suh and Hille, 2005). The importance of PIP2 regulation of cardiac $K_{ir}2.1$ channels in cardiac excitability was highlighted by the finding that an Andersen-Tawil syndrome mutation which alters the ability of PIP2 to regulate the channel can lead to ventricular arrhythmias (Ma et al., 2007). In case of cardiac $K_{ir}6.2$ channels which respond to the metabolic state of the cell, PIP2 binding to the channels reduces their affinity for ATP, apparently enabling the channels to respond to metabolic stress (Haider et al., 2007). Finally, it is important to appreciate that PIP2 levels can be rapidly modulated specifically in caveolae in response to neurohormonal stimulation such as α_1-adrenergic stimulation in cardiomyocytes (Morris et al., 2006).

The diffusible phosphoinositide IP_3 generated by PLC hydrolysis of PIP2 in caveolae may specifically regulate nearby IP_3-receptors on the sarcoplasmic reticulum. Co-localization of extrajunctional RyR2 receptors and caveolae has been demonstrated in adult rat ventricular myocytes making such localized signaling possible (Scriven et al., 2005). Predominantly type 2 IP_3-receptors are present in atrial and ventricular myocytes (Lipp et al., 2000), and IP_3 binding triggers the release of intracellular Ca^{2+} stores, generating Ca^{2+} signals that subsequently activate protein kinase cascades and contribute to Ca^{2+}-induced Ca^{2+} release (CICR) mechanisms influencing inotropy and arrhythmias (Domeier et al., 2008; Proven et al., 2006; Zima and Blatter, 2004). Thus, caveolar microdomains may provide a unique spatial microenvironment conducive for lipid-mediated signaling which both directly modulate ion channels in caveolae as well as providing localized signals that regulate nearby RyRs and hence Ca^{2+} signaling which may feedback on other caveolar signaling pathways and ion channels.

4.2 Macromolecular signalling complexes

Caveolins can serve as scaffolding proteins organizing macromolecular complexes to enable highly localized and efficient signalling. A spectrum of molecules involved in cellular signalling cascades can be found localized to caveolae including the upstream transmembrane receptors, intermediates such as G-proteins and kinases, and many targets of the signalling such as ion channels. Signalling molecules that have been localized to caveolae include G-protein coupled receptors (GPCRs), heterotrimeric G-proteins, steroid hormone receptors,

receptor tyrosine kinases, nonreceptor tyrosine kinases, protein kinases A and C, phosphatidylinositol-3-kinase/protein kinase B, MAP Kinase (p42/44 MAPK), and a range of other downstream signalling molecules (Patel et al., 2008; Steinberg, 2004). A number of different GPCRs responsible for regulation of cardiac function have been detected in caveolae including β_1-, β_2-, and α_1-adrenergic, muscarinic cholinergic (M2 and M4), and adenosine (A1) receptors (Patel et al., 2008; Steinberg, 2004). In addition, downstream molecules including $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, $G_{\beta\gamma}$ adenylyl cyclase types 5 and 6, some phospholipase C isoforms, and NOS3 have been detected in caveolae in cardiomyocytes (Patel et al., 2008; Steinberg, 2004). Thus, caveolae provide an important domain where sympathetic and parasympathetic inputs to the heart are integrated to precisely regulate cardiac function.

What enables the formation of specific macromolecular signaling complexes in caveolae? There are likely multiple interacting parts that come together to form these complexes. Cav-3 itself has a scaffolding domain which as previously described is important for binding with interacting proteins. Binding to the caveolin scaffolding domain by effector enzymes (e.g., NOS3, ACVI) has been proposed to maintain these proteins in an inactive state, and this provides a ready pool of effector molecules available for rapid activation with the appropriate stimulation (Cohen et al., 2004; Okamoto et al., 1998). Additionally, other scaffolding proteins such as membrane associated guanylate kinase (MAGUK) proteins may also contribute to formation of these complexes. For example, a MAGUK protein, SAP97, has been suggested to be instrumental in the localization of Kv1.5 to caveolae (Folco et al., 2004). Furthermore, post-translational modification of signalling proteins such as palmitoylation or myristoylation may contribute to caveolar compartmentalization (Grubb et al., 2008; Resh, 2004; Robbins et al., 1995). The unique lipid environment of the caveolae may also contribute to localization of proteins to caveolae such as binding affinity for PIP2 as PLC exhibits Additionally, it has been suggested that the phosphatidyl serine-binding annexin, A5, may help localize NCX1 to caveolae (Camors et al., 2006). Ultimately, the formation of these macromolecular signaling complexes involves a wide variety of different interacting partners which may also vary in a dynamic fashion in response to stimulation.

L-type Ca^{2+} channels in cardiomyocytes have been extensively studied for their regulation by caveolar signaling pathways. The prominent β-AR regulation of L-type Ca^{2+} channels involves the cAMP/PKA signaling cascade and contributes to the response of the myocardium to sympathetic stimulation. Localized regulation of L-type Ca^{2+} channels by β-AR stimulation was suggested by early studies in rat and frog ventricular myocytes (Jurevicius and Fischmeister, 1996; Zhou et al., 1997). It was postulated that β_2-AR regulation of $I_{Ca,L}$ was highly localized in the rat ventricular myocyte in contrast to the more diffuse β_1-AR regulation (Zhou et al., 1997), and this was supported by cell-attached single channel studies (Chen-Izu et al., 2000). A role for localized pools of cAMP in the regulation of $I_{Ca,L}$ has been demonstrated in guinea pig ventricular myocytes studied using FRET-based biosensors for cAMP (Warrier et al., 2007). Recent studies have put structure with these functional studies suggesting that localized β_2-AR regulation involves caveolar L-type Ca^{2+} channels by identifying a caveolar macromolecular signaling complex including β_2-AR, $G\alpha_s$, $G\alpha_i$, AC, PKA, PP2a, and $Ca_v1.2$ in mouse ventricular myocytes (Balijepalli et al., 2006). Furthermore, disruption of caveolae in neonatal mouse myocytes using siRNA for Cav-3 or MβCD eliminates β_2-AR but not β_1-AR stimulation of $I_{Ca,L}$ (Balijepalli et al., 2006). However, a study of adult rat ventricular myocytes showed that disruption of caveolae with MβCD resulted in a significant enhancement the β_2-AR stimulation of the $I_{Ca,L}$ without affecting the β_1-AR stimulation of $I_{Ca,L}$ (Calaghan and White, 2006). A subsequent study by the same group revealed that β_2-AR- and cAMP-mediated regulation was highly localized under control conditions, but disruption of caveolae with MβCD treatment resulted in a more global β_2-AR stimulated cAMP regulation, that is, loss of compartmentalization (Calaghan et al., 2008). These studies are consistent with a highly localized β_2-AR/AC/PKA cascade regulating $Ca_v1.2$ channels in caveolae in ventricular

myocytes, but there are differences in β_2-AR signaling compartmentalization in caveolae between neonatal and adult cardiomyocytes and across species.

Caveolae can also serve as a site for integration of cellular signaling pathways as they regulate various ion channels. Building on the example of the β-AR/AC/PKA regulation of L-type Ca^{2+} channels described above, another prominent caveolar localized enzyme that can impact this regulation is NOS3 (Feron et al., 1998). NOS3 has specifically been implicated in blunting β-adrenergic stimulation of contractility by reducing β-AR stimulation of $I_{Ca,L}$ in caveolae in mouse ventricular myocytes (Barouch et al., 2002; Massion et al., 2004). Additionally, caveolar NOS3 has been implicated in the α_1-adrenergic-mediated regulation of $I_{Ca,L}$ in cat atrial myocytes (Wang et al., 2005). In this case α_1-adrenergic receptor activates the PLC/IP3/IP3R/Ca^{2+} release pathway which leads to the activation of NOS3. The NO produced then activates guanylate cyclase to produce cGMP which acts to inhibit phosphodiesterase III leading to increased cAMP/PKA stimulating L-type Ca^{2+} channels. Thus the impact of NOS3 signaling on L-type Ca^{2+} channel activity likely depends on the other signaling pathways concurrently activated and likely the cardiac cell type studied.

The ability of caveolae to coordinate signaling is not only related to the ability of various signaling pathways to crosstalk, but also a given signaling pathway can impact multiple targets to produce an appropriate cellular response. An example of such coordinated regulation of ion channels is provided by the recent demonstration that progesterone acting via nongenomic pathways through the PI3K/Akt pathway activates NOS3, and the resulting NO release can both increase basal I_{Ks} and concurrently blunt β-AR stimulated $I_{Ca,L}$ (Nakamura et al., 2007). The combination of these effects helps control cardiac repolarization preventing excessive prolongation of the action potential duration which is associated with a risk of the ventricular arrhythmia torsades de pointes. These cellular findings correlate nicely with clinical studies showing a protective effect of the female hormone progesterone on LQTS-associated arrhythmias (Nakagawa et al., 2006; Rodriguez et al., 2001; Seth et al., 2007). These progesterone-mediated effects are consistent with the findings of different levels of nitrosylation of the L-type Ca^{2+} channels (see Murphy work)

4.3 Regulated caveolae dynamics

Regulation of the formation of caveolae and their association with the surface sarcolemma is not well understood. Subplasmalemmal pools of caveolae have been identified in some cell types, which may be able to be dynamically recruited to the surface membrane (Pelkmans and Zerial, 2005). Thus, the density of ion channels at the surface sarcolemma could rapidly be altered by the addition or subtraction of caveolae containing defined subsets of ion channels. Such a mechanism of regulation has been described for the cardiac $Na_v1.5$ channel in response to β-AR stimulation (Yarbrough et al., 2002). In this case, it was proposed that a PKA-independent direct $G_{\alpha s}$ interaction with Cav-3 promotes the presentation of $Na_v1.5$ containing caveolae to the surface membrane (Palygin et al., 2008; Shibata et al., 2006). Further investigation is required to understand this mechanism of regulation of channel density and to determine if other channels in caveolae are subject to this type of dynamic regulation in response to neurohormonal regulation.

5. Caveolae and arrhythmias

Given that a variety of different ion channels are localized to caveolae, it is likely that caveolar ion channels can contribute to the genesis of arrhythmias. Both inherited arrhythmia syndromes and acquired arrhythmias in conditions such as heart failure can theoretically involve dysregulation or alterations in the function of caveolar ion channels. The extent to which caveolar ion channels contribute to various arrhythmia syndromes is only beginning to be investigated.

5.1 Inherited arrhythmias

Inherited arrhythmia syndromes are caused by mutations in a rich variety of ion channels and associated proteins (Lehnart et al., 2007), and some of these diseases involve channels which are present in caveolae. For example, $Na_v1.5$ channels are known to have a presence in caveolae, and various mutations in $Na_v1.5$ have been implicated in type 3 long QT syndrome, Brugada syndrome, idiopathic ventricular fibrillation, sick sinus syndrome (Lehnart et al., 2007). However, the studies characterizing genetic mutations responsible for inherited arrhythmias have typically not evaluated the impact of the mutation relative to caveolae. One report has directly implicated caveolae in an inherited arrhythmia syndrome by linking mutations in *CAV3* (see Figure 3) with the congenital long QT syndrome type 9 (LQT9) (Vatta et al., 2006). When the LQT9 *CAV3* mutations were co-expressed with the cardiac $Na_v1.5$ sodium channel, an increase in late I_{Na} (gain of function effect) was observed (Vatta et al., 2006). The increase in late I_{Na} is predicted to delay cardiac repolarization and thus generate the long QT phenotype in a fashion similar to that previously described for gain of function mutations of $Na_v1.5$ responsible for the type 3 long QT syndrome (George et al., 1995; Wang et al., 1995a; Wang et al., 1995b). These observations are consistent with the reported association of $Na_v1.5$ channels and Cav-3 (Vatta et al., 2006; Yarbrough et al., 2002). The finding that mutations in *CAV3* can be proarrhythmic is further supported by findings identifying additional *CAV3* mutations (see Fig. 2) in a cohort of sudden infant death syndrome (SIDS) post-mortem samples (Cronk et al., 2007). These SIDS *CAV3* mutations also induced an increase in late Na current like the LQT9 *CAV3* mutations. The impact of the LQT9 and SIDS *CAV3* mutations on the function of other caveolar ion channels besides $Na_v1.5$ has not yet been defined and could provide further insight into the pathophysiology. The only other *CAV3* mutation reported with a cardiac phenotype is T63S *CAV3* mutation found in siblings with hypertrophic cardiomyopathy in a Japanese family. The father died prematurely of sudden cardiac death, but no genotype was available (Hayashi et al., 2004).

A wide variety of other mutations in *CAV3* have been described which are causative of skeletal myopathies including limb girdle muscular dystrophy, type 1C (LGMD-1C), in some cases of rippling muscle disease, distal myopathy, and idiopathic hyper-CKemia (Woodman et al., 2004). However, cardiac pathology is not typically observed for these *CAV3* mutations, and no propensity to arrhythmias has clearly been described. This suggests that the caveolae and compensatory mechanisms adapting to Cav-3 mutations are quite different in cardiac and skeletal muscle, but the basis for this difference is unknown.

5.2 Acquired arrhythmias

Many different kinds of arrhythmias result from acquired heart disease. In these acquired arrhythmias, cardiomyocytes may exhibit changes in the abundance or composition of caveolae, but few studies have directly examined for such alterations. In a canine tachycardia-induced cardiomyopathy model, there is an increase in Cav-3 protein levels and in morphological caveolae which is associated with increased NO production (Hare et al., 2000). Other studies have identified changes in caveolar NOS3 in hypertrophy and post-myocardial infarction (Piech et al., 2003; Ratajczak et al., 2003). A potential role of alterations in NOS3 in the generation of arrhythmias was suggested by genetic studies in mice. $NOS3^{-/-}$ mice exhibit a higher incidence of arrhythmias in a cell culture model in the presence of ouabain which was attributed to delayed afterdepolarizations (Kubota et al., 2000), and this was supported by *in vivo* mouse electrophysiology studies showing a higher incidence of inducible ventricular tachycardia in $NOS3^{-/-}$ mice (Rakhit et al., 2001). Likewise, isolated myocytes from $NOS3^{-/-}$ mice exposed to β-AR stimulation with isoproterenol exhibited an increased incidence of early afterdepolarizations and aftercontractions (Wang et al., 2008). Overexpression of NOS3 in transgenic mice conversely leads to a lower incidence of spontaneous arrhythmias in a neonatal ventricular myocyte culture model (Massion et al.,

2004). The underlying mechanism by which NOS3 regulates these effects on arrhythmogenesis likely involves alterations in β-AR stimulation of cardiac excitability specifically in the caveolar microdomain (Barouch et al., 2002). Specifically, NO produced by NOS3 is hypothesized to act to blunt β-AR stimulation of currents through L-type Ca^{2+} channels (Barouch et al., 2002; Wahler and Dollinger, 1995; Wang et al., 2008). Reduction or elimination of the NOS3-produced NO brake on β-AR stimulation of $I_{Ca,L}$ leads to greater influx of Ca^{2+} which can contribute two potent arrhythmia triggers: early afterdepolarizations and delayed afterdepolarizations (Fozzard, 1992; January and Riddle, 1989). This is consistent with data localizing the relevant molecules to caveolae including L-type Ca^{2+} channels, NOS3, β_2-AR, and multiple components of the cAMP/PKA signaling cascade (Balijepalli et al., 2006; Feron et al., 1998; Head et al., 2005; Xiang et al., 2002). In addition, Ca^{2+}/Calmodulin-mediated NOS3 regulation of I_{Ks} has also been described which importantly contributes to action potential durations and the associated risk of arrhythmias (Bai et al., 2005).

6. Conclusions and future directions

Although caveolae were morphologically identified by electron microscopy more than fifty years ago as defined invaginations of the surface membrane, it is only in the past 15 years that their composition and functional significance have begun to be understood. Caveolae are distinct in their membrane lipid composition, being highly enriched in cholesterol and sphingolipids, and caveolins are signature proteins essential for the formation of caveolae. Specifically Cav-3 is expressed in muscle cells including the cardiomyocytes. A subset of cardiac ion channels have been proposed to reside in caveolae in cardiomyocytes including HCN4, $Ca_v1.2$ $K_v1.5$ channels, $K_{ir}6.2$/Sur2a channels, $Na_v1.5$, and NCX. These caveolar ion channels are regulated by a variety of signaling pathways in a highly localized fashion in part due to the localization of multiple components of specific signaling pathways to the caveolar microdomain. Dysfunction of caveolar ion channels or their regulation have been implicated in the genesis of inherited arrhythmias such as LQT3 and LQT9 and potentially in acquired arrhythmias in conditions such as heart failure. However, understanding the composition and functional roles of caveolae in the heart as well as their contribution to arrhythmia syndromes is only beginning. Many critical questions remain to be answered.

Limitations in the techniques used to determine whether channel proteins are present in caveolae remain and need to be carefully considered. Early studies oftentimes identified proteins as localized to caveolae based on biochemical membrane fractionation studies revealing a co-distribution of the protein with Cav-3 in low density fraction sucrose gradients. Although this is supportive evidence, it is possible that these proteins reside in noncaveolar lipid rafts or are nonspecifically present in these membrane fractions. Co-immunoprecipitation studies demonstrating that proteins associate with Cav-3 are commonly employed, but these studies share the limitations of all immunoprecipitation studies of possible nonspecific associations and require careful control studies. Imaging approaches are useful, but the spatial resolution of standard light microscopy including confocal microscopy cannot definitively localize proteins to the small 50–100 nm caveolae. Electron microscopy studies can provide spatially defined localization of proteins to caveolae, but these studies are technically challenging and limited by the available specific antibodies that can be used. Approaches to disrupt caveolae using MβCD and evaluating the impact on channel localization can be helpful, but as a nonspecific chelator of cholesterol, MβCD can exert a wide range of effects in addition to disrupting caveolae which can confuse data interpretation. Reducing or eliminating Cav-3 expression is another technique using transgenic technology or siRNA approaches which may offer a more specific manner to disrupt caveolae. The most definitive studies have used a variety of techniques to demonstrate caveolar localization of the ion channels or signaling molecules to caveolae. It is likely that as additional research is performed, the list of caveolar proteins will continue to be refined.

Even more challenging than localizing a protein to caveolae, is isolating the functional properties of that protein in the caveolar microdomain in the intact cell. In the case of ion channels, determining the ionic current properties specifically of caveolar-localized channels is difficult. The loss of function studies using MβCD have the limitations previously described in specificity for caveolae. Disrupting caveolae by knocking down Cav-3 expression can be accomplished, but whether this will disrupt the function of the ion channel previously present in caveolae is not a certainty, as the channel may simply redistribute to other membrane domains. The interpretation of these results can be challenging. The most common supportive finding in caveolar channel functional studies is a dysregulation of the channels by signaling pathways following disruption of caveolae. Future efforts are needed to more specifically define the functional roles of caveolar ion channels to cardiac electrophysiology and pathology.

There are many unanswered fundamental questions regarding ion channels in caveolae and cardiac arrhythmias. How are ion channels targeted to caveolae, and how are these channels trafficked distinctly to the surface membrane? Are there multiple types of caveolae in a single cell type which may have different compositions and functional properties? For any given ion channel, what is the distribution of the channel in caveolar membranes vs. noncaveolar membranes? Is the caveolar distribution of a given ion channel dynamic and how is it regulated? How do various disease processes impact the function and regulation of caveolae that may alter the risk of arrhythmias? These are among the many interesting and important questions that will be addressed by future research.

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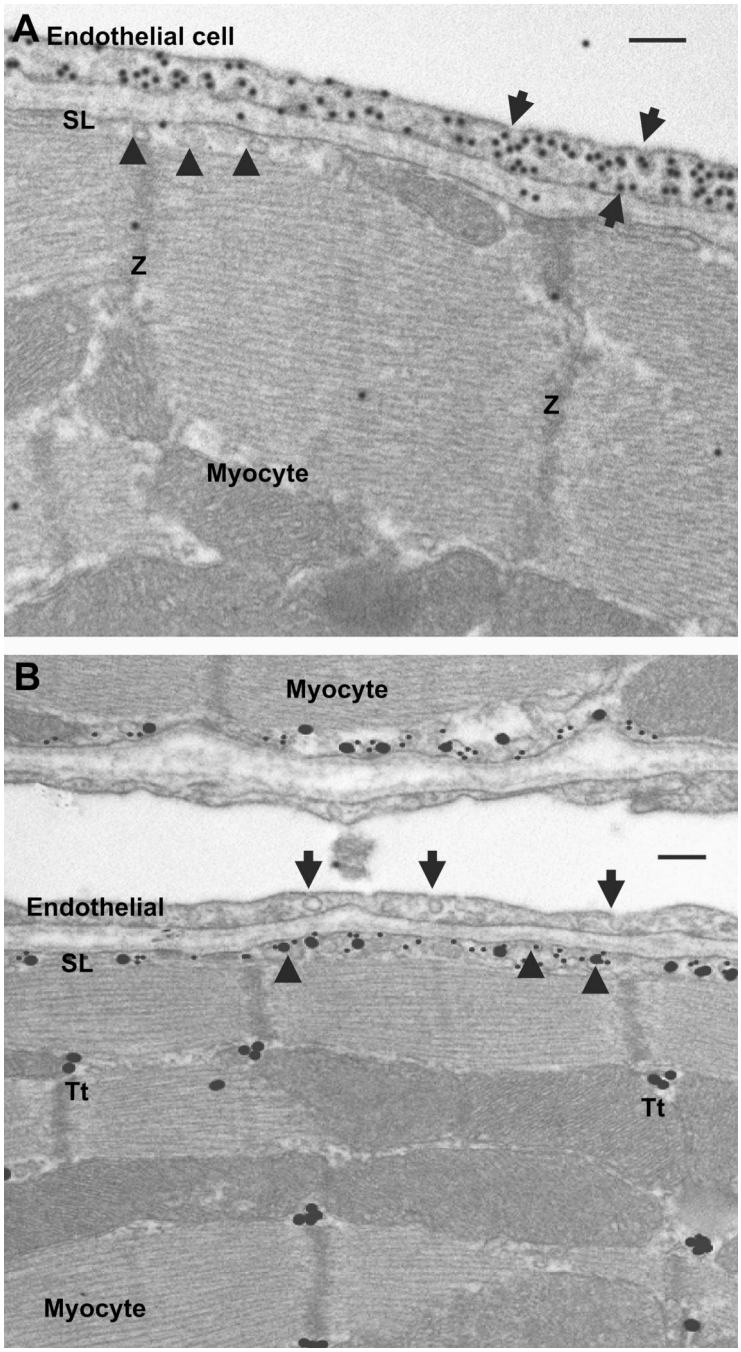

Figure 1.

Electron microscopic images of adult mouse ventricular tissue. Panel **A**, immunogold staining of caveolin-1. Staining for caveolin-1 is observed only in the caveolae (arrows) of endothelial cell. In contrast the ventricular myocyte caveolae (arrow heads) had no specific caveolin-1 staining (SL, sarcolemma; Z, z line of the myofibril). Panel **B**, immunogold co-localization of the $Ca_V1.2$ subunit of L-type Ca^{2+} channel and caveolin-3. $Ca_V1.2$ (large gold particles) and caveolin-3 (small gold particle) are co-localized relative to myocyte caveolae (arrow heads) in sarcolemma (SL) and not in the endothelial caveolae (arrows). $Ca_V1.2$ staining is also noticed in the T-tubules (Tt), where Cav3 staining is absent. Scale bar, 100 nm.

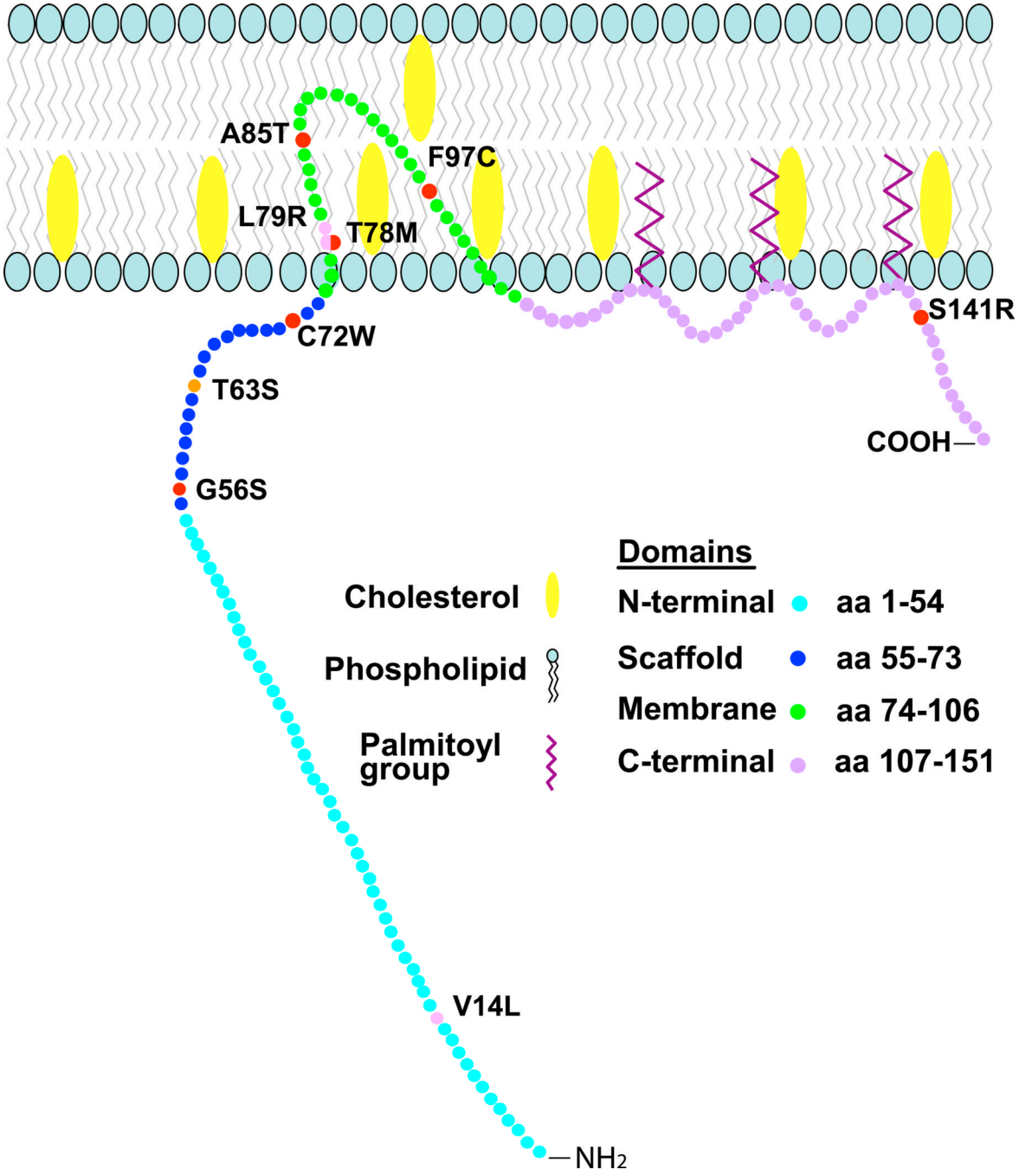

Figure 2.
Schematic of *CAV3* mutations associated with cardiac phenotype genetic diseases. Shown are locations of Cav-3 mutations identified in of Long QT syndrome type 9 (LQT9; red), sudden infant death syndrome (SIDS; pink) and hypertrophic cardiomyopathy (orange) patients. Mutation T78M in the membrane insertion region of the Cav3 was found to be associated with cases of both LQT9 and SIDS.