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Ca_v1.2 Signaling Complexes in the Heart

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Abstract

L-type Ca²⁺ channels (LTCCs) are essential for generation of the electrical and mechanical properties of cardiac muscle. Furthermore, regulation of LTCC activity plays a central role in mediating the effects of sympathetic stimulation on the heart. The primary mechanism responsible for this regulation involves β -adrenergic receptor (β AR) stimulation of cAMP production and subsequent activation of protein kinase A (PKA). Although it is well established that PKA-dependent phosphorylation regulates LTCC function, there is still much we do not understand. However, it has recently become clear that the interaction of the various signaling proteins involved is not left to completely stochastic events due to random diffusion. The primary LTCC expressed in cardiac muscle, Ca_v1.2, forms a supramolecular signaling complex that includes the β_2 AR, G proteins, adenylyl cyclases, phosphodiesterases, PKA, and protein phosphatases. In some cases, the protein interactions with Ca_v1.2 appear to be direct, in other cases they involve scaffolding proteins such as A kinase anchoring proteins and caveolin-3. Functional evidence also suggests that the targeting of these signaling proteins to specific membrane domains plays a critical role in maintaining the fidelity of receptor mediated LTCC regulation. This information helps explain the phenomenon of compartmentation, whereby different receptors, all linked to the production of a common diffusible second messenger, can vary in their ability to regulate LTCC activity. The purpose of this review is to examine our current understanding of the signaling complexes involved in cardiac LTCC regulation.

Keywords

Calcium channel; β -adrenergic receptor; A kinase anchoring protein; E type prostaglandin receptor; cardiac myocyte; protein kinase A

1. Introduction

The proximity of the constituent components of a signaling pathway often plays a critical role in ensuring the speed, efficiency, and specificity of the functional responses they produce. This is especially true for the signaling mechanisms involved in regulating many different ion channels. Ion channels forming signaling complexes with kinase(s) and phosphatase(s) is a common theme [1]. While the localization of such signaling molecules is often achieved through direct protein-protein interactions, spatial organization can also be

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achieved indirectly via scaffolding proteins as well as the targeting of the relevant control elements to specific subcellular locations or lipid domains in the plasma membrane [1–3]. The spatial restriction of signaling also extends to aspects of those pathways that involve diffusible second messengers, such as cAMP. Accordingly, signaling complexes often include receptors and enzymes such as adenylyl cyclase that are responsible for second messenger production, as well as proteins such as phosphodiesterases (PDEs), which are involved in second messenger catabolism. The primary focus of this review will be on the signaling complexes important in maintaining the fidelity of L-type Ca^{2+} channel responses involving protein kinase A (PKA) in the heart.

2. Ca^{2+} Channels in the Heart

Ca^{2+} is a potent second messenger that controls a variety of cellular functions [4, 5]. This is particularly true in the heart, where the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels plays an essential role in regulating action potential duration, triggering myocyte contraction, and controlling gene transcription [6]. Thus, they are an important target for regulating cellular function by a number of different signal transduction pathways, including those involving PKA.

3. Molecular Structure of L-Type Ca^{2+} Channels

There are four types of LTCC, $\text{Ca}_v1.1$ – 1.4 , each of which exists as a multimeric protein complex consisting of one of four different corresponding α_1 subunits ($\alpha_11.1$ – 1.4) together with auxiliary β , $\alpha_2\delta$, and γ subunits [7] (Fig. 1). The α_1 subunit forms the ion-conducting pore and defines the specific type of Ca^{2+} channel. It consists of four homologous domains (I–IV) each containing six transmembrane segments (S1–S6) and a pore forming P-loop between segments 5 and 6. $\text{Ca}_v1.2$ is the predominant LTCC expressed in ventricular myocytes, while both $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ are expressed in atrial cells as well sinoatrial and atrioventricular node cells [8].

The distal C terminus (DCT) of the $\alpha_11.2$ subunit can undergo proteolytic cleavage by the Ca^{2+} -activated protease calpain resulting in a long and a short form [9, 10]. The DCT can remain associated with the truncated $\alpha_11.2$ subunit after cleavage via a non-covalent interaction [9, 11, 12]. Expression of truncated $\alpha_11.2$ alone produces currents that are significantly greater than those produced by the full length subunit [9, 11–14], suggesting that the DCT has an autoinhibitory effect. Consistent with this idea, coexpression of the DCT as an independent polypeptide together with the $\alpha_11.2$ short form results in a decrease in the current produced by the truncated subunit alone [12]. It has been reported that the majority of $\alpha_11.2$ in heart may exist in the cleaved state [10, 15], however, see Dai et al. 2009 for evidence that 50% or more may exist in its uncleaved, full length form [1]. The C terminal fragment of $\alpha_11.2$ has also been reported to act as a Ca^{2+} channel associated transcription factor (CCAT) regulating a wide range of genes affecting neuronal signaling and excitability [16]. Whether or not it serves such a function in the heart has yet to be determined.

There are four different β subunit genes (β_1 – β_4). These can undergo alternative splicing, resulting in numerous isoforms [17]. In the cytosol, the β subunit promotes surface expression of the channel complex. Once at the membrane, the β subunit is found at the cytosolic face of the channel, where it affects voltage-dependent activation and inactivation [18–20]. The $\alpha_2\delta$ subunits are generated from a single gene product, which is proteolytically cleaved but rejoined by disulfide linkage. The α_2 subunit is extracellular, while the δ subunit consists of a single transmembrane segment with short intracellular and extracellular segments [21]. Like the β subunit, the $\alpha_2\delta$ subunits promote surface expression of the channel complex in addition to affecting channel gating [18, 22, 23]. In cardiac muscle, the

Ca_v1.2 complex also includes a γ subunit, which can affect both activation and inactivation [24].

4. Regulation of Ca_v1.2

The regulation of Ca_v1.2 has been extensively studied because of its central role in contributing to the electrical and mechanical properties of the heart. Influx of Ca²⁺ through LTCCs is responsible for maintaining membrane depolarization during the plateau of the cardiac action potential. Subsequent inactivation then allows repolarization thus affecting action potential duration. In this way LTCCs play a critical role in determining refractory period duration, thereby ensuring that electrical activation of the myocardium occurs in the appropriate sequence [25]. Both gain-of-function and loss-of-function mutations have been associated with cardiac arrhythmias [26, 27]. Influx of Ca²⁺ through Ca_v1.2 channels also has an essential function in excitation-contraction coupling by triggering the release of Ca²⁺ from type 2 ryanodine receptors (RyR2) found in the SR, which leads to subsequent contraction [6]. Consequently, β -adrenergic receptor (β AR) stimulation of LTCC activity plays a major role in the changes in both electrical and mechanical properties of the heart that occur during the fight-or-flight response [28]. Upon sympathetic stimulation, catecholamines bind to β ARs, activating the stimulatory heterotrimeric G protein G_s by inducing the exchange of GDP for GTP on G α_s . G α_s dissociates from its G $\beta\gamma$ partners to stimulate AC production of cAMP, which in turn activates PKA [29].

4.1 G Protein Coupled Receptors

Many G Protein coupled receptors (GPCRs) stimulate cAMP production in the heart, but not all produce equivalent functional responses. This disparity includes differences in the ability of these receptors to regulate LTCC activity [30–33]. Such observations are a clear demonstration that cAMP signaling is spatially restricted or compartmentalized (Fig. 2). This situation reflects the fact that cAMP and PKA are not able to diffuse freely and that GPCRs, together with other components of the cAMP/PKA signaling pathway, are not uniformly distributed throughout the cell. Moreover, it suggests that Ca_v1.2 channels are in closer proximity to some receptors than others. Consistent with this idea, there is evidence that Ca_v1.2 interacts not only with PKA, but also many other components of the cAMP signaling pathway, including certain GPCRs.

4.1.1 β -Adrenergic Receptors—Both β_1 and β_2 ARs are capable of stimulating cAMP production and enhancing sarcolemmal LTCC activity in cardiac ventricular myocytes. However, many studies suggest that only β_1 AR activation results in PKA dependent phosphorylation of non-sarcolemmal proteins, such as phospholamban and troponin I, which contribute to the increase in rate of myocyte contraction and relaxation characteristic of sympathetic stimulation [33–39]. These observations suggest that cAMP produced upon β_1 AR stimulation activates PKA more globally, whereas cAMP produced by β_2 AR stimulation is limited to activation of PKA specifically near LTCCs.

Demonstration of the locally restricted effect of β_2 AR stimulation in the heart was illustrated by the work of Chen-Izu et al. [40] using the cell attached configuration of the patch clamp technique to study regulation of LTCC activity in a patch of membrane where access to the extracellular surface was isolated from the rest of the intact cell by the recording electrode. Single LTCC activity was enhanced by activation of local β_1 or β_2 ARs within the same patch of membrane from which channel activity was being recorded. However, while activation of distal β_1 ARs outside the isolated patch of membrane was still able to enhance channel activity, activation of distal β_2 ARs was not. Analogous results have been obtained in hippocampal neurons [41]. Such observations suggest that cAMP produced by β_1 AR stimulation is able to diffuse greater distances, while cAMP produced by β_2 AR

stimulation is limited primarily to local PKA signaling domains. Contrary to this notion, MacDougall et al. [42], using FRET-based biosensors to measure responses in different subcellular compartments of intact myocytes, reported that β_2 AR production of cAMP may not be limited exclusively to PKA signaling domains. They also found evidence that localized PKA-dependent responses depends on phosphatase activity. On the other hand, Nikolaev et al. [43], using FRET-based biosensors together with scanning ion conductance microscopy, found that β_2 AR production of cAMP is specifically associated with the T-tubule network of normal adult cardiac myocytes, whereas β_1 ARs stimulating cAMP production appear to be distributed across the entire cell surface [43].

The observation that β_2 AR stimulation produces an effect limited to regulation of local LTCC function raises the possibility that the receptor might actually be part of a signaling complex that includes $\text{Ca}_v1.2$ as well as PKA. Consistent with this idea, coimmunoprecipitation studies revealed that the β_2 AR is constitutively associated with $\text{Ca}_v1.2$ along with G_s and AC [41, 44]. *In vitro* pull-down experiments with bacterially expressed fusion proteins indicate that the C terminus of the β_2 AR can directly bind to $\alpha_11.2$ [41]. How G_s and AC are linked to $\text{Ca}_v1.2$ is unclear. However, A kinase anchoring proteins (AKAPs) associate with various AC isoforms including AC5 and AC6 (see section 4.3). Such interactions could recruit one or both AC isoforms to $\text{Ca}_v1.2$. In immunoprecipitation experiments, Balijepalli et al. [44] reported that $\text{Ca}_v1.2$ preferentially associates with β_2 ARs with little or no interaction with the β_1 ARs. The lack of $\text{Ca}_v1.2$ association with β_1 ARs has also been demonstrated in neuronal tissue [45]. Nichols et al. [46], on the other hand, found that both β_1 and β_2 ARs co-immunoprecipitated with $\text{Ca}_v1.2$ from cardiac myocytes. However, the authors did not show control IgG precipitations leaving open the possibility that the β_1 AR co-immunoprecipitation with $\text{Ca}_v1.2$ was not completely specific.

4.1.2 Prostaglandin Receptors—In the adult heart of various species, stimulation of EP_2 or EP_4 prostaglandin receptors (EPRs) increases cAMP and PKA activity in the cytosol, contrasting the ability of β_1 AR stimulation to activate both cytosolic and particulate PKA [31, 32, 47–50]. Despite the fact that EPR activation stimulates cAMP production, it has been reported to have no effect on Ca^{2+} transients, myocyte contraction, or LTCC activity in guinea-pig or rat ventricular myocytes [30–32] despite the fact that both β_1 AR and EPR activation produced a more global cAMP response that could be detected by a biosensor expressed throughout the cell [31, 51]. However, only β_1 AR stimulation produced a local cAMP response that could be detected by a biosensor targeted specifically to type II PKA signaling domains. This local cAMP response correlated with the ability of β_1 AR, but not EPR, stimulation to regulate LTCC function. This suggests that LTCCs are regulated by cAMP produced specifically in type II PKA signaling domains (Fig. 2A).

The reason cAMP produced by prostaglandins is unable to reach the PKA signaling domains and regulate LTCC activity is not known. One possibility is that phosphodiesterase (PDE) activity limits the diffusion of cAMP produced by EPRs from reaching $\text{Ca}_v1.2$ signaling complexes. Despite evidence that EPR stimulation can stimulate PDE activity [52], inhibiting PDE activity does not enable these receptors to regulate LTCC function [30, 31]. It has also been hypothesized that activation of G_i by EP_3 Rs might limit the reach of the cAMP response, similar to the way that G_i limits the reach of cAMP produced by β_2 ARs (see section 4.2). However, inhibition of G_i signaling with pertussis toxin does not enable EPR activation to regulate LTCCs [31].

4.2 G Proteins

The original collision coupling model assumes signaling proteins diffuse randomly throughout the membrane, interacting only briefly [53]. However, Rodbell and coworkers provided early evidence that G proteins are actually part of larger signaling complexes [54]. Immunoprecipitation experiments have shown that $\text{Ca}_v1.2$ is part of a macromolecular signaling complex that includes G_s [41, 44]. Although antibodies to $\text{Ca}_v1.2$ did not pull down G_i , they did demonstrate an interaction with caveolin-3, which is the muscle specific caveolin typically associated with the formation of caveolae (see section 6). Caveolin-3 in turn was found to associate with G_i . The molecular basis for these G protein interactions is not yet known, but the functional significance is reflected by the important effects that both G_s and G_i coupled receptors have on $\text{Ca}_v1.2$ function. One of the best studied example involves $\beta_2\text{AR}$ regulation of the LTCC. $\beta_2\text{AR}$ s can couple not only to G_s but also to G_i [34]. The $\beta_2\text{AR}$ can thus limit cAMP production by inhibiting AC through its ability activate G_i . This mechanism appears to be an effective means of spatially restricting cAMP production and PKA-dependent regulation of various target proteins, including $\text{Ca}_v1.2$ in cardiac myocytes [35, 36, 55, 56]. This was demonstrated by Chen-Izu et al. (63), who reported that blocking G_i signaling with pertussis toxin resulted in the ability of LTCCs to be regulated by distal as well as local $\beta_2\text{AR}$ s (see section 4.1.1). It has also been demonstrated that $\beta_1\text{AR}$ s can couple to G_i as well as G_s following protein kinase C (PKC) activation [57], although the effect this has on the spatial distribution of cAMP production following stimulation of this receptor is unknown.

4.3 Adenylyl Cyclase

A $\text{Ca}_v1.2$ /PKA signaling complex that includes a GPCR and the G protein through which it acts is not sufficient to produce spatially restricted cAMP dependent responses such as those associated with βAR stimulation. Inclusion of AC is also a critical factor. Balijepalli et al. [44] found that immunoprecipitation of $\text{Ca}_v1.2$ pulled down AC5/6 in addition to $\beta_2\text{AR}$, G_s and PKA. Although the mechanism for the interaction between $\text{Ca}_v1.2$ and AC5/6 was not determined, Bauman et al. [58] used a heterologous expression system to demonstrate that AKAP5 (human AKAP79 or rodent AKAP150) is able to anchor PKA directly to both AC5 and AC6 [58, 59], the predominant isoforms in the heart [60]. It was subsequently shown that AKAP9 (Yotiao), is associated with AC activity in cardiac tissue, although it did not bind AC5 or AC6 [61]. AKAP6 (mAKAP or AKAP100), on the other hand, was found to selectively bind AC5 in cardiac myocytes, anchoring it to RyR2 [62]. However, the work of Nichols et al. [46] demonstrated that AKAP5 anchors AC5/6 to a signaling complex in cardiac myocytes that includes $\text{Ca}_v1.2$. They reported that the interaction of AKAP5 and AC5/6 with $\text{Ca}_v1.2$ was not direct, but instead via caveolin-3, which also binds a subset of $\text{Ca}_v1.2$ channels (see section 5). Through this interaction, βAR stimulation was found to preferentially phosphorylate $\text{Ca}_v1.2$ channels specifically associated with caveolin-3.

4.4 Phosphodiesterases

Hydrolysis of cAMP by PDEs can also play a critical role in restricting the effective radius of cAMP signaling and LTCC regulation in cardiac myocytes [63, 64]. Jurevicius et al. [65] elegantly demonstrated this by recording macroscopic currents generated by LTCCs at one end of an intact frog ventricular myocyte while activating βAR s in either the same end (locally) or the opposite end (distally). Under normal conditions, LTCC activity was only affected by activating local βAR s. However, stimulation of distal βAR s was able to regulate LTCCs when PDE activity was inhibited. It should be noted that LTCC responses in the frog heart are mediated exclusively by $\beta_2\text{AR}$ s [66]. This suggests that PDE activity is responsible for ensuring that $\beta_2\text{AR}$ cAMP production affects only local LTCC function. It has been hypothesized that $\beta_2\text{AR}$ stimulation can stimulate PDE activity via G_i -dependent regulation of phosphoinositide 3 kinase γ (PI3K γ) and that this is responsible for spatially restricting

the effect of β_2 AR effects on LTCC function [67]. However, there is some controversy over what PDE isoforms are regulated by PI3K γ in the heart and whether or not it affects LTCC activity [68].

The common perception is that PDEs restrict cAMP movement by acting as functional barriers to diffusion. However, computational models of cAMP signaling in cardiac myocytes suggest that this is not the case. Assuming that cAMP is able to diffuse at rates equivalent to free diffusion, PDE activity alone is not sufficient to create a gradient [48, 69]. However, if one assumes that cAMP diffusion is limited by some other mechanism, non-uniform distribution of PDEs can contribute to the generation of gradients by regulating the activity of cAMP degradation within a microdomain.

At least four different PDE families are involved in cAMP metabolism in mammalian cardiac tissue, PDE1, PDE2, PDE3, and PDE4, with each subtype represented by multiple genes products [64]. Furthermore, the relative level of expression of each isoform varies considerably among species, as does the apparent distribution between membrane and cytosolic fractions. PDE2, PDE3, and PDE4 isoforms contribute to spatially restricted cAMP signaling in cardiac myocytes [63, 64]. Furthermore, different G_s coupled receptors that stimulate LTCC activity exhibit differences in sensitivity to inhibition of PDE3 and/or PDE4 [30]. This implies that different PDEs may be associated with specific receptors as well as specific subpopulations of $Ca_v1.2$. Consistent with this idea, upon heterologous expression, different PDE4D isoforms can be recruited to β_2 AR via β -arrestin following receptor activation [70, 71]. It is not known whether β -arrestin also associates with the β_2 AR in the $Ca_v1.2$ complex in the heart, but if so, this mechanism could further contribute to the spatiotemporal restriction of cAMP signaling in the vicinity of such a complex. De Arcangelis et al. [72] demonstrated that various PDE4D isoforms are able to interact with the β_2 AR in neonatal ventricular myocytes. Furthermore, specific inhibition of PDE4D8 and PDE4D9 was found to enhance β AR stimulation of the spontaneous contraction rate in these cells. However, it is unclear what role if any regulation of LTCC activity plays in these responses.

Association with AKAPs is another means of achieving PDE localization [3]. The PDE4D3 isoform can be recruited to various subcellular compartments by directly or indirectly binding to AKAP6 [73, 74], AKAP9 (AKAP350) [75], and AKAP12 (gravin or AKAP250) [76]. These AKAPs act in part to assemble PKA and PDE4D into a complex in which localized phosphorylation of PDE4D by PKA results in its stimulation [73, 77].

PDE4B and PDE4D are also part of a signaling complex with $Ca_v1.2$ in mouse ventricular myocytes [78]. Whether or not this interaction is direct or perhaps involves an AKAP was not determined. However, β AR stimulation of LTCC activity was only enhanced in myocytes from PDE4B knockout animals. On the other hand, β AR stimulation of intracellular Ca^{2+} transients and myocyte contraction were enhanced in both PDE4B and PDE4D knockout mice. This is consistent with the idea that PDE4D is part of the RyR2 signaling complex [79].

4.5 Protein Kinases

4.5.1 Protein Kinase A—The role of PKA-dependent phosphorylation in regulation of cardiac LTCCs was first established over thirty years ago [28, 80]. Subsequent biochemical studies suggested the C terminus of the $\alpha_11.2$ subunit as the likely location of PKA-dependent phosphorylation. This was supported by work reporting that the truncated or $\alpha_11.2$ short form is not phosphorylated *in vitro* [81]. Furthermore, deletion of the $\alpha_11.2$ DCT has also been reported to cause a loss of β -adrenergic regulation [82]. However, the small size of the current observed in myocytes from transgenic animals expressing the

truncated channel protein could have potentially obscured the ability to observe a PKA-dependent response. Early on, serine-1928 in the DCT was identified as the main target for PKA-dependent phosphorylation *in vitro* [15, 83] (Fig. 1). Such observations led to speculation that phosphorylation of this residue releases the autoinhibitory effect that the DCT has on the rest of the channel [1]. In support of this idea, both C-terminal truncation of α_1 1.2 and phosphorylation by PKA produce a hyperpolarizing shift in the voltage-dependence of channel gating [9, 11–13, 84, 85]. C-terminal truncation and PKA phosphorylation also have effects on modal gating of the channel (see section 4.6) [86, 87]. However, difficulties in reliably and reproducibly reconstituting regulation of Ca_v 1.2 in heterologous expression systems left doubt about the role of serine-1928 [7, 13, 88, 89]. It has been suggested that in exogenous expressions systems, differences in basal phosphorylation might have preempted subsequent stimulatory responses in some studies, which could explain the inconsistencies [1]. However, the role that this residue plays was brought further into question by Ganesan et al. [90], who used an endogenous expression system to conclude that serine-1928 is not essential for PKA-mediated stimulation of Ca_v 1.2 activity. They found that in cardiac myocytes in which native LTCC activity was inhibited, expression an α_1 1.2 subunit in which serine-1928 was mutated to an alanine produced a Ca^{2+} current that was still sensitive to β AR stimulation. Perhaps the most definitive test for the role of serine-1928 phosphorylation was provided by Lemke et al. [91], who mutated serine-1928 to alanine in knock-in mice and found that stimulation of the LTCC current by the β AR agonist isoproterenol or the AC agonist forskolin was unaffected.

More recently, Fuller et al. [92] provided new evidence supporting the idea that PKA-dependent phosphorylation does indeed regulate Ca_v 1.2 channel function by interfering with the autoinhibitory effect of the DCT fragment. But rather than involving serine-1928, they found that it actually requires phosphorylation of serine-1700, which is located in the proximal C-terminus of the truncated α_1 1.2 subunit where it interfaces with the DCT peptide (Fig. 1). A key to reconstitution was found to be careful titration of AKAP expression. However, these studies were conducted using the truncated α_1 1.2 subunit expressed together with a separate DCT fragment. Whether or not phosphorylation of serine-1700 plays a similar role in regulating the uncleaved α_1 1.2 subunit has not been determined. Previous studies have demonstrated that mutation of serine-1928 in the full length subunit prevents PKA-dependent regulation [93, 94]. It is conceivable that the presence of both cleaved and uncleaved forms of the channel found in native cardiac myocytes could obfuscate the potential role of serine-1928, especially in light of evidence that LTCCs exist as oligomers [95].

Evidence that PKA phosphorylates at least one β subunit isoform has also led some to hypothesize that Ca_v 1.2 regulation requires coexpression of α_1 1.2 and β subunits [96, 97]. It was reported that PKA can phosphorylate serines-459, -478, and -479 of the cardiac β_2 subunit splice variant β_{2a} [98]. Mutating serines-478 and -479, but not -459, to alanines was reported to eliminate current stimulation by PKA [96]. However, even though serines-478 and -479 are conserved in most other β_2 splice variants, they are absent in β_1 , β_3 , and β_4 subunits. Therefore, because α_1 1.2 can interact with several different β subunits [14, 99] and not all have corresponding phosphorylation sites, the role that β subunits may play PKA dependent regulation of LTCCs remains uncertain [100]. In fact, Brandmayr et al. [101] used transgenic mouse models to demonstrate that deletion of the C terminal portion of the β_2 subunit, which contains the putative phosphorylation sites, did not alter the ability of β AR stimulation to regulate LTCC activity.

It has also been suggested that PKA-dependent phosphorylation of ahnak, a 700-kDa protein that is reported to bind Ca_v 1.2 β_2 subunits, may explain how channel activity is regulated [102]. It has been hypothesized that ahnak prevents the β_2 subunit from interacting with

$\alpha_1 1.2$, but following α_1 phosphorylation the β_2 and $\alpha_1 1.2$ subunits are then able to come together, causing an increase in $\text{Ca}_v 1.2$ current.

4.5.2 Protein Kinase C—Activation of PKC via G_q linked α_1 -adrenergic, endothelin, or angiotensin receptors has been reported to stimulate, inhibit, or both stimulate and inhibit LTCC activity in the heart [7, 103]. PKC has been reported to inhibit cloned $\text{Ca}_v 1.2$ channels consisting of $\alpha_1 1.2$, $\beta 1b$, and $\alpha_2 \delta_1$ by phosphorylating threonine residues -27 and -31 in the amino terminus [104]. The mechanism for PKC stimulation of cardiac LTCC function is less clear. While it has been shown that serine-1928 can be phosphorylated by PKC [105], this is much less prominent than PKA phosphorylation of that residue [106]. Evidence suggests that at least some of the PKC effects on LTCC function involve recruitment of the enzyme to adaptor proteins known as receptors for activated C kinase (RACKs) [107]. However, PKC has also been shown to interact directly with $\alpha_1 1.2$ [105] as well as indirectly through AKAP5 [108].

4.6 Protein Phosphatases

Phosphatases are necessary to ensure the reversibility of kinase dependent phosphorylation. Perhaps the first evidence that phosphatases are part of a signaling complex associated with cardiac LTCCs came from electrophysiological studies looking at the effect of phosphatase inhibitors on rundown or spontaneous loss of single channel activity recorded in isolated patches of membrane. Ono and Fozzard demonstrated that rundown could be reversed by exposure of the intracellular surface of the membrane patch to the phosphatase inhibitor okadaic acid [109]. Okadaic acid also increases currents through cardiac LTCCs reconstituted in planar lipid bilayers [110]. Several pieces of evidence indicate that protein phosphatase type 2A (PP2A) and in some systems type 1 (PP1) are involved in reversing the effects PKA phosphorylation has on LTCCs [88]. For example, purified PP1 and PP2A inhibit whole cell LTCC currents that have been upregulated by β AR stimulation in isolated cardiac myocytes [111, 112]. Conversely, inhibition of PP1 and PP2A reverse or prevent PKA-mediated increases in LTCC currents [113, 114].

A more detailed analysis has provided some evidence for the idea that PP1 and PP2A differentially regulate different modes of channel gating, suggesting that there may be more than one phosphorylation site regulating LTCC activity. As discussed above, $\text{Ca}_v 1.2$ exhibits modal gating: in mode 0 the channel open probability is near zero, in mode 1 the channel exhibits an increase in open probability due to bursts of brief openings, and in mode 2 the channel exhibits very long openings [115]. β -Adrenergic stimulation induces a switch from mode 0 to modes 1 and 2 [87]. Experiments using different inhibitors suggest PP1 and PP2A may be specific for regulating the transition between these different modes of gating [115, 116].

These observations suggest that a phosphatase is closely associated with $\text{Ca}_v 1.2$. In fact, PP2A coimmunoprecipitates with $\text{Ca}_v 1.2$ in neuronal preparations and reverses PKA-mediated phosphorylation of serine-1928 on $\alpha_1 1.2$ [117]. PP2A actually binds to two regions of the $\alpha_1 1.2$ subunit: residues 1795–1818 and 1965–1971 [118, 119].

Initial studies did not indicate that inhibition of protein phosphatase type 2B (PP2B or calcineurin) had any effect on LTCC currents in cardiac myocytes [114, 120] or neurons [121]. However, more recent work shows that PP2B regulates $\text{Ca}_v 1.2$ activity in cardiac, neuronal, and smooth muscle cells [94, 122, 123]. AKAP5 recruits PP2B to $\text{Ca}_v 1.2$ in neurons [94], and PP2B coimmunoprecipitates with $\text{Ca}_v 1.2$ in cardiac myocytes [46, 123]. Furthermore, PP2B binds to the N and C-termini of the $\alpha_1 1.2$ subunit [123]. At the C terminus, the interaction involves residues 1943–1971, which are adjacent to the phosphorylation site at serine-1928 [118, 123]. Whereas this direct interaction is readily

detectable by co-immunoprecipitation, the AKAP5-mediated interaction is not, as detergent used to solubilize $\text{Ca}_v1.2$ disrupts the AKAP5-PP2B interaction (Patriarchi and Hell, unpublished observation). Interestingly, even though PP2B was able to dephosphorylate $\text{Ca}_v1.2$, expression of a constitutively active form of the phosphatase actually increased the size of the endogenous LTCC current [123], which is different from the inhibitory action of AKAP5-anchored PP2B reported for neuronal $\text{Ca}_v1.2$ [94]. PP2B bound to the C-terminus of $\alpha_11.2$ might dephosphorylate a site of an unidentified kinase that otherwise inhibits channel activity whereas AKAP5-anchored PP2B likely acts by dephosphorylating stimulatory PKA sites.

The demonstration that $\text{Ca}_v1.2$ is part of a signaling complex that includes more than one type of phosphatase supports the idea that multiple phosphorylation sites, both stimulatory and inhibitory, are involved in regulating LTCC activity.

5. A Kinase Anchoring Proteins

PKA is an inactive tetramer consisting of two regulatory (inhibitory) (R) and two catalytic (C) subunits. Distinct genes encode four R ($\text{RI}\alpha$, β and $\text{RII}\alpha$, β) and three C subunits ($\text{C}\alpha$, β , and γ). Suppression of C subunit catalytic activity is released following the binding of two molecules of cAMP to each R subunit [124]. It is now widely accepted that AKAPs play an essential role in orchestrating many different cAMP-dependent signaling events by tethering PKA and other regulatory enzymes together to form signaling complexes [125, 126]. PKA binding to AKAPs involves an amphipathic α helix that binds RII and in some cases RI dimers [127–129]. AKAPs, in turn, are recruited to specific subcellular locations through lipid membrane interactions or specific interaction with other proteins [130–132]. At least forty-three AKAP family members have been identified, with multiple isoforms generated by alternative splicing [132]. Many AKAPs were originally named based on their apparent molecular weight. This nomenclature has led to complications due to variations in electrophoretic mobility of different orthologs. In this review, we refer to AKAPs by their gene name, giving their other common names in parentheses.

At the time when there were inconsistencies in the ability to reconstitute PKA-dependent regulation of $\text{Ca}_v1.2$ channel function in heterologous expression systems, it had already been shown that AKAPs were necessary for PKA regulation of other types of ion channels in different systems [1]. Based on such observations, Hosey and colleagues [93] suggested that this might also be true for PKA-dependent regulation of cardiac LTCCs. Consistent with this idea, they reported that $\alpha_11.2$ could be phosphorylated by PKA in HEK293 cells stably expressing wild type AKAP5 (human AKAP79, rodent AKAP150), but not an AKAP5 mutant unable to bind PKA. They also demonstrated that Ht31, a peptide derived from the AKAP RII docking site that disrupts PKA binding [133], blocked PKA-dependent regulation of LTCC in native cardiac myocytes. In neurons, it has been shown that AKAP5 binds to three different sites on $\alpha_11.2$ (Fig. 1) [134]. Although this AKAP was thought to be absent or in low abundance in the heart, recent studies clearly show its presence [1, 46]

The first AKAP found to be associated with $\text{Ca}_v1.2$ was the microtubule associated protein MAP2B [106]. However, MAP2B is expressed primarily in nerve tissue. On the other hand, Hulme et al. [135] demonstrated that AKAP7 (AKAP15 or AKAP18) is abundant in heart, where it coimmunoprecipitates and colocalizes with $\text{Ca}_v1.2$. Furthermore, it had been shown that PKA-dependent regulation of $\text{Ca}_v1.2$ currents could be reconstituted in HEK293 cells in the presence but not the absence of AKAP7 [131]. Finally, a peptide derived from the LZ-like motif that mediates AKAP7 binding to $\text{Ca}_v1.2$ was found to block β -adrenergic stimulation of L-type Ca^{2+} currents in native cardiac myocytes [135]. However, it is

important to keep in mind that this peptide could also have displaced AKAPs other than AKAP7.

The above results suggest that both AKAP5 and AKAP7 play a role in regulating LTCC in cardiac myocytes. If this is true, the question remains as to how preventing the interaction of either one of these AKAPs alone was able to completely block PKA-dependent stimulation of the current in native myocytes. Part of the answer may lie in the fact that there is overlap in the docking sites. In fact one of the AKAP5 binding sites on $\alpha_11.2$ overlaps with the leucine zipper (LZ)-like motif that anchors AKAP7 [94, 134, 135] (Fig. 1).

More recently, Nichols et al. [46] examined the role of AKAP5 in sympathetic regulation of cardiac LTCC function using a knockout mouse model. They found that in the absence of this AKAP, the ability of β -adrenergic stimulation to enhance cytosolic Ca^{2+} transients in cardiac myocytes was blocked. Yet, unexpectedly, β -adrenergic stimulation of the LTCC current appeared to remain intact. This suggested that AKAP5 is not essential for regulation of cardiac LTCC activity. However, biochemical analysis using serine-1928 as a “readout” revealed that in wild type animals, β -adrenergic stimulation only led to phosphorylation of the subpopulation of LTCCs associated with caveolin-3, but in knockout animals β -stimulation preferentially phosphorylated those LTCCs not associated with caveolin-3. According to their results, AKAP5 targets AC, PP2B and PKA to a signaling complex associated with caveolin-3, which includes a subset of $\text{Ca}_v1.2$ (Fig. 2B). This facilitates the localized production of cAMP necessary for PKA-dependent phosphorylation of $\text{Ca}_v1.2$ associated with caveolin-3 as well as the SR proteins RyR2 and phospholamban. The authors suggest that in the absence of AKAP5, AC is redistributed to where it can contribute to β AR regulation of LTCCs not associated with caveolin-3, but regulation of RyR2 and phospholamban is lost. The authors also demonstrated that deleting the PKA binding domain of AKAP5 did not affect the ability of β AR stimulation to enhance Ca^{2+} transients. This finding suggests that PKA must be anchored to this signaling complex by other means as well. One possibility is through AKAP7, which has been reported to interact with the LZ-like domain on $\text{Ca}_v1.2$ [135]. However, it has recently been shown that PKA-dependent regulation of LTCC currents is intact in cardiac myocytes from AKAP7 knockout animals [136]. The fact that disrupting leucine zipper interactions blocks LTCC regulation suggests that some other leucine-zipper containing AKAP may be involved. Possible candidates include AKAP6 [137], AKAP9 [138], and AKAP13 (AKAP-Lbc) [139].

6. Lipid Rafts, Caveolae, and Caveolin-3

An alternative mechanism for the assembly of signaling complexes associated with $\text{Ca}_v1.2$ is through the colocalization of proteins in specific membrane domains [2]. The tight packing of sphingolipids and cholesterol in the plasma membrane creates microdomains called lipid rafts. These cholesterol rich domains can assemble signaling proteins with ion channels [140–142]. Proteins associated with lipid rafts are resistant to detergent (Triton X-100) solubilization and can be found in the low density membrane fractions obtained by sucrose density gradient centrifugation [143].

Caveolae are a morphologically distinct subset of lipid rafts that appear as 50–100 nm invaginations in the plasma membrane of many different cell types, including cardiac myocytes [140, 142, 144]. Caveolae are also specifically associated with the membrane protein caveolin [145]. There are three different types of caveolins: caveolin-1, -2, and -3. Caveolin-3 is sometimes referred to as the muscle specific caveolin, as it is found primarily (although not exclusively) in cardiac, skeletal, and smooth muscle. Caveolar proteins are typically associated with low density membrane fractions also containing caveolin-3. However, there is evidence that in cardiac myocytes not all caveolin-3 is associated with

lipid rafts [146]. Some, but not all, $\text{Ca}_v1.2$ are present in caveolar fractions of the plasma membrane [44, 46, 147] along with varying amounts of other proteins associated with the cAMP/PKA signaling cascade. These include β_1 - and β_2 ARs, AC5/6, G_s , G_i , PP2A, and the type II R subunit of PKA [32, 44, 46, 146, 148]. As described in previous sections of this review, many of these components of the cAMP/PKA signaling pathway have been tied to $\text{Ca}_v1.2$ via protein-protein interactions, either directly or indirectly. In some cases, the common connection may actually be through caveolin-3, by virtue of its caveolin scaffolding domain (CSD), which is a 20 amino acid sequence found in the membrane proximal N terminus [145, 149]. This may be important in forming signaling complexes in T-tubule membrane domains that are in close proximity to the junctional SR (Fig. 2B), where caveolae appear to be absent [150]. Another possibility is that some proteins are targeted to lipid rafts and caveolae by post-translational modifications such as myristoylation and/or palmitoylation. These include the β_2 AR, caveolins, G proteins, AKAP5, AKAP7, and the $\text{Ca}_v1.2$ β_{2a} subunit [99, 151–155].

Cyclodextrins deplete membrane of cholesterol and disrupt caveolae [156] thereby altering responses associated with these signaling platforms [142]. Rybin et al. [148] first suggested that caveolae play a role in creating the local response associated with β_2 ARs in cardiac myocytes. They found the majority of β_2 ARs in neonatal ventricular myocytes in caveolar membrane fractions and that disrupting caveolae by depleting membrane cholesterol enhanced cAMP production in response to β_2 AR activation. Cholesterol depletion enhanced the sensitivity of the LTCC response to β_2 AR stimulation [33], an effect similar to that produced by pertussis toxin block of G_i signaling, suggesting that caveolar signaling complexes are involved in G_i attenuation and localization of β_2 -adrenergic responses. The increase in sensitivity could also be due to disruption of the interaction between caveolin-3 and AC, which has an inhibitory effect on cyclase activity [148, 157].

Balijepalli et al. [44] demonstrated that caveolin-3, the β_2 AR, G_s , G_i , AC5/6, PKA, and $\text{Ca}_v1.2$ coimmunoprecipitate with each other. Furthermore, disruption of caveolae by cholesterol depletion or siRNA knockdown of caveolin-3 prevented β_2 -adrenergic stimulation of the LTCC current in neonatal mouse ventricular myocytes when G_i signaling was first blocked by pertussis toxin [44]. These observations indicate that caveolin-3 and more generally caveolae play some role in either the formation of the β_2 AR- $\text{Ca}_v1.2$ complex or its colocalization with other signaling components.

Other studies have demonstrated that lipid rafts and/or caveolae are also important in β_1 AR regulation of LTCCs. Agarwal et al. [32] found that cholesterol depletion enhanced β_1 AR stimulation of LTCC function in adult cardiac myocytes. This correlated with the ability of cholesterol depletion to enhance cAMP signaling in microdomains associated specifically with type II PKA signaling as measured using FRET-based biosensors. Cholesterol depletion did not alter the β_1 AR sensitivity of global cAMP responses. The authors also demonstrated that cholesterol depletion had no effect on more global cAMP response produce by EPR activation. Those results correlated with the fact the β_1 ARs were found in caveolar and non-caveolar fractions of the plasma membrane, while EPRs were only found in non-caveolar fractions [32], suggesting that PKA-dependent regulation of $\text{Ca}_v1.2$ involves localized production of cAMP by receptors in a signaling complex associated with caveolae/lipid rafts (Fig. 2A).

7. Summary

PKA-dependent phosphorylation plays an essential role in LTCC regulation. However, the exact mechanism for this regulatory effect is still an area of active investigation. Current evidence supports the idea that it may involve phosphorylation of one or more residues on

α_1 1.2, which then disrupts the autoinhibitory effect of the distal C terminal region of this subunit.

Another important aspect of LTCC regulation is the interaction of $\text{Ca}_v1.2$ with PKA. There is evidence that AKAP5 and AKAP7 can directly tether PKA to $\text{Ca}_v1.2$, while AKAP5 may also link PKA to $\text{Ca}_v1.2$ indirectly via caveolin-3. However, the precise roles of AKAP5 and AKAP7 have yet to be resolved. An intriguing possibility is that different AKAPs may be involved in regulating different subpopulations of LTCCs under different conditions and they can substitute for each other in corresponding KO animals.

In addition to PKA, regulation of $\text{Ca}_v1.2$ also depends on close association with AC and a GPCR. AKAPs appear to be important in targeting AC to $\text{Ca}_v1.2$ signaling complexes, while receptors such as the β_2 AR are physically linked to a subpopulation of $\text{Ca}_v1.2$ that is associated with caveolin-3. Evidence supporting a similar interaction between $\text{Ca}_v1.2$ and the β_1 AR is mixed. However, functional studies support the notion that at least some LTCCs are regulated via a localized cAMP response produced by β_1 ARs associated with lipid rafts. This highlights the potential importance of signaling complexes formed not only by protein-protein interactions, but also by colocalization of signaling proteins in specific membrane domains.

The fact that different receptors associated with cAMP production do not have the same effects on LTCC function is quite remarkable. EPRs produce cAMP without affecting channel function, β_2 ARs appear to affect only local channels, while β_1 ARs seem to affect both local and distal channels. The exact mechanism for this behavior is not well understood, but there is now evidence that this may involve the inclusion of PDEs as part of the $\text{Ca}_v1.2$ signaling complex.

Finally, reversal of PKA-dependent responses depends on phosphatase activity, and we now know that the $\text{Ca}_v1.2$ is actually capable of interacting with more than one type of phosphatase. This supports the interesting possibility that multiple phosphorylation sites are involved in regulating $\text{Ca}_v1.2$ activity.

In conclusion, rather than stochastically finding their targets, it is becoming increasingly clear that many, perhaps most, signaling proteins are linked in different ways to their ultimate targets, $\text{Ca}_v1.2$ being the prototypical one for ion channel regulation.

Abbreviations

LTCC	L-type Ca^{2+} channel
βAR	β -adrenergic receptor
EPR	E type prostaglandin receptor
RyR2	type 2 ryanodine receptor
AC	adenylyl cyclase
PDE	phosphodiesterase
PP	protein phosphatase
AKAP	A kinase anchoring protein
PKA	protein kinase A
DCT	distal C terminus

SR	sarcoplasmic reticulum
GPCR	G protein coupled receptor
SERCA2a	type 2a sarcoplasmic reticulum Ca ²⁺ ATPase
PLN	phospholamban
CAV-3	caveolin-3

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Highlights

Ca^{2+} influx by L-type Ca^{2+} channels trigger Ca^{2+} release and cardiac contraction

We discuss how VGCC are regulated by GPCR via cAMP/PKA

VGCC form signaling complexes with $\beta_2\text{AR}$, Gs, adenylyl cyclase, and PKA

VGCC also associate with the protein phosphatases PP2A and PP2B

These complexes regulate channel activity in a highly localized manner

Figure 1.

The $\text{Ca}_V1.2$ signaling complex includes the $\alpha_11.2$ subunit, β_2 subunit, A kinase anchoring proteins 5 and 7 (AKAP5 and AKAP7), protein kinase A (PKA), protein phosphatases 2A and 2B (PP2A and PP2B), the β_2 -adrenergic receptor ($\beta_2\text{AR}$), adenylyl cyclase 5/6 (AC5/6), stimulatory G protein (G_s), and phosphodiesterases 4B and 4D (PDE4B and PDE4D). PKA phosphorylation sites are indicated in red; sites of interaction with binding partners are indicated by corresponding color-coded segments or brackets. Site cleaved by calpain indicated by scissors. See text for details.

figure 2A

figure 2B

Figure 2.

Ca_v1.2 is regulated by cAMP confined to specific signaling domains. **Panel A:** β₁- and β₂-adrenergic receptors (βARs) associated with Ca_v1.2 and caveolin-3 (CAV-3) signaling complexes in caveolae can stimulate cAMP production that regulates channel activity. E type prostaglandin receptors (EPRs) and βARs found outside of these signaling complexes can stimulate global changes in cAMP concentration that are not accessible to Ca_v1.2.

Panel B: βARs associated with Ca_v1.2 and caveolin-3 at dyadic junctions, where caveolae are not present, can stimulate cAMP production that regulates channel activity as well as the activity of the type 2 ryanodine receptor (RyR2) and phospholamban (PLN). βARs not associated with CAV-3 and adenyl cyclase (AC) are not able to stimulate cAMP production and regulates channel activity. In addition to the Ca_v1.2 signaling complex described in figure 1, CAV-3 associates with the inhibitory G protein (G_i), and AC5/6

associates with AKAP5 and PP2B. AKAPs also form signaling complexes with PLN and the type 2a sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a) as well as RyR2. See text for details.