

# Regulation of Cardiac L-Type Calcium Channels by Protein Kinase A and Protein Kinase C

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**Abstract**—Voltage-dependent L-type  $\text{Ca}^{2+}$  channels are multisubunit transmembrane proteins, which allow the influx of  $\text{Ca}^{2+}$  ( $I_{\text{Ca}}$ ) essential for normal excitability and excitation-contraction coupling in cardiac myocytes. A variety of different receptors and signaling pathways provide dynamic regulation of  $I_{\text{Ca}}$  in the intact heart. The present review focuses on recent evidence describing the molecular details of regulation of L-type  $\text{Ca}^{2+}$  channels by protein kinase A (PKA) and protein kinase C (PKC) pathways. Multiple G protein-coupled receptors act through cAMP/PKA pathways to regulate L-type channels.  $\beta$ -Adrenergic receptor stimulation results in a marked increase in  $I_{\text{Ca}}$ , which is mediated by a cAMP/PKA pathway. Growing evidence points to an important role of localized signaling complexes involved in the PKA-mediated regulation of  $I_{\text{Ca}}$ , including A-kinase anchor proteins and binding of phosphatase PP2a to the carboxyl terminus of the  $\alpha_{1\text{C}}$  ( $\text{Ca}_v1.2$ ) subunit. Both  $\alpha_{1\text{C}}$  and  $\beta_{2\text{a}}$  subunits of the channel are substrates for PKA in vivo. The regulation of L-type  $\text{Ca}^{2+}$  channels by Gq-linked receptors and associated PKC activation is complex, with both stimulation and inhibition of  $I_{\text{Ca}}$  being observed. The amino terminus of the  $\alpha_{1\text{C}}$  subunit is critically involved in PKC regulation. Crosstalk between PKA and PKC pathways occurs in the modulation of  $I_{\text{Ca}}$ . Ultimately, precise regulation of  $I_{\text{Ca}}$  is needed for normal cardiac function, and alterations in these regulatory pathways may prove important in heart disease. (*Circ Res.* 2000;87:1095-1102.)

**Key Words:** L-type calcium channel ■ protein kinase C ■ protein kinase A ■ heart ■ regulation ■ phosphorylation

The influx of  $\text{Ca}^{2+}$  ions through voltage-dependent L-type  $\text{Ca}^{2+}$  channels plays an essential role in cardiac excitability and in coupling excitation to contraction. The depolarizing current through L-type  $\text{Ca}^{2+}$  channels ( $I_{\text{Ca}}$ ) contributes to the plateau phase of the cardiac action potential as well as to pacemaker activity in nodal cells. This influx of  $\text{Ca}^{2+}$  triggers the release of intracellular stores of  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum, and the ensuing intracellular  $\text{Ca}^{2+}$  transient results in activation of the myofilaments. L-type channels can also impact on other cellular processes modulated by intracellular  $\text{Ca}^{2+}$  such as gene expression and excitation-secretion coupling. Alterations in density or function of L-type  $\text{Ca}^{2+}$  channels have been implicated in a variety of cardiovascular diseases, including atrial fibrillation,<sup>1,2</sup> heart failure,<sup>3-6</sup> and ischemic heart disease.<sup>7</sup>

Cardiac L-type  $\text{Ca}^{2+}$  channels are regulated by a variety of neurotransmitters, hormones, and cytokines. In fact, the first description of currents carried by this channel revealed its regulation by epinephrine.<sup>8</sup> Sperelakis and Schneider<sup>9</sup> and Reuter and Scholz<sup>10</sup> independently hypothesized that  $\beta$ -adrenergic receptor (AR)-mediated stimulation of cardiac L-type  $\text{Ca}^{2+}$  channels was due to phosphorylation of the channel by cAMP-dependent protein kinase A (PKA). Extensive electrophysiology experimentation over the subsequent 2 decades has supported the hypothesis; however, the molecu-

lar details have been slow to follow. The scarcity of this transmembrane protein as well as difficulty in reconstituting regulation in heterologous expression systems has limited progress. Other signaling pathways have also been suggested to regulate the channel by phosphorylation, but the details are even less clear. For example, activation of protein kinase C (PKC) has resulted in widely variable effects on L-type channel activity. The purpose of the present review is to describe recent advances in the understanding of the regulation of L-type  $\text{Ca}^{2+}$  channels by PKA- and PKC-mediated pathways focusing on features that provide specificity and localization to this signaling. Excellent general reviews on the structure and function of L-type  $\text{Ca}^{2+}$  channels are available.<sup>11-14</sup>

## Structure of L-Type $\text{Ca}^{2+}$ Channels

Voltage-dependent  $\text{Ca}^{2+}$  channels are multimeric protein complexes present in many cell types throughout the body. The  $\alpha_1$  subunit is the main functional component of the channel complex. It is composed of 4 homologous domains (I-IV), each containing 6 transmembrane segments (S1-S6) as schematically shown in Figure 1. The  $\alpha_1$  subunit contains the voltage sensor for the channel, which is primarily formed by the positively charged arginine and lysine residues in the S4 segments. The P loops between S5 and S6 line the pore of

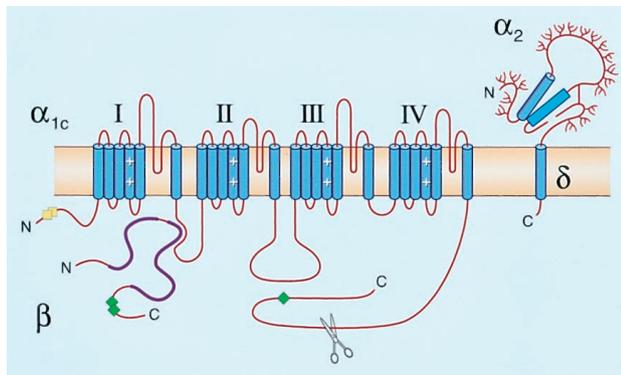
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**Figure 1.** Proposed transmembrane topology and subunit composition of L-type  $\text{Ca}^{2+}$  channel. Shown is the pore-forming  $\alpha_{1c}$  subunit consisting of 4 homologous repeated domains (I–IV), each composed of 6 transmembrane segments as described in text. The cytoplasmic  $\beta$  subunit is formed by 2 highly conserved domains indicated in purple, and the amino-terminal portion of the second conserved domain interacts with the I–II loop of  $\alpha_{1c}$ . The  $\delta$  subunit has a single transmembrane segment with a short cytoplasmic C terminus and is linked by a disulfide bond to the extracellular, glycosylated  $\alpha_2$  subunit. PKA phosphorylation sites of proven functional significance are shown as green diamonds at Ser1928 on  $\alpha_{1c}$  and Ser478 and Ser479 on  $\beta_{2a}$ . PKC phosphorylation sites of proven functional importance at Thr27 and Thr31 on  $\alpha_{1c}$  are indicated by yellow squares.

the channel.<sup>15,16</sup> At least 10 different  $\alpha_1$ -subunit genes have been identified, which provide unique functional properties to  $\text{Ca}^{2+}$  channels present in different cell types.<sup>17</sup> In cardiac muscle, L-type  $\text{Ca}^{2+}$  channels are primarily encoded by the  $\alpha_{1c}$  gene ( $\text{Ca}_v1.2$ ) with possible contribution by  $\alpha_{1D}$  ( $\text{Ca}_v1.3$ ).<sup>18,19</sup> In vivo, a substantial portion of  $\alpha_{1c}$  undergoes proteolytic processing about 400 to 500 residues away from its C terminus, but the C-terminal fragment stays associated with the channel complex.<sup>20–23</sup>

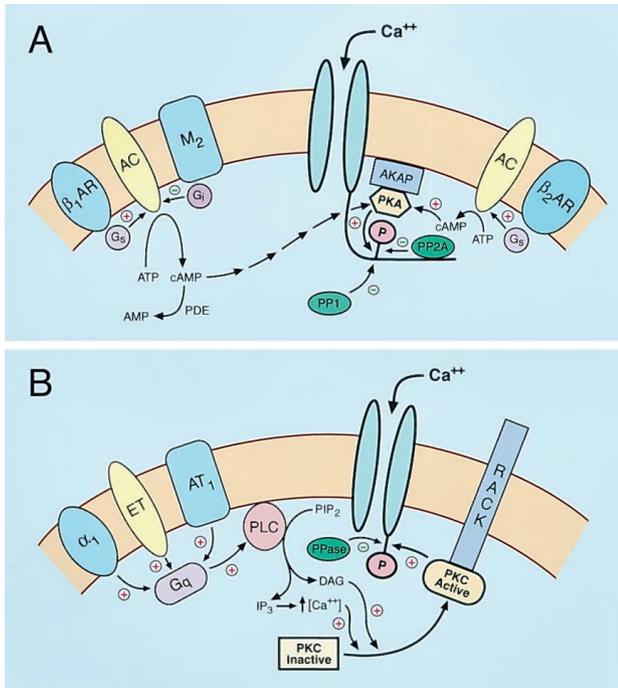
Cardiac L-type  $\text{Ca}^{2+}$  channels are also composed of auxiliary subunits, including  $\beta$  and  $\alpha_2$ - $\delta$ . Additionally, a  $\gamma$  subunit has been found in  $\text{Ca}^{2+}$  channels in skeletal muscle and brain,<sup>24–26</sup> but it remains unclear as to whether cardiac L-type  $\text{Ca}^{2+}$  channels contain a  $\gamma$  subunit.<sup>27</sup> Four distinct genes encode cytoplasmically localized  $\text{Ca}^{2+}$  channel  $\beta$  subunits, each having multiple splice variants.<sup>28</sup> The  $\beta$  subunits are important in trafficking of the channel complex to the surface membrane as well as in modifying its gating properties.<sup>28–31</sup> Although the  $\beta_{2a}$  subunit may be the predominant isoform in heart, there appears to be significant species variation, and multiple isoforms are expressed.<sup>32,33</sup> The  $\alpha_2$ - $\delta$  subunits are created from a precursor protein by proteolytic cleavage.<sup>34</sup> Both fragments remain linked via a disulfide bridge.  $\delta$  is an integral membrane protein with a single transmembrane region, a short intracellular sequence, and a larger extracellular portion, which is differentially glycosylated.<sup>35</sup>  $\alpha_2$  is an extracellular, glycosylated protein.<sup>35</sup> Three  $\alpha_2$ - $\delta$  genes have been identified.<sup>36,37</sup> This subunit has also been implicated in modifying the gating properties of the channel as well as the expression level of the channel complex.<sup>29,37,38</sup> Therefore, a rich variety of different subunit isoforms can combine to produce voltage-dependent  $\text{Ca}^{2+}$  channels in a cell-specific and potentially disease-modulated fashion.

## Regulation by PKA

Multiple G protein-coupled receptors in the heart act through cAMP/PKA pathways to regulate many cellular proteins, including the L-type  $\text{Ca}^{2+}$  channel (Figure 2A). These receptors are coupled to heterotrimeric G proteins, which either stimulate ( $G_s$ ) or inhibit ( $G_i$ ) adenylyl cyclase (AC). An increase in AC activity leads to increased cellular cAMP, which binds to the regulatory subunits of cAMP-dependent protein kinase (PKA), liberating the catalytic subunits to phosphorylate their substrates on specific serine and threonine residues. This cascade is counterbalanced by phosphodiesterases that degrade cAMP into 5'-AMP as well as serine-threonine phosphatases. Multiple laboratories have provided extensive evidence demonstrating robust upregulation of  $I_{Ca}$  by the  $\beta\text{AR}/\text{cAMP}/\text{PKA}$  pathway, and these pioneering electrophysiological studies have been reviewed well elsewhere.<sup>13,14,39</sup> In addition,  $\beta$ -adrenergic activation of  $G_s$  has been suggested to directly stimulate  $I_{Ca}$  independently of PKA,<sup>40</sup> but the role of this regulation in normal physiology is controversial.<sup>41</sup> The present review will focus on more recent experiments dissecting out the molecular details of PKA-mediated upregulation of channel function.

Most initial studies on the stimulation of cardiac L-type  $\text{Ca}^{2+}$  channel by  $\beta\text{AR}$  signaling focused on the  $\beta_1\text{AR}$ , the predominant  $\beta\text{AR}$  in the normal adult mammalian heart. These studies have clearly demonstrated a cAMP/PKA-dependent stimulation of  $I_{Ca}$ .  $\beta_2\text{AR}$  stimulation also increases  $I_{Ca}$  in certain cardiac myocyte preparations depending on the species, developmental stage, and presence of disease.<sup>42,43</sup> Whereas both  $\beta_1\text{AR}$  and  $\beta_2\text{AR}$  are positively coupled to  $G_s$ , cAMP levels, and L-type  $\text{Ca}^{2+}$  channel activity,  $\beta_2\text{AR}$  can in some cases stimulate  $I_{Ca}$  without significantly elevating total cellular cAMP.<sup>44</sup> This finding, as well as the lack of  $\beta_2\text{AR}$  effects on PKA-mediated phosphorylation of phospholamban and troponin I, led to the suggestion that regulation of L-type  $\text{Ca}^{2+}$  channels by  $\beta_2\text{AR}$  was due to highly localized elevations in cAMP around the channel.<sup>45</sup> In amphibian ventricular myocytes, which contain almost exclusively  $\beta_2\text{AR}$ s, regulation of  $I_{Ca}$  is spatially restricted.<sup>46</sup>  $\beta_2\text{AR}$ s couple not only to  $G_s$  but also to  $G_i$ . The latter pathway has been suggested to play a role in spatially restricting  $\beta_2\text{AR}$  signaling.<sup>47</sup> However, some studies have not been able to demonstrate  $\beta_2\text{AR}$  regulation of  $I_{Ca}$ .<sup>48,49</sup> There are multiple other  $G_s$ -coupled receptors in the heart that can upregulate  $I_{Ca}$ , including histamine receptors ( $H_2$ ) and glucagon receptors.<sup>14,39</sup> The specifics of their regulation of  $I_{Ca}$  will likely differ in detail, but less information is available for these receptors.

The muscarinic  $M_2$  receptor represents the best-studied example of a  $G_i$ -coupled receptor that regulates  $I_{Ca}$ .<sup>50</sup> In general, most  $G_i$ -coupled receptors appear not to alter basal  $I_{Ca}$  levels but dramatically inhibit the  $\beta\text{AR}$  stimulation of  $I_{Ca}$ . Initial studies suggested that this effect was due to  $G_i$ -mediated inhibition of AC and lowering cAMP levels. However, in the case of muscarinic  $M_2$  receptor-mediated inhibition of  $I_{Ca}$ , other mechanisms are likely in place such as activation of phosphatases<sup>51</sup> and a debatable role of NO and stimulation of cGMP-dependent phosphodiesterase.<sup>52,53</sup> Interestingly,  $\beta_1\text{AR}$ - and  $\beta_2\text{AR}$ -stimulated responses may exhibit differential sensitivity to muscarinic inhibition.<sup>54</sup> Multiple



**Figure 2.** Signaling cascades regulating L-type  $\text{Ca}^{2+}$  channels. A, Schematic of the cAMP/PKA cascade regulating L-type channels. Stimulation of  $\beta_1\text{AR}$  or  $\beta_2\text{AR}$  leads to  $\text{G}_s$ -mediated activation of AC and increased production of cAMP, which stimulates PKA, as described in text. PKA can then phosphorylate the channel at multiple potential sites indicated schematically by the single P in the diagram. The PKA phosphorylated site(s) is then sensitive to the phosphatases PP1 and PP2A. Whereas  $\beta_1\text{AR}$  regulation causes more global increases in cAMP,  $\beta_2\text{AR}$  stimulation can result in highly localized cAMP level changes and regulation. Regulatory proteins may be localized to the channel by an AKAP for PKA and by binding of PP2A to the C terminus of the channel. Muscarinic  $\text{M}_2$  receptors can oppose the  $\beta\text{AR}$  upregulation of  $I_{\text{Ca}}$  by acting through  $\text{G}_i$  to inhibit AC. B, PLC/PKC signaling cascade regulating L-type  $\text{Ca}^{2+}$  channels. Activation of  $\alpha_1$ -adrenergic, ET, or  $\text{AT}_1$  receptors stimulates  $\text{G}_q$  with resulting activation of PLC, which leads to the production of diacylglycerol and activation of PKC. PKC is proposed to target to the membrane by binding a RACK protein in the vicinity of the L-type  $\text{Ca}^{2+}$  channel, which it then phosphorylates (see text for details). A Ser/Thr phosphatase counterbalances this phosphorylation.  $\text{IP}_3$  indicates inositol trisphosphate;  $\text{PIP}_2$ , phosphatidylinositol 4,5 biphosphate.

other  $\text{G}_i$ -coupled receptors have been implicated in  $I_{\text{Ca}}$  regulation, including adenosine ( $\text{A}_1$ ) receptors, opiate receptors, and atrial natriuretic factor receptors.<sup>14</sup>

An alternative mechanism of PKA-mediated stimulation of L-type  $\text{Ca}^{2+}$  channels occurs as a result of strong depolarization. This process of voltage-dependent facilitation is hypothesized to be caused by a voltage-dependent conformational change in the channel, making it amenable to PKA-dependent phosphorylation.<sup>55</sup> This finding suggested that PKA may be in close proximity to the channel, and in the case of skeletal muscle, an A-kinase anchor protein (AKAP) associating PKA with the channel has been shown to be essential for this regulation.<sup>56</sup> Although state-dependent regulation of the channel has been observed in native ventricular myocytes,<sup>57,58</sup> it has only been variably reproduced in heterologous systems. The neuronal splice variant,  $\alpha_{1\text{C-c}}$ , expressed in

*Xenopus* oocytes has demonstrated pronounced voltage-dependent facilitation that requires PKA and  $\beta$ -subunit coexpression.<sup>59</sup> In contrast, studies in mammalian HEK293 cells expressing cardiac isoforms of  $\alpha_{1\text{C}}$  have demonstrated voltage-dependent facilitation, but it is independent of PKA.<sup>60,61</sup> The reasons for these apparently distinct results, as well as the molecular details of voltage-dependent facilitation of L-type  $\text{Ca}^{2+}$  channel activity, remain largely unknown.

### Biochemical and Functional Characterization of Channel Phosphorylation by PKA

Evidence for a direct phosphorylation of L-type channels by PKA did not become available until it was recognized that the full-length form of  $\alpha_{1\text{C}}$  can be proteolytically truncated at its C terminus.<sup>21</sup> The proteolytic cleavage is mediated in neurons and possibly in the heart by the  $\text{Ca}^{2+}$ -dependent protease calpain.<sup>22</sup> Only the long but not the short form of  $\alpha_{1\text{C}}$  is effectively and stoichiometrically phosphorylated by PKA in vitro.<sup>21,62</sup> Ser1928, which is located in the C-terminal portion that is cleaved off the full-length form (Figure 1), is the only detectable phosphorylation site on  $\alpha_{1\text{C}}$ <sup>20</sup> and is phosphorylated in vivo.<sup>20,62,63</sup> In heart, the prevailing isoform detectable by immunoblotting is the short form.<sup>20</sup> However, the long form is also present, and biochemical and functional evidence indicates that the C-terminal fragment remains tethered to the channel.<sup>23,64</sup> Electrophysiological studies utilizing heterologous expression systems for  $\alpha_{1\text{C}}$  suggested that no other  $\text{Ca}^{2+}$  channel subunit is absolutely required for stimulation of channel activity by PKA.<sup>55,65</sup> Furthermore, mutation of Ser1928 to alanine in  $\alpha_{1\text{C}}$  prevented phosphorylation and upregulation of the channel by PKA.<sup>64</sup>

$\alpha_2$ - $\delta$  is primarily extracellular, and phosphorylation by PKA or PKC is not detectable.<sup>21,63</sup> In contrast,  $\text{Ca}^{2+}$  channel  $\beta$  subunits serve as substrates for multiple kinases in vitro and in intact cells.<sup>13,64</sup> Application of the  $\beta\text{AR}$  agonist isoproterenol in vivo resulted in phosphorylation of 1 or more PKA sites of the cardiac L-type channel  $\beta$  subunits.<sup>66,67</sup> PKA phosphorylates 3 sites of  $\beta_{2a}$  (Ser459, Ser478, and Ser479) in vitro (Figure 1).<sup>68</sup> To test the functional relevance of these phosphorylation sites,  $\beta_{2a}$  was coexpressed with a C-terminally truncated version of  $\alpha_{1\text{C}}$  that lacks Ser1928. Channel activity could be increased by PKA when wild-type  $\beta_{2a}$  was present, indicating that phosphorylation of the  $\beta$  subunit can contribute to the upregulation of channel activity.<sup>69</sup> Mutation of Ser478/Ser479 to alanines but not of Ser459 on  $\beta_{2a}$  prevented upregulation of channel activity.<sup>69</sup> These results indicate that phosphorylation of either Ser478, Ser479, or both contributes to channel regulation by PKA at least in the presence of C-terminally truncated  $\alpha_{1\text{C}}$ .

AKAPs target PKA to various substrates to provide fast and specific signaling.<sup>70–72</sup> When PKA is prevented from binding to AKAPs by a peptide derived from one of the interaction sites, its regulation of skeletal muscle ( $\text{Ca}_v1.1$ ) and cardiac L-type channels is blocked.<sup>56,64</sup> PKA-mediated  $\alpha_{1\text{C}}$  phosphorylation can be reconstituted in HEK293 cells by coexpression of the channel with wild-type AKAP79 but not an AKAP79 mutant deficient in binding of PKA.<sup>64</sup> Recently, association of PKA with  $\alpha_{1\text{C}}$  has been demonstrated in the brain.<sup>63</sup> This interaction may be mediated by microtubule-

associated protein MAP2B,<sup>63</sup> which is the first identified AKAP.<sup>73</sup> Because MAP2B is not expressed in the heart, another AKAP may recruit PKA to cardiac L-type channels. One candidate is mAKAP (AKAP100), which localizes to the region of the transverse tubules and junctional sarcoplasmic reticulum,<sup>74</sup> similar to the predominance of L-type channels in the transverse tubules.<sup>75</sup> Another possibility is AKAP15, which acts as the adaptor protein for PKA association with the skeletal muscle L-type channel<sup>76</sup> and is expressed in the heart.<sup>77</sup>

The functional effects of phosphorylation of cardiac L-type  $\text{Ca}^{2+}$  channels have been examined in single-channel studies. The functional properties of the  $\text{Ca}^{2+}$  channels determine the whole-cell  $I_{\text{Ca}}$  by the equation  $I_{\text{Ca}} = N \times f_{\text{active}} \times p_o \times g \times \Delta V$ , where  $N$  is the total number of L-type  $\text{Ca}^{2+}$  channels,  $f_{\text{active}}$  is the fraction of these channels that are available to open during a depolarization,  $p_o$  is the probability of an active channel to be open,  $g$  is the single-channel conductance, and  $\Delta V$  is the difference between the test potential and the reversal potential for the channel. Single Ca channel currents recorded on consecutive depolarizations have demonstrated a variety of gating patterns that can most simply be divided into blank sweeps (no openings) and active sweeps. The blank sweeps are clustered together in time, as are the active sweeps. One prominent effect of PKA activation is to decrease the number of blank sweeps or increase  $f_{\text{active}}$ . It was hypothesized that phosphorylation of the channel by PKA was necessary for the channels to become active.<sup>78,79</sup> Herzig et al<sup>80</sup> developed a model suggesting that the availability of channels to open could indeed be controlled by a single phosphorylation event. In addition, the activity of the channel during active traces can be markedly increased by PKA stimulation due to increase in  $p_o$  resulting from changed modes of active gating.<sup>81</sup> The relative importance of increased  $f_{\text{active}}$  and  $p_o$  in  $\beta\text{AR}$  stimulation of  $I_{\text{Ca}}$  has been debated and likely varies in different experimental preparations. No changes in single-channel conductance, reversal potential, or the number of channels in the patch have been observed in response to  $\beta\text{AR}$  or PKA stimulation of the channels.

Dynamic regulation of channel activity requires that phosphorylation be readily reversible by phosphatases. The Ser/Thr phosphatases PP1 and PP2A but not PP2B or PP2C have been demonstrated to regulate L-type channels stimulated by PKA.<sup>55,82,83</sup> Experiments with phosphatase inhibitors that differentially inhibit PP1 and PP2A suggest the existence of 2 different phosphorylation sites governing the 2 major changes in gating of L-type  $\text{Ca}^{2+}$  channels observed in response to  $\beta\text{AR}$  stimulation. In rabbit and guinea pig ventricular myocytes, a phosphorylation site sensitive to PP1 regulates the availability of channels ( $f_{\text{active}}$ ), whereas a distinct phosphorylation site sensitive to PP2A controls modal gating during active sweeps.<sup>58,84</sup> However, the case may be different in amphibian ventricular myocytes.<sup>85</sup> Furthermore, rundown of L-type channel activity in inside-out patches obtained from rabbit ventricular myocytes is strongly slowed by an inhibitor of PP1 and PP2A,<sup>83</sup> suggesting that PP1 or PP2A may be linked to the plasma membrane in close proximity to the channel. We recently found that PP2A is directly bound to  $\alpha_{1\text{C}}$  in rat brain and reverses phosphoryla-

tion of Ser1928.<sup>86</sup> Overall, these studies have provided evidence of single L-type  $\text{Ca}^{2+}$  channel complexes being modulated by at least 2 distinct PKA-mediated phosphorylation events and that PKA and PP2A may be highly localized to the channel complex. Investigations have not yet linked the identified PKA phosphorylation sites with specific changes in channel gating in native cells.

### Regulation by PKC

The PKC family of kinases also plays an essential role in the regulation of the L-type  $\text{Ca}^{2+}$  channel in the heart. Multiple  $G_q$  protein-coupled receptors, including endothelin (ET),  $\alpha_1$ -adrenergic, and angiotensin II receptors, trigger the signaling cascade leading to activation of PKC (Figure 2B).<sup>87</sup> Activated  $G_q$  stimulates phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate ( $\text{PIP}_2$ ), generating inositol trisphosphate and diacylglycerol (DAG).<sup>88</sup> DAG, phosphatidylserine, and in some cases  $\text{Ca}^{2+}$  collectively activate PKC.

Initial studies of the modulation of  $I_{\text{Ca}}$  by neurohormones linked to PKC have demonstrated a variety of results. For example, ET-1 resulted in clear increases,<sup>89,90</sup> decreases,<sup>91</sup> or no change in basal  $I_{\text{Ca}}$ .<sup>92,93</sup> Some authors have even demonstrated biphasic effect on  $I_{\text{Ca}}$ , ie, a decrease followed by a more sustained increase.<sup>94–96</sup> The range of effects may be due to differences in experimental conditions, species, and methods of studying  $I_{\text{Ca}}$ . Techniques that preserve the cytoplasmic environment, such as the perforated-patch whole-cell approach or cell-attached single-channel method, may be necessary to demonstrate an upregulation of  $I_{\text{Ca}}$  in response to  $\alpha_1$ -adrenergic stimulation, arginine vasopressin, and ET-1.<sup>90,94,95,97,98</sup> In addition, an upregulation of  $I_{\text{Ca}}$  is consistent with the positive inotropic effects and increased intracellular  $\text{Ca}^{2+}$  transients observed in response to many of these neurohormones.<sup>94,99</sup>

Conflicting findings have also resulted from studies of direct activators of PKC, such as dioctanoylglycerol ( $\text{diC}_8$ ) and 1-oleoyl-2-acetyl-*sn*-glycerol, as well as phorbol esters.<sup>100–105</sup> Furthermore, the complexity of the response of  $I_{\text{Ca}}$  to phorbol esters has been demonstrated in studies of neonatal rat ventricular myocytes and adult canine ventricular myocytes showing a biphasic effect on  $I_{\text{Ca}}$  with an initial stimulation followed by an inhibition.<sup>101,103</sup> In some preparations, PKC-independent effects of phorbol esters and DAG analogues on  $I_{\text{Ca}}$  have been observed.<sup>102,106</sup> We recently demonstrated a PKC-independent inhibition of  $I_{\text{Ca}}$  by bath application of  $\text{diC}_8$  but showed in parallel that photorelease of intracellular caged  $\text{diC}_8$  caused a robust PKC-dependent stimulation of  $I_{\text{Ca}}$ .<sup>90</sup> Some PKC inhibitors have also been implicated in directly blocking  $I_{\text{Ca}}$  independently of their effects on PKC.<sup>107</sup> In summary, experiments utilizing direct activators of PKC have demonstrated a range of effects on  $I_{\text{Ca}}$ , not all of which are PKC-dependent.

The ultimate effect of stimulation of PKC on  $I_{\text{Ca}}$  may be closely related to the isoform(s) of PKC activated by a particular signaling pathway or chemical. The PKC isoforms are expressed in developmentally regulated, species-dependent, and disease-specific fashion in the heart.<sup>108–110</sup> Activation of PKC involves translocation of the enzyme to

specific targets, and different isozymes show different patterns of subcellular localization on activation, corresponding to the subcellular localization of the specific substrate proteins. Interestingly, PKC $\epsilon$  translocates to cross-striated regions in ventricular myocytes, which places it near T-tubules where L-type Ca<sup>2+</sup> channels are localized.<sup>111,112</sup> The membrane targeting of PKC isozymes is in part due to interactions with specific anchoring proteins referred to as RACKs (receptors for activated C kinases).<sup>113</sup> The amino-terminal regulatory region of PKC contains the membrane-targeting motifs that interact with RACKs in an isoform-specific manner. Peptides derived from these amino-terminal regions of PKC can be used as isoform-selective translocation inhibitors.<sup>113</sup> A recent study has demonstrated that peptides derived from the corresponding region of PKC $\beta$  specifically block the inhibition of  $I_{Ca}$  by phorbol esters in rat ventricular myocytes, suggesting a role for conventional PKC isoforms in this regulation.<sup>114</sup> It is possible that distinct isoforms of PKC may have opposing effects on L-type Ca<sup>2+</sup> channels, as previously suggested for the effect of phorbol esters on the chronotropic state of neonatal rat ventricular myocytes.<sup>115</sup>

### Molecular Targets for PKC Regulation of L-Type Ca<sup>2+</sup> Channels

PKC-activating pathways can clearly modulate the L-type Ca<sup>2+</sup> channel in cardiac muscle; however, the substrate(s) for PKC and the underlying molecular mechanisms of this regulation remain largely unknown. Biochemical studies in vitro have demonstrated that both the  $\alpha_{1C}$  and  $\beta_{2a}$  subunits of the L-type Ca<sup>2+</sup> channel can be substrates for PKC.<sup>116</sup> When the recombinant rabbit cardiac  $\alpha_{1C}$  was expressed in *Xenopus* oocytes, phorbol 12-myristate 13-acetate (PMA) treatment resulted in an increase followed by a gradual decrease in  $I_{Ca}$ .<sup>117,118</sup> This regulation occurred whether the auxiliary subunits were coexpressed or not.<sup>118</sup> In contrast, channel activity of the human cardiac  $\alpha_{1C}$  subunit expressed in *Xenopus* oocytes was only inhibited by application of PMA, and this inhibition required coexpression of the  $\beta_{1a}$  subunit.<sup>119</sup> It was suggested that the difference in the amino terminus of the rabbit and human clone were responsible for the distinct effects,<sup>119</sup> and recent experiments confirmed that the unique 46 amino acids of the N terminus of the rabbit clone are necessary for PKC-mediated upregulation of  $I_{Ca}$ .<sup>120</sup> It was proposed that PKC stimulates  $I_{Ca}$  by removing the tonic inhibitory effect of the long (rabbit) N terminus on  $I_{Ca}$ . In striking contrast, currents carried by the rabbit heart  $\alpha_{1C}$  expressed in TSA-201 cells are markedly inhibited by PKC.<sup>121</sup> Mutagenesis of threonines at amino acids 27 and 31 in rabbit  $\alpha_{1C}$  demonstrated that these residues are the targets for PKC responsible for the inhibition of  $I_{Ca}$ .<sup>121</sup> Why expressed recombinant L-type channels demonstrate such contrasting regulation in *Xenopus* oocytes compared with mammalian TSA-201 cells is unknown. Important questions remain regarding the regulation of  $I_{Ca}$  in the intact heart by PKC.

### Integrating the Signals/Crosstalk

The regulation of cardiac  $I_{Ca}$  by various signaling pathways has typically been examined by studying each pathway in isolation. In the intact organism, a dynamic mix of cellular

signals regulates the function of the channel. Even in the apparently simple case of a single biologically relevant neurotransmitter, norepinephrine, multiple adrenergic receptor subtypes and their associated signaling cascades are activated in the cardiac myocyte. For example,  $\alpha_1$ ARs activate PLC/PKC-dependent signaling, whereas  $\beta$ ARs activate cAMP/PKA-dependent signaling, and both of these pathways have been shown to stimulate  $I_{Ca}$  in most physiological preparations. However, the combination of  $\alpha_1$ AR and  $\beta$ AR activation on  $I_{Ca}$  is not simply additive, as  $\alpha_1$ AR activation strongly blunts the increase in  $I_{Ca}$  by  $\beta$ AR stimulation.<sup>122</sup> Likewise, activation of ET and angiotensin receptors, which are associated with stimulation of PKC, also strongly antagonize the effect of  $\beta$ AR stimulation of  $I_{Ca}$ .<sup>92,123,124</sup> Transgenic overexpression of G $\alpha_q$  and resulting activation of PKC has also been shown to blunt  $\beta$ -adrenergic stimulation of  $I_{Ca}$ .<sup>125</sup> Crosstalk likely occurs at various levels of the signaling cascades to produce these counterregulatory effects, and in some cases it may occur at the level of the channel itself.

There is also evidence for crosstalk with other signaling pathways regulating  $I_{Ca}$ . For example, in human atrial myocytes, tyrosine kinase stimulates  $I_{Ca}$  only after PKC is activated.<sup>126</sup> In guinea pig ventricular myocytes, the tyrosine kinase inhibitor, genistein, increases the sensitivity of  $I_{Ca}$  to  $\beta$ AR stimulation.<sup>127</sup> The status of the cytoskeletal system in the cells can even impact PKA-mediated regulation of  $I_{Ca}$ .<sup>128</sup> Understanding the many interactions between the various signaling cascades and their ultimate impact on channel function is just beginning.

### Conclusions and Future Directions

Given the critical role of the L-type Ca<sup>2+</sup> channel in multiple cellular functions, it is not surprising that this channel is extensively regulated by a variety of signaling pathways. Investigations over the last three decades have defined that the marked upregulation of  $I_{Ca}$  by  $\beta$ AR stimulation results from activation of the cAMP/PKA signaling cascade. However, the molecular details of this regulation have only recently started to be revealed with the discovery of functionally important PKA phosphorylation sites on  $\alpha_{1C}$  and  $\beta_{2a}$ . Many important questions remain, including whether additional phosphorylation sites are involved; how these phosphorylation sites interact; what role the truncated C terminus, including Ser 1928, plays in this regulation; what the functional effects of each site on channel gating are; which sites are important in the intact heart; and how this regulation changes in disease. Additionally, evidence is accumulating for a localized signaling complex that targets regulation to the L-type Ca<sup>2+</sup> channel, including AKAPs to localize PKA and direct binding of PP2a to the C terminus of the  $\alpha_{1C}$  subunit. The composition of these signaling complexes and their functional importance will be exciting areas of future investigation.

PKC regulation of L-type Ca<sup>2+</sup> channels is even more of a mystery. There is clear evidence that activation of PKC can both stimulate and inhibit  $I_{Ca}$  depending on the cells studied and experimental conditions. It seems likely that different PKC isoforms may be activated by different signaling mechanisms, resulting in distinct targeting of the isoforms involved

in this regulation. Likewise, different splice variants of the channel subunits may be critical, especially with regard to the amino terminus of  $\alpha_{1c}$ . Future studies are likely to take advantage of improved tools, including isoform-specific inhibitors, and activators of PKC. Ultimately, understanding the details of these regulatory pathways will provide insights into the role of the L-type  $\text{Ca}^{2+}$  channel in normal physiology and disease.

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