Regulation of Cardiac L-Type Ca\(^{2+}\) Channel Ca\(_{1,2}\) Via the β-Adrenergic-cAMP-Protein Kinase A Pathway

Old Dogmas, Advances, and New Uncertainties

Sharon Weiss, Shimrit Oz, Adva Benmocha, Nathan Dascal

Abstract: In the heart, adrenergic stimulation activates the β-adrenergic receptors coupled to the heterotrimeric stimulatory G\(_{\text{S}}\) protein, followed by subsequent activation of adenyl cyclase, elevation of cyclic AMP levels, and protein kinase A (PKA) activation. One of the main targets for PKA modulation is the cardiac L-type Ca\(^{2+}\) channel (Ca\(_{1,2}\)) located in the plasma membrane and along the T-tubules, which mediates Ca\(^{2+}\) entry into cardiomyocytes. β-Adrenergic receptor activation increases the Ca\(^{2+}\) current via Ca\(_{1,2}\) channels and is responsible for the positive ionotropic effect of adrenergic stimulation. Despite decades of research, the molecular mechanism underlying this modulation has not been fully resolved. On the contrary, initial reports of identification of key components in this modulation were later refuted using advanced model systems, especially transgenic animals. Some of the cardinal debated issues include details of specific subunits and residues in Ca\(_{1,2}\) phosphorylated by PKA, the nature, extent, and role of post-translational processing of Ca\(_{1,2}\), and the role of auxiliary proteins (such as A kinase anchoring proteins) involved in PKA regulation. In addition, the previously proposed crucial role of PKA in modulation of unstimulated Ca\(^{2+}\) current in the absence of β-adrenergic receptor stimulation and in voltage-dependent facilitation of Ca\(_{1,2}\) remains uncertain. Full reconstitution of the β-adrenergic receptor signaling pathway in heterologous expression systems remains an unmet challenge. This review summarizes the past and new findings, the mechanisms proposed and later proven, rejected or disputed, and emphasizes the essential issues that remain unresolved. (Circ Res. 2013;113:617-631.)

Key Words: adrenergic ■ calcium channels ■ cyclic AMP-dependent protein kinases ■ heart ■ molecular mechanism

Contraction and relaxation of the heart are regulated by its electric activity. L-Type voltage–dependent Ca\(^{2+}\) channels (L-VDCC), predominantly of the Ca\(_{1,2}\) type, are the main Ca\(^{2+}\) entry pathway in ventricular cardiomyocytes; L-VDCCs of the Ca\(_{1,3}\) type prevail in pacemaker cells and embryonic myocytes. Ca\(^{2+}\) entry activates the ryanodine receptors (RyR), rapidly elevating Ca\(^{2+}\) concentration through calcium-induced calcium release, which triggers contraction. Heartbeat is tightly regulated by autonomic and hormonal control. Activation of the sympathetic nervous system, for example, in the fight-or-flight response, increases the heartbeat rate, contractility, and relaxation rate (positive chronotropic, inotropic, and lusitropic effects, respectively). This is caused by catecholamine binding to β-adrenergic receptors (β-AR), which are G-protein–coupled receptors that trigger GDP–GTP exchange at the stimulatory G-protein subunit G\(_{\text{S}}\). Activated G\(_{\text{S}}\)-GTP separates from GDP and activates adenyl cyclase (AC), resulting in elevated cyclic AMP (cAMP) levels and activation of the cAMP-dependent protein kinase (protein kinase A [PKA]), which phosphorylates multiple target protein, among them L-VDCC, troponin I, and phospholamban.

Mammalian cardiomyocytes express 3 β-AR subtypes, β1- to β3-AR, primarily the β1-AR (≈80%) and substantially β2-AR. β1-AR is coupled exclusively to G\(_{\text{S}}\), whereas β2-AR is coupled to both G\(_{\text{S}}\) and the inhibitory Gi/o. Activity of β1-AR, located at the plasma membrane, produces elevation in cAMP throughout the cell, whereas β2-AR produces spatially restricted cAMP increase, probably close to the T-tubules. 

Increased Ca\(^{2+}\) entry into the myocytes via the cardiac Ca\(_{1,2}\), mediated by both β1- and β2-AR, substantially contributes to the positive inotropic effect of β-AR activation (Figure 1). This process is central to normal cardiac physiology and is involved in pathophysiological changes occurring in cardiac hypertrophy and heart failure. Detailed characterization of the β-AR–activated, PKA-mediated regulation of L-VDCC is, therefore, crucial for understanding normal and pathological cardiac physiology. Moreover, neuronal Ca\(_{1,2}\) is regulated by PKA similarly to cardiac Ca\(_{1,2}\), although isoform-specific or regulatory protein-specific details may vary.

β-AR enhancement of cardiac Ca\(_{1,2}\) has been extensively studied for >30 years, and the accumulated evidence...
leaves no doubt that the β-AR effect is mediated via PKA. Remarkably, the actual molecular mechanism and details of PKA regulation of the channel remain unclear. Many experimental results and proposed mechanisms have not withstood the test of time. Fundamental issues remain unresolved.

1. Which L-VDCC subunit (α1C or β) is phosphorylated by PKA, mediating the functional regulation of the channel.
2. A definitive identification of specific residues involved in the functional effects of PKA is still lacking.
3. The role and extent of post-translational processing of CaV1.2 need to be better understood. The cleaved distal C terminus (dCT) of α1C seems essential for the β-adrenergic modulation of CaV1.2, yet it is not clear whether the full-length nontruncated channel is also modulated and how.
4. The role and importance of auxiliary proteins, in particular AKAP5 (AKAP79/150) and AKAP7 (AKAP15/18), remain unclear.
5. The complete β-AR cascade, where activation of the G-protein–coupled receptor regulates the channel, has never been reconstituted in heterologous systems. In the best case, the reconstituted cascade starts with the activation of AC.

In particular, major controversies arose from the attempts to reproduce the physiological observations in heterologous expression systems, which is crucial for detailed studies of molecular mechanisms of ion channel regulation. Recent studies in transgenic mice rejected accepted schemes that prevailed a decade ago and presented in prominent reviews. Some of the disputed findings and mechanisms are summarized in Table 1 and will be referred to throughout the article. This review focuses on achievements and controversies in our understanding of β-AR-PKA regulation of cardiac CaV1.2 and attempts to sort out the unresolved issues and to suggest directions for further research.

Nonstandard Abbreviations and Acronyms

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<th>Nonstandard Abbreviations and Acronyms</th>
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<td>AC</td>
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<td>AKAP</td>
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<td>CaV1.2</td>
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L-Type Calcium Channel

L-VDCCs (CaV1.1–CaV1.4) play critical roles in excitation–contraction coupling in cardiac, skeletal, and smooth muscle, regulate Ca2+ homeostasis, secretion, tissue development, excitability, and excitation–transcription coupling, and are involved in learning and memory in the brain. L-VDCCs are multisubunit proteins containing, as a minimum, α1 (the pore-forming subunit) and the auxiliary CaV1.2, β and δ subunits. α1 is composed of 4 homologous transmembrane domains and cytoplasmic N terminus (NT) and C terminus (CT). The CACNA1C gene encodes the α1 subunit of CaV1.2, α1C, which is expressed in cardiac and smooth muscle and in the brain; multiple isoforms are present because of alternative splicing. Furthermore, post-translational proteolytic cleavage of α1C yields a dCT-truncated α1C form of ≈210 kDa, which in most cases can be detected along with a full-length ≈240-kDa protein. The site of proteolytic cleavage in the homologous α1S of the skeletal muscle, Ala1664, has been determined with high precision. The corresponding residue in α1C of the cardiac CaV1.2 is Ala1800 (Figure 2), predicting a ≈35-kDa cleaved dCT polypeptide. The cleaved dCT seems to remain associated with the main α1C, tonically inhibiting it.

The CaV1.2 subunit binds with high affinity to an α1C interaction domain in loop 1 (Figure 2), and it is profoundly important for VDCC biogenesis and regulation. It elevates macroscopic Ca2+ current (ICa) by increasing surface density of α1C and the open probability (P0), regulates inactivation, and enhances activation by shifting the current–voltage curve (I–V curve) to hyperpolarized voltages.

β-AR stimulation and PKA activation increase ICa via CaV1.2 in cardiomyocytes ≈3-fold, mainly by increasing
Table 1. Pros and Cons of Contested Findings and Mechanisms of β-AR Modulation

<table>
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<tr>
<th>Study</th>
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<tr>
<td><strong>191</strong></td>
<td>Xenopus oocytes expressing full-length cardiac (\alpha_{1C})</td>
<td>Injected cAMP enhanced (I_{\text{Ca}}) only when (\beta)-subunit was coexpressed. Mechanism: β subunit is essential for PKA regulation.</td>
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<td>192,144 Xenopus oocytes</td>
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<td><strong>169,168</strong></td>
<td>CHO cells (CCAR2317 line) transfected with full-length (\alpha_{1C}) without the β subunits or AKAP proteins</td>
<td>Two species of (\alpha_{1C}) were found: 250 and 200 kDa. cAMP analogs or PKA catalytic subunit greatly increased (I_{\text{Ca}}) and phosphorylation of the 250-kDa (\alpha_{1C}), but not 200-kDa (\alpha_{1C}). The effect was blocked by okadaic acid. Mechanism: full-length (\alpha_{1C}) subunit is sufficient to mediate effect of PKA.</td>
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<td>173</td>
</tr>
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<td><strong>164</strong></td>
<td>HEK cells transfected with full-length (\alpha_{1C}), (\beta_{2}) and AKAP5 when desired</td>
<td>Forskolin enhanced WT (\alpha_{1C}) (I_{\text{Ca}}) (but not the (\alpha_{1C}) mutant Ser1928Ala) although by &lt;50%, only in the presence of AKAP5. Mechanism: full-length (\alpha_{1C}) subunit is sufficient for PKA modulation</td>
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<td>26</td>
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<td><strong>77</strong></td>
<td>tsA cells (a HEK-derived line) transfected with a dCT-truncated (\alpha_{1C}) (at a.a. 1904) and (\beta_{2}) without AKAPs</td>
<td>Identified Ser478 and Ser479 of the (\beta_{2}) subunit as sites essential for the PKA enhancement of (I_{\text{Ca}}). Mechanism: phosphorylation of (\beta_{2}) subunit mediates increase in (I_{\text{Ca}})</td>
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<td>173</td>
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<td><strong>170</strong></td>
<td>BHK6 (hamster kidney cell line) stably expressing Ca(\text{a}<em>{\beta}) and (\alpha</em>{2\delta}) transfected with (\alpha_{1C})</td>
<td>Forskolin enhanced currents by ≈50% and PKI abolished forskolin-mediated enhancement. A mutation of Ser1928 to Ala also abolished forskolin-mediated enhancement. Mechanism: phosphorylation of Ser1928 on (\alpha_{1C}) mediates increase in (I_{\text{Ca}}).</td>
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<td>24</td>
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</table>

AKAP indicates A kinase anchoring protein; β-AR, β-adrenergic receptors; dCT, distal C terminus; \(I_{\text{Ca}}\), Ca\(^{2+}\) current; Ca\(_{1.2}\), L-type Ca\(^{2+}\) channel PKA, protein kinase A; PKI, heat-stable PKA-inhibiting protein; and WT, wild type.
the channel’s $P_o$ because of a shift of Ca$^{2+}$ channel gating from inactive mode (mode 0) and a low $P_o$-mode (mode 1) to mode 2 characterized by high $P_o$ with clusters of long and frequent openings. A similar modal shift also occurs after activation of Ca-/calmodulin-dependent kinase II (CaMKII), coexpression of the CaVβ subunit, and during voltage-dependent facilitation (VDF) after strong depolarization (see below). The mechanistic and structural basis of phenomenologically similar changes in gating, caused by diverse regulatory agents, is not known and presents a fascinating challenge for future research. β-AR stimulation and PKA also increase channels’ sensitivity to depolarization, as reflected in a hyperpolarizing shift in the I–V curve. In addition, β-adrenergic modulation of CaV1.2 reduces voltage-dependent and enhances Ca$^{2+}$-dependent inactivation.

### Criteria for Regulation of Target Proteins by PKA-Mediated Phosphorylation

In view of the difficulties in determining the mechanism of β-AR-PKA regulation of CaV1.2, we feel that it is important to reiterate the criteria for establishing that the regulation is mediated by phosphorylation of the target protein. Classical criteria (here briefly summarized as 1–3) were formulated in the 1980s, and others have become accepted in research practice with the advance of pharmacology and molecular genetics.

1. The first messenger/agonist should activate AC and cause a rise in intracellular cAMP in cells.
2. Exogenously added cAMP and PKA catalytic subunit (PKA-CS) should mimic the effects of the agonist. Dephosphorylation should reverse the functional effects of PKA.
3. The target protein should be a substrate for PKA-CS, and dephosphorylated by a protein phosphatase.
4. The requirement for inhibition of the physiological response (in our case, increase in I_\text{ca}) by specific PKA inhibitors is becoming consistently imposed.
5. Putative PKA phosphorylation sites should be identified in the primary protein sequence and confirmed using biochemical methods.
6. The reconstitution criterion: to confirm that the putative phosphorylation sites (and any auxiliary proteins) are physiologically relevant, the β-AR/cAMP/PKA regulation of the target protein should be reconstituted in a heterologous model cell system when all the participating proteins are expressed. Elimination of phosphorylation site(s) in the protein by mutagenesis should abolish modulation by PKA.

For many physiological regulations, the role of phosphorylation has been established by fulfilling most or all of the above criteria. However, in the specific case of Ca_\text{v1.2}, criterion 6 turned out to be difficult to fulfill and the results proved controversial. Therefore, recent studies have been using an additional, particularly rigorous criterion:

7. Transgenic animals or manipulated native cells expressing the target protein lacking putative phosphorylation sites (or auxiliary proteins proposed to be crucial for the regulation) should not exhibit the physiological response to PKA and adrenergic stimulation.

These guidelines are by no means stiff or obligatory and are presented to simplify the reading of this review and, potentially, to provide a flexible framework to guide future studies.

The Classical Period: β-AR Regulation of L-Type VDCC is Mediated by PKA

PKA is a tetramer of 2 regulatory (PKA-R) and 2 catalytic subunits. When inactive, the regulatory and catalytic subunits are bound, and on cAMP addition PKA-R binds cAMP and PKA-CS is released. This PKA holoenzyme + cAMP ↔ (PKA – CS) + (PKA – R – cAMP). When PKA is active, it has a lower affinity for cAMP and dissociates into PKA-R and PKA-CS. PKA can also undergo autophosphorylation, which can increase its activity.

Early works, corroborated by many recent studies, found a β-AR–induced activation of AC followed by a rise in cAMP in the heart and in cultured cardiomyocytes, showed enhancement of L-VDCC currents in cardiomyocytes by cAMP, PKA-CS, phosphodiesterase, and protein phosphatase inhibitors, and demonstrated negation of these effects by protein phosphatases. Purified PKA-CS has been shown to activate purified Ca_\text{v1.1} (the L-VDCC of the skeletal muscle) and cardiac Ca_\text{v1.2}, after reconstitution in lipid bilayers and liposomes, suggesting that the target for PKA regulation is the channel itself. These studies satisfied criteria 1 and 2 listed above.

Both α_\text{IC} and β Subunits are Phosphorylated by PKA

Once Ca_\text{v1.2} subunits were cloned and splice variants analyzed, several putative PKA phosphorylation sites have been identified in α_\text{IC} and Ca_\text{v1.2} subunits. Initial studies did not observe PKA phosphorylation of α_\text{IC} in membranes of cardiomyocytes or after partial purification of the protein. However, in 1996, Ser1928 (numbering by rabbit α_\text{IC}) was identified as the main PKA site in α_\text{IC}. Ser1928 was found to be the only site phosphorylated by PKA in vitro in rabbit heart α_\text{IC}, using site-specific antibodies and by monitoring direct PKA phosphorylation of α_\text{IC} expressed and purified from Saccharomyces cerevisiae. Finally, phosphorylation of Ser1928, in the full-length α_\text{IC}, only, in response to β-AR stimulation was confirmed in vivo in intact cardiomyocytes using site-specific antibodies. Moreover, studies that used biochemical and recombinant DNA methods also identified Ser1928 as a strong substrate for PKC and for CGMP-dependent protein kinase, and also for protein kinase D (formerly called PKCp).

Recently, Emrick et al identified Ser1575 and Thr1579 of Ca_\text{v1.1} (α_\text{IC}) as in vitro and in vivo targets for PKA in the skeletal muscle and then demonstrated the phosphorylation of the homologous Ser1700 and Thr1704 in cardiac α_\text{IC}.

Ca_\text{v1.2} is phosphorylated both in vitro by PKA-CS on Ser459, Ser478, Ser479, and Ser296 (numbering by rabbit α_\text{IC}) in response to β-AR stimulation. In summary, these studies satisfied criteria 3 and 5: α_\text{IC} and β subunits of Ca_\text{v1.2} are PKA targets; the functional importance of the various phosphorylation sites is discussed below.

PKA Inhibitors and β-AR Regulation

The use of protein kinase inhibitors (criterion 4 above) is both an essential step and a potent diagnostic tool in establishing the involvement of a protein kinase in a biological regulation. In principle, the functional effect of a kinase can be mediated either by phosphorylation of the target protein or by a direct interaction with it. A phosphorylation mechanism can be strongly supported by the use of inhibitors acting at the active (catalytic) site of the kinase. In contrast, inhibitors of kinase activation, for example, cAMP analogs such as adenosine-3’,5’-cyclic phosphorothioate (Rp-cAMPS), which prevent dissociation of PKA subunits, cannot discriminate between the 2 mechanisms. Unfortunately, most synthetic inhibitors acting at the evolutionary conserved catalytic site, such as the widely used H-89, are not entirely PKA specific. For PKA, a widely used choice is the highly specific protein inhibitor initially purified from rabbit skeletal muscle and heart, termed heat-stable cAMP-dependent protein kinase inhibitor, or heat-stable PKA-inhibiting protein (PKI). PKI and PKI-derived peptides not only inhibit the catalytic activity of PKA by interacting with the PKA-CS but also suppress the dissociation of PKA-CS from PKA-R. The K_i of PKI for PKA-CS is ≈0.5 to 5 nmol/L. However, proteolysis of PKI and PKI-derived peptides in living cells is common. Also, in functional studies, the inhibitor’s final concentration within the cell and in the channel’s microenvironment is difficult to estimate. This often serves an excuse for using high doses of inhibitors to obtain positive results (inhibition of physiological regulation). Such results must be interpreted with caution because of limited specificity of most inhibitors. Moreover, in view of batch variability and potential loss of potency of many compounds in transportation and storage, negative
results (absence of a compound’s effect) need to be verified by demonstrating an effect of the same batch of the compound on an established target.

The β-AR-induced increase in I\textsubscript{\text{Ca}} is highly sensitive to PKA inhibitors. Direct intracellular perfusion (dialysis) of the myocytes through the patch pipette often yielded incomplete inhibition of the β-AR effect: 50% to 70% with 1 μmol/L PKI, 0.5 mmol/L Rp-cAMPS, or a cocktail of PKA and phosphorylation inhibitors.87–90 Other works reported full inhibition of effects of β-AR agonists in frog and rat ventricular cardiomyocytes by dialyzing cells with 2 to 5 mmol/L Rp-cAMPS, 16 μmol/L PKI (which is >3 orders of magnitude higher than its K\textsubscript{i}), 20 μmol/L PKI peptide (PKI\textsubscript{40–44}, K=36 mmol/L),91–93 or with 1 μmol/L H-89 applied externally.84 Although the latter studies used rather high doses of inhibitors, the accumulated data strongly support the view that a major part and possibly all of the β-AR enhancement of cardiac Ca\textsubscript{i,1.2} is mediated by activation of PKA.

Nevertheless, initial reports of a residual β-AR enhancement of I\textsubscript{\text{Ca}} in the presence of PKA inhibitors prompted a search for additional mechanisms. Yatani et al\textsuperscript{95,96} and Pelzer et al\textsuperscript{97} reported a PKI-resistant enhancement of Ca\textsubscript{i,1.2} activity in excised patches of cardiomyocytes by the nonhydrolyzable GTP analog GTP\textsubscript{yS} and by GTP\textsubscript{yS}-activated G\textsubscript{\gammaS}, Yatani and Brown\textsuperscript{98} proposed that a fast phase of the β-AR regulation of L-type Ca\textsuperscript{2+} channels is mediated by a direct, membrane-delimited effect of G\textsubscript{\gammaS}, whereas a slow phase is mediated by the PKA phosphorylation. In contrast, using a fast perfusion system, Hartzell et al\textsuperscript{99} and Méry et al\textsuperscript{100} observed a monophasic slow isoproterenol (Iso) effect which was fully blocked by PKA inhibitors, both in frog and mammalian cardiomyocytes, and refuted the idea of any physiological role for direct regulation. Yatani et al\textsuperscript{101} or of channel rundown in ATP-free solutions used.89,90

**Is PKA Involved in Basal I\textsubscript{\text{Ca}} or Rundown of Ca\textsubscript{\text{i,1.2}}?**

**Basal I\textsubscript{\text{Ca}} in the Absence of Hormonal Regulation**

Extracellular application of PKI-containing liposomes blocked slow action potentials and the effect of Iso\textsuperscript{102} in cardiomyocytes. This led Sperelakis et al\textsuperscript{103,104} to propose the hypothesis, which became quite popular and was supported by studies in other cell types\textsuperscript{105} that the L-VDCC needs to be basally phosphorylated by PKA to become functional. However, smaller or no effects on basal I\textsubscript{\text{Ca}} were seen in later works that used cell dialysis of inhibitors through the patch pipette or extracellular incubation with cell-permeable inhibitors. The results are divergent, from no effect of Rp-cAMPS (0.1–2 mmol/L in the pipette) and H-89 in adult mouse cardiomyocytes\textsuperscript{89,106,107} through a minor ≈20% reduction in basal I\textsubscript{\text{Ca}} by dialysis of purified PKI protein (2.9 μmol/L) in adult guinea pig myocytes,\textsuperscript{88} to 20% to 55% inhibition in late versus early embryonic mouse cardiomyocytes, respectively, after extracellular application of 15 μmol/L of a cell-permeable PKI peptide.\textsuperscript{72} Interestingly, reduction in basal I\textsubscript{\text{Ca}} caused by PKA inhibitors\textsuperscript{72} or protein phosphatase PP2A\textsuperscript{106} was correlated with a decrease in phosphorylation of Ser1928, monitored with a site-specific antibody.\textsuperscript{72,106} In myocytes of atrium and sinoatrial node PKA inhibitors reduced basal I\textsubscript{\text{Ca}} by 25% to 36%,\textsuperscript{107,108} but the contribution of Ca\textsubscript{\text{i,1.3}} cannot be excluded. In all, the accumulated evidence suggests a minor role for PKA in the maintenance of basal I\textsubscript{\text{Ca}} in healthy adult ventricular cardiomyocytes.

And yet, basal I\textsubscript{\text{Ca}} is strongly enhanced by phosphatase inhibitors.81 Therefore, phosphorylation must play a role unless phosphatases regulate the channel exclusively by direct interactions. Unlike the effect of Iso, the enhancement of basal I\textsubscript{\text{Ca}} by phosphatase inhibitors is not blocked by PKA inhibitors.92,104 Thus, for basal I\textsubscript{\text{Ca}}, a phosphatase(s) seems to oppose phosphorylation by kinases other than PKA. PKC and CaMKII have been proposed as candidates. CaMKII inhibitors reduced basal activity of Ca\textsubscript{\text{i,1.2}}\textsuperscript{109} but this was not observed by others.\textsuperscript{104,110} Navedo et al\textsuperscript{111,112} demonstrated PKC-dependent constitutive activity of Ca\textsubscript{\text{i,1.2}} in smooth muscle within spatially organized clusters. In adult ventricular cardiomyocytes, PKC inhibitors Ro 31-8220 and Gö 6976 decreased steady-state I\textsubscript{\text{Ca}} and also blunted the effect of a phosphatase inhibitor\textsuperscript{104}; however, a PKC inhibitory peptide was without effect.92 In all, basal I\textsubscript{\text{Ca}} in mammalian cardiomyocytes is regulated by a balanced action of serine/threonine kinases and phosphatases\textsuperscript{104,106,113}, the kinases involved remain to be identified. Notably, phosphorylation and dephosphorylation also regulate the level of the membrane phosphatidyl inositol biphosphate, a ubiquitous regulator of ion channels and transporters.114 Phosphatidyl inositol biphosphate is involved in the maintenance of the basal I\textsubscript{\text{Ca}} of some VDCCs,\textsuperscript{115–117} and its role in cardiac Ca\textsubscript{\text{i,1.2}} awaits further study.

**Rundown**

Regulation of basal I\textsubscript{\text{Ca}} of L-VDCCs by PKA has been initially linked to the phenomenon of rundown—a decrease in channel activity with time during recording in dialyzed cells and in particular in excised patches. Rundown is not observed in cell-attached patches or sharp-electrode recordings that do not involve dilution of cell interior, indicating that nonmembrane ingredients are involved.101 Channel proteolysis and (de)phosphorylation have been the most widely proposed potential mechanisms. ATP slows the rundown, but the nonhydrolyzable ATP\textsubscript{yS} does not prevent rundown; therefore, dephosphorylation cannot be the only cause.101 Initially, cAMP and PKA-CS were reported to reverse rundown of L-VDCCs in cardiomyocytes and other cells.103,118,119 However, some of these results could be because of PKA-induced activation of Ca\textsubscript{\text{i,1.2}} by a mechanism unrelated to the rundown. Later works showed that rundown can be reversed by cytosolic extracts from cardiomyocytes and other cells. Calpastatin (an endogenous inhibitor of the protease calpain) together with additional high molecular weight cytosolic factors\textsuperscript{120,121} or the addition of calmodulin together with ATP has been reported to prevent the rundown of Ca\textsubscript{\text{i,1.2}}\textsuperscript{110,122,123} (calmodulin is VDCC regulator, which is constitutively associated with Ca\textsubscript{\text{i,1.2}} and tightly regulates its function both directly and via CaMKII).124 A recent study in neurons demonstrated Ca\textsuperscript{2+}-dependent rundown of Ca\textsubscript{\text{i,1.2}} through endocytosis mediated largely by Ca/...
calmodulin-induced displacement of α-actinin.\textsuperscript{125} It seems that the rundown is not the opposite of the regular basal $I_{Ca}$, but the result of a collapse of the multiprotein complex within which the channel operates and of the phosphorylation–dephosphorylation balance of the channel or associated proteins.

Reconstitution of PKA Regulation in Heterologous Models and Rigorous Tests of Proposed Mechanisms in Transgenic Animals

Proteolytic Cleavage of dCT of $\alpha_{1C}$

The dCT seems to be a critical structural element in PKA regulation. Transgenic mice expressing $\alpha_{1C}$ truncated at amino acid (a.a.) 1820 or 1905 (rabbit $\alpha_{1C}$ count) died at birth; embryonic cardiomyocytes showed a greatly reduced $I_{Ca}$, probably because of enhanced Ca\textsubscript{v}1.2 degradation, heart hypertrophy, and loss of β-AR regulation of the remaining small $I_{Ca}$.\textsuperscript{126,127}

Although the latter result supports a role for dCT in β-AR regulation, it is uncertain whether other β-adrenergic regulations and vital functions were not disrupted in these defective hearts.

dCT also serves as a transcription factor that migrates to the nucleus and autoregulates the expression of $\alpha_{1C}$ in neurons, cardiomyocytes, and smooth muscle.\textsuperscript{41,128,129} In heart and smooth muscle, the dCT reduces the expression of $\alpha_{1C}$.\textsuperscript{41,129}

In the heart, blockade of Ca\textsubscript{v}1.2 results in a dCT-dependent protein upregulation which is regulated by cytoplasmic Ca\textsuperscript{2+} concentrations.\textsuperscript{129–132}

Heterologous expression in nonexcitable cells normally yields only the full-length, and completely functional, $\alpha_{1C}$ protein.\textsuperscript{44,133–136} However, in some works, both full-length and truncated $\alpha_{1C}$ can be seen in transfected human embryonic kidney (HEK) cells.\textsuperscript{41,128} Removal (by mutagenesis) of the dCT from heterologously expressed $\alpha_{1C}$ increases current amplitude,\textsuperscript{127,138} and coexpression of the dCT as a separate peptide reverses this effect and reduces $I_{Ca}$. Removal of dCT affects Ca\textsubscript{v}1.2 gating similarly to PKA: it causes a hyperpolarizing shift in the I–V curve and increases its $P_{o}$.\textsuperscript{44,137,139}

Accordingly, it has been proposed that the dCT folds over another part of the channel to inhibit activity, and that phosphorylation–dephosphorylation balance of the channel or associated proteins.

### Table 2. Proteolytic Processing of dCT of $\alpha_{1C}$

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<thead>
<tr>
<th>Study</th>
<th>Preparation</th>
<th>% of dCT Truncated-$\alpha_{1C}$</th>
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<tr>
<td>37</td>
<td>Rat brain</td>
<td>&gt;60% truncated</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>Rabbit cardiomyocytes</td>
<td>≈80% truncated</td>
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<td>36</td>
<td>Rabbit cardiomyocytes</td>
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<td>By calibration of antibodies and comparison with HEK cells</td>
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<tr>
<td>193</td>
<td>Rat fetal and adult ventricles</td>
<td>No or minor truncation</td>
<td></td>
</tr>
<tr>
<td>129</td>
<td>Adult and embryonic mouse cardiomyocyte</td>
<td>Fully truncated</td>
<td></td>
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<tr>
<td>15</td>
<td>Rat heart</td>
<td>50% truncated</td>
<td>\textsuperscript{15}, Figure 7</td>
</tr>
<tr>
<td>41</td>
<td>Rat and human cerebral arteries</td>
<td>≈30% truncated</td>
<td>\textsuperscript{41}, Figure 1B</td>
</tr>
</tbody>
</table>

dCT indicates distal C terminus.

β-Adrenergic Regulation of L-Type Ca\textsuperscript{2+} Channel

In the majority of popular heterologous expression systems, even when no positive PKA regulation of Ca\textsubscript{v}1.2 was reported, introduction of PKI and less specific inhibitors or protein phosphatases usually reduced $I_{Ca}$.\textsuperscript{42,144–146} In Xenopus oocytes, Rp-cAMPS and H-89 reduced basal $I_{Ca}$ by 20% to 30%, mutating Ser1928 to Ala reduced basal $I_{Ca}$ and abolished the regulation by PKA inhibitors.\textsuperscript{147} Similarly, Ser1928...
sustained basal ICa in heterologously expressed human α1C in HEK cells.75 Disruption of AKAP-PKA interaction by a specific peptide, Ht-31, reduced basal ICa of CaV1.2 expressed in HEK cells, suggesting a role for an unidentified endogenous AKAP. Notably, this effect of Ht-31 was absent when Ser1928 was mutated to Ala.148 Thus, it seems that phosphorylation of Ser1928 by one or several kinases, maybe in conjunction with an AKAP regulation, plays a role in sustaining basal ICa of full-length α1C in heterologous systems. Also, phosphorylation of Thr1704 has been implicated in basal ICa in HEK cells.76 The physiological relevance of these findings is unclear because the role of PKA in basal ICa may be different in cardiomyocytes. The option that phosphorylation of Ser1928 may be related to the basal regulation by other protein kinases has not been studied sufficiently.

**Reconstitution of the VDF of CaV1.2**

Ca2+ entry during an action potential or a depolarizing pulse induces Ca2+-dependent inactivation of L-VDCCs.149 In cardiomyocytes, there is also an opposite process referred to as facilitation or positive staircase, whereby the amplitude of Ca2+ current increases in a manner dependent on stimulus strength and frequency.150,151 Facilitation is believed to contribute to the increase in Ca2+ influx during increased cardiac work, and probably comprises several components. One component is a Ca2+-dependent facilitation, which involves CaM and, at least in part, CaMKII.124,153,154 A second, Ca2+-independent mechanism, called VDF, takes place when either Ba2+ or Ca2+ carry a depolarizing prepulse because of a shift to mode 2 gating.54,155

VDF in neuronal (CaV2.1) channels is mainly because of depolarization-induced dissociation of G-protein Gγ subunits from the α1C subunit of the channel (reviewed in Dolphin et al156 and Tedford et al157). However, the mechanism of VDF of CaV1.2 is not well understood and does not seem to involve interaction with G-protein subunits.158

Sculthorpeanu et al reported the reconstitution of CaV1.2 VDF in a CHO-derived CCAR3217 cell line expressing the full-length α1C without αδ or CaV1.2αβ. VDF required active PKA, was abolished by PKI and was occluded by long exposures to PKA. The authors proposed that VDF is fully dependent on a voltage-regulated PKA phosphorylation and dephosphorylation of α1C. Reconstitution of VDF in heterologously expressed CaV1.2 was confirmed by other groups; however, the results and conclusions were often controversial. In Xenopus oocytes, depolarizing prepulses increased ICa ≤3-fold with no need for cAMP or PKA injection; this effect was partially blocked by H-7 (a broad protein kinase inhibitor), PKI, and Rp-cAMPs and unaffected by cAMP or PKA-CS.159,160 Bourinet et al159 proposed that only PKA-phosphorylated channels are subject to VDF rather than PKA phosphorylation is voltage-dependent. Dai et al160 reconstituted VDF in HEK cells and conclusively demonstrated that, in this preparation, it is not affected by the presence or block of PKA or by the addition of AKAP5 and equally occurs with either full-length or dCT-truncated α1C. Somewhat paradoxically, VDF was inhibited by the αδ subunit160 rendering the physiological relevance of the reconstituted VDF less certain. Interestingly, VDF reconstituted in oocytes and HEK cells crucially depended on coexpression of the CaV1.2 subunit,18,159,161 although a weak, PKA-, PKI-, and G-protein–independent VDF was reported in CHO cells expressing the smooth muscle α1C, alone.162 In all, the bulk of reported results support a major role for the CaV1.2 subunit in the VDF of CaV1.2 reconstituted in heterologous systems. The reliance of VDF on CaV1.2 subunit, its independence of dCT and AKAPs, and insensitivity to PKA blockers does not support a crucial role for PKA. The exact molecular mechanism of VDF in CaV1.2 awaits further study.

**Regulation by PKA Activators or PKA-CS**

Numerous unsuccessful attempts were made during the past 2 decades to reconstitute the complete β-AR–induced regulation of L-VDCC (beginning with the application of an agonist) using heterologously expressed cloned (cDNA derived) receptors, channels, and other proteins of the signaling cascade. However, there are several reports of regulation of cloned CaV1.2 by the AC activator forskolin, cAMP or its analogs, or PKA-CS. The results are diverse, depicting various sites on different subunits of the channel as being crucial for regulation of CaV1.2 by PKA phosphorylation (Table 1).

In the majority of studies, the heterologously expressed full-length α1C, with or without coexpressed β and δ subunits, was not regulated by PKA-CS or PKA activators.42,144,145,163,164 It has been initially proposed that the expressed CaV1.2 is fully phosphorylated which explains why PKA cannot augment ICa, whereas inhibition of PKA or dephosphorylation by added phosphatases can reduce basal ICa.144,145 In a 1995 review, Charnet et al165 presented a summary of reconstitution attempts in Xenopus oocytes, CHO, and HEK cells. They particularly emphasized that, in contrast to channels expressed from separate CaV1.2 subunit gene products, ICa was enhanced 1.5- to 3-fold by β-AR stimulation when total cardiac RNA was injected into oocytes.166,167 Charnet et al165 proposed that an additional protein(s) (missing link) may be needed for the full reconstitution of the PKA effect.

Despite the above, several groups have reported reconstitution of PKA regulation in heterologous systems expressing full-length α1C (Table 1). A particular CHO-derived cell line constitutively expressing full-length α1C alone (CCAR3217) was used in 2 studies, which reported a large enhancement of ICa (>3-fold) by cAMP, dibutyryl-cAMP, and PKA-CS.168,169 Interestingly, 2 molecular sizes of the expressed α1C were observed, suggesting α1C cleavage or truncation in these cells. Activation of PKA increased phosphorylation of the full-length α1C (250 kDa) but not of the truncated form (200 kDa).169 The presence of endogenous auxiliary VDCC subunits or AKAPs in this cell line has not been characterized. In a baby hamster kidney (BHK6) cell line stably expressing CaV1.2 and transiently expressing α1C, forskolin enhanced ICa.170 The enhancement was blocked by PKI and by mutating Ser1928, although the forskolin-induced shift in the I–V curve persisted in the Ser1928Ala mutant.171 No data are available on the possible proteolytic cleavage of α1C in this system. In all, a spontaneous cleavage of the dCT and possibly the presence of endogenous AKAPs or other missing link proteins may have assisted the reconstitution of the PKA effect in these studies. As summarized in Table 1, the discrepancies between these and other works have never been resolved.
An influential and widely cited study reported the reconstitution of PKA regulation of Ca$_{1.2}$ containing the full-length $\alpha_{c}$, coexpressed with AKAP5 in HEK cells.$^{164}$ PKA activation by forskolin or a cAMP analog increased channel phosphorylation and caused a 50% increase in IC$_{a}$ and a shift in I–V curve (however, $\approx 50\%$ of the cells were irresponsive). This regulation was absent in the Ser1928Ala mutant of $\alpha_{c}$ or when AKAP5 was not coexpressed.$^{164}$ In a follow-up study in HEK cells, Bünnemann et al$^{172}$ reported a 2-fold PKA-CS–induced increase in IC$_{a}$ of $\alpha_{c}$ truncated at a.a. 1905 ($\alpha_{c}$Δ1905), missing both the Ser1928 and the DCRD. The regulation did not require AKAP but crucially depended on the presence of intact Ca$_{\beta_{2}}$ and was abolished by double mutation of the strong PKA phosphorylation sites in Ca$_{\beta}$, Ser478, and Ser479. The authors proposed that the phosphorylation of Ca$_{\beta}$ on these serines underlies most of the PKA-induced enhancement in IC$_{a}$, whereas the AKAP-dependent phosphorylation of Ser1928 in $\alpha_{c}$ mediates some of the increase in IC$_{a}$ and the shift in the I–V curve.$^{172}$ In contrast, subsequent studies were unsuccessful in reproducing the robust PKA-induced regulation of either full-length or dCT-truncated $\alpha_{c}$, even in the presence of AKAPs, in HEK cells or Xenopus oocytes. At best, small increases (15%–20%) in IC$_{a}$ were observed$^{160,161,172}$ (and our unpublished results). Despite these reservations, the 2 studies$^{77,164}$ have led to a prevailing concept of a molecular mechanism in which PKA, anchored at the CT of $\alpha_{c}$ via an AKAP, regulates Ca$_{1.2}$ by phosphorylating Ser1928 in $\alpha_{c}$ and 2 serines in Ca$_{\beta}$ after activation by cAMP.$^{181}$

Recent studies in cardiomyocytes refuted the role of Ser1928 and phosphorylation of the $\beta$ subunit but confirmed the necessity of the dCT (Table 1). In the first study,$^{173}$ adult cardiomyocytes were virally transfected with a mutated Ca$_{\beta_{2}}$ subunit lacking 2 prominent phosphorylation sites and 3 distinct GFP-labeled constructs of a dihydropyridine-insensitive $\alpha_{c}$: wild type (WT), a Ser1928Ala mutant, and the $\alpha_{c}$Δ1905-truncated channel. The Iso-induced increase in IC$_{a}$ was abolished in the $\alpha_{c}$Δ1905-$\alpha_{c}$, but persisted in Ser1928Ala-$\alpha_{c}$, suggesting that the dCT was essential for Iso-induced activation, but phosphorylation of Ser1928 was not.$^{173}$ Notably, the Iso-induced increase in IC$_{a}$ was $\approx 50\%$, substantially smaller than the $> 3$-fold increase of the endogenous IC$_{a}$ recorded in the same cells. This difference could be because of overexpression of the $\alpha_{c}$ subunit$^{171}$ or the use of Ca$_{\beta_{2}}$ instead of Ca$_{\beta_{2}A}$, which is the dominant Ca$_{\beta}$ in the heart.

A second study$^{30}$ analyzed the role of Ser1928 in vivo using transgenic mice with a knockin mutation of Ser1928Ala. $\beta$-AR regulation of heart rate and contraction, IC$_{a}$ density, and the I–V relationship, as well as Iso- or forskolin-mediated 2.5- to 3-fold increase in IC$_{a}$ were indistinguishable from the WT mice. Protein levels of $\beta$1-AR and PKA subunits, as well as their coimmunoprecipitation with Ca$_{1.2}$ (indicating the correct formation of the signaling complex), were also intact. Noteworthy, Western blots showed only a full-length 250-kDa $\alpha_{c}$, in both WT and Ser1928Ala mice.$^{31}$ This raises the interesting question whether the full-length $\alpha_{c}$ was the isoform involved in the $\beta$-AR regulation in these mice.

The crucial role of phosphorylation of Ca$_{\beta}$ by PKA regulation was also overturned by recent studies using transgenic mice and transfected cardiomyocytes. First, PKA-induced enhancement of IC$_{a}$ was preserved in cardiomyocytes infected with a Ca$_{\beta}$, lacking the crucial phosphorylation sites Ser459, Ser478, and Ser479.$^{174}$ Second, transgenic mice with Ca$_{\beta_{2}}$, truncated to remove PKA phosphorylation sites Ser478 and Ser479 (according to rabbit $\beta_{2}$ count) demonstrated an unaltered $\beta$-adrenergic stimulation of cardiomyocytes by Iso.$^{26}$ Finally, the effect of Iso in mice lacking both Ser1928 in $\alpha_{c}$ and the 2 strong phosphorylation sites of Ca$_{\beta_{2}}$ was the same as in the WT mice.$^{26}$ These results strongly suggest that PKA phosphorylation of neither Ser1928 in $\alpha_{c}$, nor the prominent serines 478 and 479 in Ca$_{\beta_{2}}$ are essential for the Iso-induced enhancement of IC$_{a}$.

The confusion from all of the data collected is rather overwhelming (Table 1). A recent seminal study by Fuller et al$^{42}$ may help resolve the conundrum. They reconstituted PKA modulation in tsA cells (an HEK-derived cell line) by expressing a complex of a dCT-truncated $\alpha_{c}$, the dCT as a separate polypeptide, $\alpha_{c}$$\delta$, Ca$_{\beta}$, and AKAP7. IC$_{a}$ of $\alpha_{c}$Δ1800 (truncated at a.a. 1800) was not enhanced by activated PKA irrespective of AKAP expression. In contrast, when $\alpha_{c}$Δ1800 was expressed together with the separate dCT, IC$_{a}$ was increased and Ser1700 became phosphorylated by forskolin-activated PKA, as well as by Ca$^{2+}$-activated CaMKII. The functional regulation of IC$_{a}$ required coexpression of AKAP7 in a precisely titrated range while avoiding overexpression. Analysis of phosphorylation site mutants indicated that Ser1700 and Thr1704 participate in sustaining basal IC$_{a}$, whereas Ser1700 is the site underlying the reduction of basal IC$_{a}$ was $\approx 50\%$, substantially smaller than the $> 3$-fold increase in IC$_{a}$ and the shift in the I–V curve. $^{30}$
of Ro 31-8220 for PKCα inhibition is 33 nmol/L; at 1 µmol/L, PKCα is inhibited by 97% and PKA by 70% in vitro. The concentration of 1 µmol/L of Ro 31-8220 was used in most experiments (eg, when directly probing the phosphorylation sites using site-specific antibodies and for the assessment of the role of the phosphorylation sites regulating the basal ICa). Although 50 µmol/L Ro 31-8220 had almost no effect on ICa, with 1 µmol/L of this blocker one may expect a more robust inhibition of various PKC isoforms. PKC may play a role in basal ICa (see above) and PKC activation increases ICa with a possible involvement of Ser1928; therefore, its role in some of the effects of Ro 31-8220 on basal ICa cannot be excluded.

**Requirement for AKAPs and Other Auxiliary Proteins in the β-Adrenergic Modulation of α1C**

cAMP/PKA compartments, or microdomains, are important in β-AR signaling. Microdomains may form in caveolae or membrane rafts, or within multiprotein complexes held together by scaffold proteins. AKAPs specifically tether the PKA-R dimers to a PKA substrate or microdomain, but they also act as scaffolds supporting multiple protein–protein interactions. The function of such complexes is controlled by many factors and includes feedback mechanisms achieved by interactions. The function of such complexes is controlled by many factors and includes feedback mechanisms achieved by phosphodiesterases, protein phosphatases, and additional factors.

Disruption of AKAP–PKA interactions with specific peptides inhibits PKA regulation of CaV1.2 in neurons, smooth muscle, and cardiomyocytes. The reduced β-AR modulation of cardiomyocytes from CaV1.2 dCT−/− transgenic mice (CT truncated at a.a. 1796) was correlated with reduced surface expression of CaV1.2 and localization of AKAP7. Reconstitution of cAMP-PKA activation and L-VDCC modulation in heterologous expression systems seems to require AKAPs. These finding strongly suggested a crucial role of AKAPs, in particular AKAP5 and AKAP7, in PKA regulation of CaV1.2. Yet again, recent studies with transgenic mice do not clearly support this notion.

First, knockout of AKAP5 did not abolish the β-AR regulation of CaV1.2. In fact, Icα was increased by Iso or forskolin to the same extent in cardiomyocytes from WT or AKAP5−/− mice. However, cardiomyocytes, which report the release of Ca2+ from the sarcoplasmic reticulum (SR) via RyR2, were not responsive to β-AR activation in the AKAP5−/− mice. This was probably because of the disruption of the AKAP5-containing signaling complex which comprises, in addition to AKAP, AC, PKA, protein phosphatase 2B, CaV1.2, and β-ARs. This complex generates a microdomain of cAMP that is juxtaposed to the RyR2, thus affecting the amplitude and duration of calcium transients. Interestingly, the authors observed that in wild-type myocytes, only CaV1.2 channels associated with caveolin-3 were phosphorylated by PKA (on Ser1928, as shown with a site-specific antibody) after β-AR stimulation. In the AKAP5−/− mice, noncaveolin-associated α1C was phosphorylated. These findings contradict an earlier observation that only β-AR is associated with caveolin-3 and CaV1.2; disruption of caveolae fully eliminated the β-AR regulation of Cav1.2 via β2-AR but not via β1-AR. The 2 works used different assays for the final effect of the β-AR stimulation, phosphorylation of Ser1928, or the increase in ICa which could partially account for the discrepant conclusions on the type of β-AR involved.

Even more strikingly, a full knockout of AKAP7 in mice, and even a double knockout of both AKAP5 and AKAP7, did not impair the β-AR regulation of CaV1.2, the phosphorylation of phospholamban and CaV1.2 itself on Ser1928, or the association of PKA-RII with CaV1.2 (Table 1). The authors did not detect compensation by several known AKAPs, but proposed that, in mice, an unidentified AKAP anchors PKA to the vicinity of CaV1.2 and phospholamban. It would be interesting to test whether PKA association with Cav1.2 is affected by AKAP5-D36 (which lacks PKA-R binding site, does not affect its expression, and retains functional binding sites of other regulatory proteins). Using AKAP5-D36 will most likely eliminate compensation by other AKAPs and will allow scrutinizing its role in this signaling cascade. In all, the recent studies with transgenic mice reveal an additional layer of complexity of CaV1.2 regulation in the β-AR cascade, and to date do not support an essential role for AKAP5 or AKAP7 for the β-AR and PKA-induced enhancement of CaV1.2 activity in the heart.

Another protein that was proposed to take part in the enhancing effect of PKA on L-VDCC is Ahnak, a 700-kDa protein that binds CaV1β. This interaction is reduced when Ahnak is phosphorylated by PKA. Application of an unphosphorylated mutated (Ile5326Thr) Ahnak fragment to native cardiomyocytes enhanced L-VDCC current by 60%. Haase proposed that PKA-catalyzed phosphorylation of Ahnak itself, or together with CaV1β, releases the inhibitory control imposed by Ahnak and allows CaV1β to interact with CaV1C. However, follow-up studies in Ahnak-deficient mice revealed that the responsiveness of the isolated transgenic hearts was similar to WT. Thus, Ahnak is not essential for the β-adrenergic stimulation of L-VDCC, but it may play a modulatory role.

**Concluding Remarks and Perspectives**
The textbook description of CaV1.2 regulation by β-ARs in the heart involves activation of cAMP, PKA, and eventually an enhancement of CaV1.2 current by phosphorylation of the channel’s subunits. Latest studies overturned previously accepted mechanisms and revealed unexpected complexities. Several points remain certain: much or all of the β-AR regulation of CaV1.2 in the heart is mediated by PKA; phosphorylation (probably not by PKA alone) and dephosphorylation play important roles in the regulation of intrinsic basal activity of the channel; phosphorylation of a cellular target by PKA must be a major event in channel regulation because β-AR effects are strongly attenuated by PKA inhibitors and enhanced by inhibitors of protein phosphatases. The target is strongly believed to be 1 or 2 of the channel subunits; however, the identity of the phosphorylation sites remains uncertain; phosphorylation of a missing link protein has not been excluded.

Biochemical and cell biology studies had unequivocally established Ser1928, located in the dCT of α1C, as a major
amino acid residue phosphorylated by PKA in the full-length, nontruncated α1C. Numerous studies indicate that Ser1928 is a plausible candidate for coordination of action of protein kinases and phosphatases at least in the regulation of basal channel activity. However, it seems to play no role in mediating the β-AR–induced enhancement of Ca2+ influx. The meaning and the function of the pronounced increase in phosphorylation of Ser1928 during the fight-or-flight β-adrenergic response remains an open question. Similarly, Caβ subunit is an excellent PKA substrate and has been repeatedly implicated in the β-AR regulation of CaV1.2; but these concepts have been overturned by studies in transgenic animals and cardiomyocytes. We do not know whether Caβ subunits participate in any way in the β-AR regulation.

The dCT, produced by the post-translational proteolytic cleavage of α1C, has a dramatic influence on channel activity and on β-AR regulation of CaV1.2. Therefore, it is important to identify the protease that cleaves α1C in the heart, and to estimate the extent and the regulation of channel proteolysis at the dCT in native heart, using scrupulous preparative protocols and new site-specific antibodies. It is important to examine whether prevention of the proteolytic cleavage in vivo, for example, by a mutation in the cleavage site in α1C, would eliminate the β-AR regulation of CaV1.2.

The newly proposed molecular mechanism15 that involves an intricate coordination of channel activity by its truncated dCT and the phosphorylation of Ser1700 and Thr1704 of α1C by PKA is supported by recent results from Catterall laboratory suggesting that transgenic mice with the double mutation of Ser1700 and Thr1704 in α1C and protein interaction inhibitors is a powerful and diversified approach to address these options.

It is usually assumed that all the effects of PKA-CS are mediated by phosphorylation of target residues in CaV1.2, but other possibilities such as a direct interaction have not been ruled out. Hypothetically, PKA may modulate CaV1.2 by a direct interaction or via an AKAP but in a phosphorylation-independent manner. There are many examples in different signaling pathways, including those regulating VDCCs. The phosphatase calcineurin has been shown to regulate CaV1.2 by a direct interaction16; CaMKII is activated after direct binding to CaV1.2. CaMKII directly interacts with several cytosolic segments of α1C, and was hypothesized to regulate the channel’s function.15 Extensive use of specific phosphorylation and protein interaction inhibitors is a powerful and discriminatory approach to address these options.

We now begin to understand the complexity of the β-AR-PKA-CaV pathway, which includes a large and intricate signaling complex of interacting proteins, many of which may affect the performance of the pathway. Experiments in transgenic mice may soon provide definite answers as to the role of unique phosphorylated amino acids proposed to mediate the regulation. In contrast, when addressing the roles of auxiliary proteins, compensation may compromise the insights from full-protein knockout approach. This could be the case with Ahnak, AKAP5, and AKAP7, and potentially other proteins that have not yet been sufficiently explored. Multiple and inducible knockouts may help to overcome compensation. We further propose that, within the β-AR-PKA-CaV pathway, there may be several subpathways that compensate each other when one of them is rendered non-functional. Such redundancy would safeguard the highly important β-AR regulation of heartbeat. Thus, the possibility remains that Caβ, Ahnak, AKAP5, and AKAP7 are key players in different subpathways, or they carry out modulatory functions in the yet unidentified main pathway.

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References


Adrenergic Regulation of the Ca Channel in the Guinea-pig Heart. Interaction with the Catalytic Subunit of the Protein Kinase.

The involvement of cAMP-dependent protein kinases.


of action of some commonly used protein kinase inhibitors.


duBell WH, Rogers TB. Protein phosphatase 1 and an opposing protein kinase regulate steady-state L-type Ca2+ current in mouse myocardial cells. J Physiol. 2004;556:79–93.


Hao LY, Xu JJ, Minobe E, Kameyama A, Kameyama M. Calmodulin kinase II activation is required for the maintenance of basal activity of...


Bénitah JP. ‘Ca(2+)-induced Ca(2+) entry’ or how the L-type Ca2+ channel remodels its own signalling pathway in cardiac cells. Prog Biophys Mol Biol. 2012;110:1661–1677.


Richard S, Perrier E, Fauconnier J, Perrier R, Pereira L, Goméz AM, Bénitah JP. ‘Ca2+-induced Ca2+ entry’ or how the L-type Ca2+ channel remodels its own signalling pathway in cardiac cells. Prog Biophys Mol Biol. 2006;90:118–135.


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Regulation of Cardiac L-Type Ca$^{2+}$ Channel CaV1.2 Via the β-Adrenergic-cAMP-Protein Kinase A Pathway: Old Dogmas, Advances, and New Uncertainties
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