The quest to identify the mechanism underlying adrenergic regulation of cardiac Ca\(^{2+}\) channels

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ABSTRACT
Activation of protein kinase A by cyclic AMP results in a multi-fold upregulation of Ca\(_V\)1.2 currents in the heart, as originally reported in the 1970’s and 1980’s. Despite considerable interest and much investment, the molecular mechanisms responsible for this signature modulation remained stubbornly elusive for over 40 years. A key manifestation of this lack of understanding is that while this regulation is readily apparent in heart cells, it has not been possible to reconstitute it in heterologous expression systems. In this review, we describe the efforts of many investigators over the past decades to identify the mechanisms responsible for the β-adrenergic mediated activation of voltage-gated Ca\(^{2+}\) channels in the heart and other tissues.

The phrase “fight or flight” was coined by Walter Bradford Cannon at Harvard Medical School in 1915 in his book Bodily Changes in Pain, Hunger, Fear and Rage [1]. He described how both physical trauma and psychological emergencies have the same effects on the body through the release of catecholamines (e.g. epinephrine and norepinephrine) into the bloodstream. He further described some of those effects including dilation of skeletal muscle blood vessels, constriction of skin blood vessels, and release of glucose into the bloodstream. Additionally, he and others described the physiologic effects on the heart, including increased heart rate, contractility and relaxation (chronotropy, inotropy and lusitropy, respectively). It was not until the discovery of the β-adrenergic receptor in the mid 1980’s by Robert Lefkowitz and his colleagues [2] that we began to understand how epinephrine and norepinephrine are able to exert those effects on the heart. The heart contains β\(_1\) and β\(_2\) adrenergic receptors. These G-proteincoupled receptors, once bound by epinephrine or norepinephrine, activate a G-protein signaling cascade that ends in activation of protein kinase A (PKA), which phosphorylates a number of proteins involved in excitation-contraction coupling to increase chronotropy, inotropy and lusitropy.

The cardiac L-type Ca\(^{2+}\) channel, also known as Ca\(_V\)1.2, plays a critical role in excitation-contraction coupling. The Ca\(_V\)1.2 channels are characterized by their high voltage-activating and slow inactivating properties. They contain at minimum a pore-forming subunit (α\(_{1C}\)) with 4 homologous transmembrane domains consisting of 6 transmembrane segments with a membrane-associated pore loop between transmembrane segments 5 and 6, and cytoplasmic N- and C-termini (Figure 1(a)). An auxiliary β subunit (β\(_{2B}\) in heart) binds to the α-interacting domain in the I–II loop with high affinity (Figure 1(a)) and plays roles in surface expression of α\(_{1C}\) and channel availability [3]. Normal cardiac excitation-contraction coupling occurs when initiation of the cardiac action potential activates Ca\(_V\)1.2 channels, which reside in the transverse tubules (T-tubules) of the cardiomyocyte [4–6]. This leads to Ca\(^{2+}\)-induced Ca\(^{2+}\) release whereby the ryanodine receptor releases Ca\(^{2+}\) from the sarcoplasmic reticulum (SR). This Ca\(^{2+}\) then binds troponin C, permitting the cross-linking of myofilaments. The majority of the Ca\(^{2+}\) is then removed from the cytosol via the sarcoplasmic-endoplasmic reticulum Ca\(^{2+}\)-ATPase.
(SERCA), modulated by phospholamban (PLB), and the Na⁺-Ca²⁺ exchanger.

Upon activation of the β-adrenergic signaling cascade, PKA is activated and directly phosphorylates several targets in the heart including the ryanodine receptor [7], troponin-I [8] and phospholamban [9], leading to increased Ca²⁺ release from the SR and thereby increased myofilament cross-linking, increased Ca²⁺ re-uptake into the SR, and increased myofilament relaxation. Importantly, activation of β-adrenergic receptors also leads to an increase in Caᵥ1.2 current and availability [10,11]. The mechanism through which β-adrenergic signaling exerts this effect has been the subject of debate. Activation of PKA is a universally accepted requirement for regulation of the channel [12–14], but relevant functional target sites have continued to been elusive for more than 40 years [15]. Although the obvious targets of phosphorylation are Caᵥ1.2 α or β subunits (Figure 1(a)) which have been the focus of most major studies to date, the data have been contradictory and controversial. Full reconstitution of the β-adrenergic receptor signaling pathway in heterologous expression systems remained an unmet challenge [16] implying critical gaps in our understanding of the process. Here, we provide a historical perspective on the quest to discover the mechanisms of adrenergic regulation of cardiac Caᵥ1.2, and describe how new technology led to a shift in that paradigm.

**Heterologous reconstitution of adrenergic regulation**

Numerous attempts have been made to reconstitute adrenergic regulation of Caᵥ1.2 by PKA activation in heterologous expression systems, done in a variety of models including Xenopus oocytes and various cell lines. Early attempts at reconstitution included work by Kameyama and Nakayama who solubilized cardiac Ca²⁺ channels into artificial lipid bilayers after mixing purified sarcolemma and membrane proteins with soybean phospholipid to form proteoliposomes [17]. They demonstrated that Ca²⁺ current from proteoliposomes incubated with activated PKA and Mg²⁺-ATP was 5 times larger than the controls, implying that activation of Caᵥ1.2 occurs via direct phosphorylation of a channel subunit or a protein that

![Figure 1. Schematic of Caᵥ1.2 and proposed model of β-adrenergic regulation of Caᵥ1.2. (a) Diagram showing rabbit cardiac α₁C and β₂B subunits. GK, guanylate kinase domain; SH3, Src homology 3 domain. (b) No adrenergic agonist – basal state model (left): Rad associates with Caᵥ1.2 β subunit and the plasma membrane, thereby inhibiting channel activity. Adrenergic agonist – stimulated state model (right): PKA phosphorylation of Rad reduces the affinity of Rad with the membrane and with the Caᵥβ subunit. Phosphorylated Rad leaves the “neighborhood” of the Ca²⁺ channel resulting in increased Ca²⁺ influx (green circles). Adapted from [59].](image)
remains closely associated with the channel. In oocytes, reconstitution of adrenergic regulation of CaV1.2 was accomplished by injection of total cardiac mRNA [18]. The injected mRNA encoded not only the α1C subunit, but also other protein(s) that could enable PKA regulation of CaV1.2 to occur. In retrospect, these studies may have revealed the importance of an as of yet unidentified protein in the regulation of CaV1.2 current. Other studies reported successful reconstitution simply using α1C and/or β subunits [19–22]. The concept that AKAPs, which bind and sequester PKA to specific subcellular locations, was also considered in these studies [23–26].

The above results were not without some controversy as researchers had difficulty reproducing reconstitution results. In an attempt to categorically identify essential protein components, Xenopus oocytes expressing cardiac CaV1.2 channels with various combinations of auxiliary subunits were studied [27]. No increase in current through α1C was shown after addition of cAMP or the catalytic subunit of PKA. Perez-Reyes et al. showed that only after PKA inhibition by H-89, a forskolin-mediated increase in current was observed in HEK cells co-transfected α1 and β2A subunits. This suggested a basal-state phosphorylation of the expressed channel or an associated protein in these cells [28]. In a comprehensive study of both CHO cells stably expressing α1C and HEK cells transiently expressing α1C and β2 subunits, neither inhibition of endogenous PKA, inhibition of protein phosphatases, or internal dialysis of PKA significantly affected peak barium current through α1C [29]. Mikala et al. suggested that α1C is unable to be regulated by phosphorylation in HEK cells after showing that a high concentration of H-89, a high concentration of the catalytic subunit of PKA, or 8-Br-cAMP had no effect on the activity of the wild-type channel [30].

A role for proteolytic cleavage of the C-terminus of α1C was also proposed as an essential process for adrenergic signaling. Different sizes of α1C have been detected in skeletal muscle [31,32], brain [33,34], and cardiac sarcolemmal membranes [35]. Truncation of 46% to 70% of the C-terminus of α1C results in increased activity of the channel [36], suggesting an inhibitory activity of this region. Cleavage of the C-terminus of α1C was proposed to be essential for PKA regulation of the channel because the release of the autoinhibitory action of the C-terminus by phosphorylation of Ser1700 was required [37]. Without cleavage, release of the autoinhibition could not occur. In the heart, however, C-terminal cleavage is absolutely not required for β-adrenergic stimulation of CaV1.2. We showed this by generating transgenic mice with inducible expression of proteolytic-resistant α1C [38].

Identifying and testing PKA phosphorylation sites

After the cloning of α1C [39], the sequence was subsequently analyzed for putative phosphorylation sites. Early site-specific identification efforts showed potential PKA phosphorylation of Ser1627 and Ser1700 [40]. Other suggested sites of regulation included Ser1829 [41] and Ser1142 [42] and Ser1928 [35,43]. The concept that Ser1928 mediates activation of CaV1.2 was further supported through heterologous expression of a Ser1928 mutant that prevented upregulation of CaV1.2 [23]. Other investigators failed to reproduce PKA modulation of Ca2+ currents in heterologous expression systems [29,30]. Phosphorylation sites have also been mapped to the Ca2+ channel β subunit at Ser459, Ser478 and Ser479 [44–46].

Given the challenges associated with heterologous expression systems and improvements in the development of genetically-altered mice, many investigators turned to ventricular myocytes to study mechanisms responsible for adrenergic regulation of CaV1.2. The dogma that phosphorylation of Ser1928 by PKA is required for β-adrenergic stimulation of CaV1.2 was tested by the O’Rourke group in 2006 through adenoviral gene transfer to express mutant α1C subunits in guinea pig cardiomyocytes [47]. Interestingly, an alanine point mutation at Ser1928 did not significantly reduce the response of α1C to isoproterenol. Two years later, Lemke et al. confirmed that phosphorylation of Ser1928 is not required for adrenergic regulation of CaV1.2 via generating a knock-in mouse with an alanine substitution at Ser1928 [48].
To test whether PKA phosphorylation of the β subunit was relevant in cardiomyocytes, β subunit with alanine substitutions at the three putative phosphorylation sites was expressed in cardiomyocytes. These sites were shown to be unessential for adrenergic stimulation of Cav1.2 [47,49]. As definitive proof that these sites were not required, a knock-in mouse expressing a β2 subunit truncated prior to these phosphorylation sites displayed normal PKA modulation of Cav1.2, thus ruling out involvement of any putative C-terminal phosphorylation sites in mediating β-adrenergic regulation of Cav1.2 [50].

As a heteromultimeric complex, Cav1.2 natively associates with a variety of auxiliary proteins, and several of these proteins have the ability of modulating channel activity. Neuroblast differentiation-associated protein ahnak has been shown to associate with Cavβ2 and this association decreases after PKA phosphorylation of both ahnak and the β subunit [51]. While it was initially proposed that phosphorylation and subsequent release of ahnak from β2 allowed for greater binding of β2 to α1C leading to enhanced Ca2+ current during adrenergic activation, later studies showed that ahnak-deficient cardiomyocytes displayed preserved adrenergic modulation of Cav1.2 [52].

**Additional phosphorylation sites**

After phosphorylation of Ser1928 was shown to be non-essential for β-adrenergic regulation of Cav1.2 in the heart, phosphorylation of Ser1700 and Thr1704 was proposed [37,53]. To expedite the process of studying multiple phosphorylation sites in mice, we developed a transgenic approach that enabled doxycycline-inducible expression of FLAG-tagged, dihydropyridine (DHP)-resistant Cav1.2 channels in mice [54]. The transgenic and endogenous Cav1.2 currents are distinguishable by application of nisoldipine, a Ca2+ channel dihydropyridine-antagonist [54]. This approach not only circumvented any potential developmental abnormalities caused by constitutive expression of the transgene, but also prevented high levels of basal transgenic current which might mask any observed adrenergic regulation of the channel [49,55]. We first generated transgenic mice with inducible expression of a DHP-resistant (T1066Y/Q1070M) N-terminal 3X FLAG-epitope-tagged α1C, designated pseudo-WT (pWT α1C). The resultant DHP-resistant Ca2+ current responded normally to adrenergic stimulation, nearly doubling after superfusion of isoproterenol in cardiomyocytes. By creating additional transgenic mice with alanine-substitutions of Ser1700 and Thr1704, residues proposed as essential phosphorylation sites based on experiments in tsA-201 cells [37], we found that phosphorylation of Ser1700 and Thr1704 is not required [54].

Independently, the Catterall group generated a mutant knock-in mouse line with alanine substitutions at Ser1700 and Thr1704 [56]. They reported that phosphorylation of Ser1700 alone or in combination with Thr1704 is required, basing their conclusion upon an unconventional metric: the difference in absolute current amplitude rather than the fold-increase after isoproterenol, which is the standard analysis. Their metric is valid only if the density of Ca2+ channels at the surface was unchanged, yet basal Ca2+ currents were substantially reduced [56,57]. Hofmann and colleagues subsequently created S1700A/T1704A knock-in mice and concluded that isoproterenol stimulated Ca2+ current in the control and mutant S1700A/T1704A cardiomyocytes to the same extent [58]. Furthermore, Hofmann’s group recalculated Catterall’s data and showed that in both groups’ knock-in mice, the β-adrenergic stimulation for wild-type and mutant channels were equivalent [58]. This confirmed our initial findings [54], which we have further substantiated with additional transgenic mice [59,60].

The failure to find any single α1C site as essential for β-adrenergic modulation of Cav1.2 led us to hypothesize that a combination of sites in α1C is required. After identifying conserved PKA consensus sequences in α1C of five species (mouse, rat, rabbit, guinea pig and human), we generated α1C transgenic mice (“22-mutant α1C”) in which we replaced 17 conserved consensus PKA phosphorylation sites that were not previously studied, and 5 conserved PKA/CaMKII phosphorylation sites known to be non-essential including Ser1700 and Thr1704 [35,47,50,54]. Surprisingly, we found that none were necessary [38]. This led us to then hypothesize that the functionally-relevant PKA targets in α1C might be different amongst
inhibit all high-voltage-activated Ca\(^{2+}\) channels [64,65], was decreased from the neighborhood of Ca\(_V\)1.2.

Rad-knockout mice display an increased maximum Ca\(^{2+}\) current, and the Ca\(^{2+}\) channels activate at lower voltages, mimicking the effects of \(\beta\)-adrenergic receptor stimulation [66,67]. Other studies, however, led to expectations that Rad is not directly involved in adrenergic regulation of Ca\(_V\)1.2 since adenoviral-induced overexpression of Rad [68] and Rem [69] in cultured cardiomyocytes ablated Ca\(_V\)1.2 and attenuated adrenergic regulation. The proximity labeling experiments suggested, however, that Rad was the missing link.

The robust heterologous reconstitution of PKA regulation of Ca\(_V\)1.2 currents has been long pursued as a crucial step in identifying and validating the mechanism. To reconstitute PKA regulation in a heterologous expression system, we carefully considered experimental conditions. We used perforated patch clamp techniques, to preserve the intracellular milieu, and a voltage ramp, rather than step protocols, which required the use of Ba\(^{2+}\) rather than Ca\(^{2+}\) to minimize inactivation [59]. A step protocol, however, was also effective. Perhaps most importantly, we co-expressed a limited amount of Rad with \(\alpha_{1C}\) and \(\beta_{2B}\) subunits in HEK293 T cells, adding 3- to 6-fold less Rad than Ca\(_V\)1.2 subunits. We reasoned that excess Rad could eliminate Ca\(^{2+}\) current.

In HEK293T cells transfected with only \(\alpha_{1C}\) plus \(\beta_{2B}\), superfusion of forskolin over 1–3 minutes had no impact on Ba\(^{2+}\) current. Applying forskolin to cells expressing \(\alpha_{1C}\) with \(\beta_{2B}\) and Rad, in contrast, increased the maximal conductance and shifted the \(V_{50}\) for activation. Forskolin also increased the maximal conductance of Ca\(_V\)1.2 channels comprised of 35-mutant \(\alpha_{1C}\) with 28-mutant \(\beta_{2B}\) and Rad, implying that similar to cardiomyocytes, phosphorylation of \(\alpha_{1C}\) and \(\beta_{2B}\) subunits is not required [59]. Rad is also a potential PKA target [70]. Using mass spectrometry, we identified three phosphorylation sites on Rad: Ser25, Ser38 and Ser300. We were unable to detect non-phosphorylated or phosphorylated peptides containing Ser272, but we presumed that it was also a PKA phosphorylation site based upon prior biochemical studies [70]. Alanine-substitutions of these four serine residues in Rad prevented the

Rad is the PKA target

Cognizant of the possibility that an unknown protein may mediate adrenergic regulation, we took an unbiased approach and sought to determine the comprehensive interactome of Ca\(_V\)1.2 in the heart. Proteomic analysis of membrane-signaling complexes from native tissue by traditional means alone is challenging, however, as many interactions are labile under harsh purification conditions. We turned to proximity labeling and quantitative proteomics [61–63]. As channel regulation only reliably manifests in native tissues, we extended this method to native tissues and whole organs of transgenic mice. We generated transgenic mice with doxycycline-inducible, cardiomyocyte-specific expression of DHP-resistant-\(\alpha_{1C}\) or \(\beta_{2B}\) proteins with ascorbate-peroxidase (APEX2) and a V5 epitope conjugated to the N-termini, enabling biotin-labeling of proteins within ~20 nm of the Ca\(^{2+}\) channels [59]. After exposure to isoproterenol, the enrichment of Rad, a member of the Rad and Gem/Kir Ras-related GTP-binding protein (RGK) family of GTP-binding proteins known for their capacity to

these five species. To test this, we mutated all 51 conserved and non-conserved serine and threonine residues within the 35 intracellular PKA consensus phosphorylation sites of rabbit \(\alpha_{1C}\) to alanine (“35-mutant \(\alpha_{1C}\)”). In cardiomyocytes, the nisoldipine-insensitive 35-mutant Ca\(^{2+}\) currents were both up-regulated and activated at more negative potentials in response to isoproterenol or forskolin [59].

Similarly, we mutated to alanine all 37 conserved and non-conserved serine and threonine residues within 28 PKA-consensus phosphorylation sites of human \(\beta_{2B}\) (“28-mutant \(\beta_{2B}\)). Cardiomyocytes expressing GFP-tagged 28-mutant \(\beta_{2B}\) displayed isoproterenol- or forskolin-induced stimulation of Ca\(_V\)1.2 current amplitude. Finally, we crossed 35-mutant \(\alpha_{1C}\) with 28-mutant \(\beta_{2B}\) transgenic mice. These mutant channels also displayed a normal isoproterenol- or forskolin-induced increase in peak Ca\(^{2+}\) current [59]. These results indicate that \(\beta\)-adrenergic stimulation of Ca\(_V\)1.2 does not involve direct phosphorylation of \(\alpha_{1C}\) or \(\beta_{2}\) subunits.
forskolin-induced increase in maximal conductance. We found that alanine substitutions at Ser272 and Ser300 within the C-terminal polybasic membrane region of Rad prevented both the forskolin-induced increase in current amplitude and the hyperpolarizing shift in \( V_{50} \) [59].

Rad can inhibit \( \text{Ca}_V^{1.2} \) via \( \text{Ca}_V^{1.2}\beta \)-binding-dependent and independent (\( \alpha_{1C} \)-dependent) mechanisms [71,72]. Mutating residues on Rad or on \( \beta_2B \) that prevented interaction between Rad and \( \beta_2B \) [71,73] prevented the forskolin-induced increase in the maximal conductance and the hyperpolarizing shift in the I–V curve. Thus, the interaction between Rad and \( \beta \) subunits is essential for cAMP–PKA regulation of \( \text{Ca}_V^{1.2} \) in HEK cells [59]. These results are consistent with our findings that the \( \beta \) subunit association with \( \alpha_{1C} \) is absolutely required for \( \beta \)-adrenergic stimulation of \( \text{Ca}_V^{1.2} \) currents in cardiomyocytes [60].

Conclusions

In cardiomyocytes, \( \beta \)-adrenergic signaling stimulates cAMP production and subsequently activates PKA. PKA phosphorylation of Rad leads to its displacement from \( \text{Ca}_V^{1.2}\beta \), thereby releasing the channel from an inhibited state (Figure 1(b)). The PKA-mediated phosphorylation of an inhibitor and subsequent release of the inhibitor from the complex is reminiscent of the effect of PKA phosphorylation of phospholamban on SERCA [74]. The mechanism appears to be transferable to other voltage-gated \( \text{Ca}^{2+} \) channels, such as \( \text{Ca}_V^{2.2} \) and \( \text{Ca}_V^{1.3} \), which also bind \( \beta \) subunits, and are regulated by forskolin when Rad or Rem are co-expressed [59]. We hypothesize that phosphorylation of the C-terminus of Rad (or Rem) alters the electrostatic interactions with the plasma membrane, as well as the interactions with \( \beta \) subunits, thereby releasing the channel from an inhibited state. The identification of this mechanism could offer opportunities to develop specific modulators of the sympathetic nervous system modulation of \( \text{Ca}^{2+} \) currents for patients with heart failure for instance. Future projects will also explore the role of Rad phosphorylation using phosho-mutant knock-in mice, and how the neighborhood of the \( \text{Ca}^{2+} \) channels change in disease states.

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Disclosure statement

The authors declare no competing interests.

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