




## The quest to identify the mechanism underlying adrenergic regulation of cardiac $\text{Ca}^{2+}$ channels

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### ABSTRACT

Activation of protein kinase A by cyclic AMP results in a multi-fold upregulation of  $\text{Ca}_v1.2$  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  $\beta$ -adrenergic mediated activation of voltage-gated  $\text{Ca}^{2+}$  channels in the heart and other tissues.

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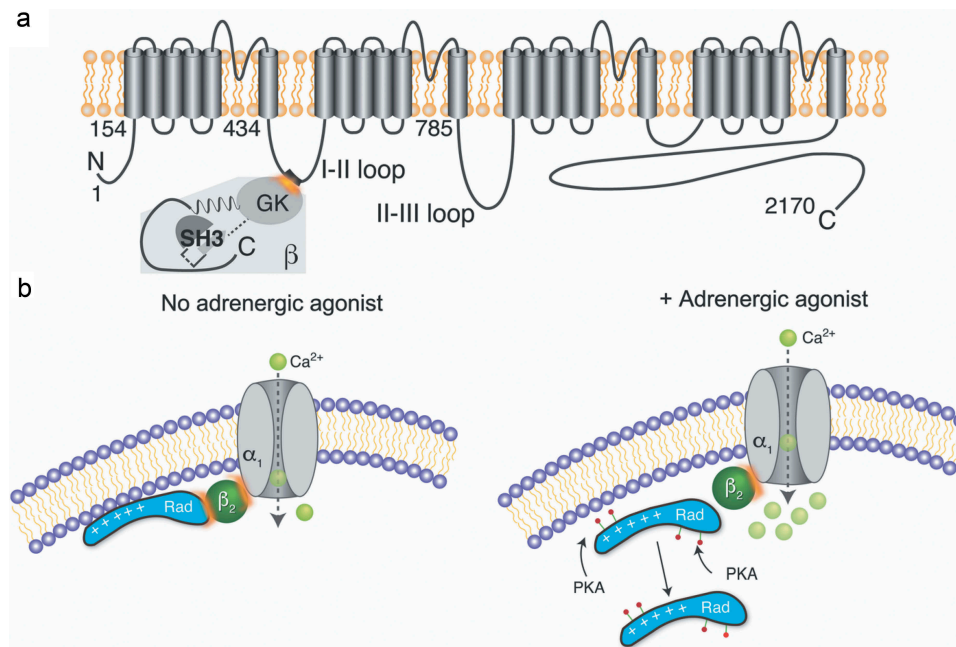
### KEYWORDS

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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  $\beta$ -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  $\beta_1$  and  $\beta_2$  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  $\text{Ca}^{2+}$  channel, also known as  $\text{Ca}_v1.2$ , plays a critical role in excitation-contraction coupling. The  $\text{Ca}_v1.2$  channels are characterized by their high voltage-activating and slow inactivating properties. They contain at minimum a pore-forming subunit ( $\alpha_{1C}$ ) 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  $\beta$  subunit ( $\beta_{2B}$  in heart) binds to the  $\alpha$ -interacting domain in the I-II loop with high affinity (Figure 1(a)) and plays roles in surface expression of  $\alpha_{1C}$  and channel availability [3]. Normal cardiac excitation-contraction coupling occurs when initiation of the cardiac action potential activates  $\text{Ca}_v1.2$  channels, which reside in the transverse tubules (T-tubules) of the cardiomyocyte [4–6]. This leads to  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release whereby the ryanodine receptor releases  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum (SR). This  $\text{Ca}^{2+}$  then binds troponin C, permitting the cross-linking of myofilaments. The majority of the  $\text{Ca}^{2+}$  is then removed from the cytosol via the sarcoplasmic-endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase



**Figure 1.** Schematic of  $Ca_v1.2$  and proposed model of  $\beta$ -adrenergic regulation of  $Ca_v1.2$ . (a) Diagram showing rabbit cardiac  $\alpha_{1C}$  and  $\beta_{2B}$  subunits. GK, guanylate kinase domain; SH3, Src homology 3 domain. (b) No adrenergic agonist – basal state model (left): Rad associates with  $Ca_v1.2$   $\beta$  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  $Ca_v\beta$  subunit. Phosphorylated Rad leaves the “neighborhood” of the  $Ca^{2+}$  channel resulting in increased  $Ca^{2+}$  influx (green circles). Adapted from [59].

(SERCA), modulated by phospholamban (PLB), and the  $Na^+-Ca^{2+}$  exchanger.

Upon activation of the  $\beta$ -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  $Ca^{2+}$  release from the SR and thereby increased myofilament cross-linking, increased  $Ca^{2+}$  re-uptake into the SR, and increased myofilament relaxation. Importantly, activation of  $\beta$ -adrenergic receptors also leads to an increase in  $Ca_v1.2$  current and availability [10,11]. The mechanism through which  $\beta$ -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 be elusive for more than 40 years [15]. Although the obvious targets of phosphorylation are  $Ca_v1.2$   $\alpha$  or  $\beta$  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  $\beta$ -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  $Ca_v1.2$ , 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  $Ca_v1.2$  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  $Ca^{2+}$  channels into artificial lipid bilayers after mixing purified sarcolemma and membrane proteins with soybean phospholipid to form proteoliposomes [17]. They demonstrated that  $Ca^{2+}$  current from proteoliposomes incubated with activated PKA and  $Mg^{2+}$ -ATP was 5 times larger than the controls, implying that activation of  $Ca_v1.2$  occurs via direct phosphorylation of a channel subunit or a protein that

remains closely associated with the channel. In oocytes, reconstitution of adrenergic regulation of  $\text{Ca}_V1.2$  was accomplished by injection of total cardiac mRNA [18]. The injected mRNA encoded not only the  $\alpha_{1C}$  subunit, but also other protein(s) that could enable PKA regulation of  $\text{Ca}_V1.2$  to occur. In retrospect, these studies may have revealed the importance of an as of yet unidentified protein in the regulation of  $\text{Ca}_V1.2$  current. Other studies reported successful reconstitution simply using  $\alpha_{1C}$  and/or  $\beta$  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  $\text{Ca}_V1.2$  channels with various combinations of auxiliary subunits were studied [27]. No increase in current through  $\alpha_{1C}$  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  $\alpha_1$  and  $\beta_{2A}$  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  $\alpha_{1C}$  and HEK cells transiently expressing  $\alpha_{1C}$  and  $\beta_2$  subunits, neither inhibition of endogenous PKA, inhibition of protein phosphatases, or internal dialysis of PKA significantly affected peak barium current through  $\alpha_{1C}$  [29]. Mikala et al. suggested that  $\alpha_{1C}$  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  $\alpha_{1C}$  was also proposed as an essential process for adrenergic signaling. Different sizes of  $\alpha_{1C}$  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  $\alpha_{1C}$  results in increased activity of the channel [36], suggesting an inhibitory activity of this region. Cleavage of the

C-terminus of  $\alpha_{1C}$  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  $\beta$ -adrenergic stimulation of  $\text{Ca}_V1.2$ . We showed this by generating transgenic mice with inducible expression of proteolytic-resistant  $\alpha_{1C}$  [38].

### Identifying and testing PKA phosphorylation sites

After the cloning of  $\alpha_{1C}$  [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  $\text{Ca}_V1.2$  was further supported through heterologous expression of a Ser1928 mutant that prevented upregulation of  $\text{Ca}_V1.2$  [23]. Other investigators failed to reproduce PKA modulation of  $\text{Ca}^{2+}$  currents in heterologous expression systems [29,30]. Phosphorylation sites have also been mapped to the  $\text{Ca}^{2+}$  channel  $\beta$  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  $\text{Ca}_V1.2$ . The dogma that phosphorylation of Ser1928 by PKA is required for  $\beta$ -adrenergic stimulation of  $\text{Ca}_V1.2$  was tested by the O'Rourke group in 2006 through adenoviral gene transfer to express mutant  $\alpha_{1C}$  subunits in guinea pig cardiomyocytes [47]. Interestingly, an alanine point mutation at Ser1928 did not significantly reduce the response of  $\alpha_{1C}$  to isoproterenol. Two years later, Lemke et al. confirmed that phosphorylation of Ser1928 is not required for adrenergic regulation of  $\text{Ca}_V1.2$  via generating a knock-in mouse with an alanine substitution at Ser1928 [48].

To test whether PKA phosphorylation of the  $\beta$  subunit was relevant in cardiomyocytes,  $\beta$  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  $\text{Ca}_v1.2$  [47,49]. As definitive proof that these sites were not required, a knock-in mouse expressing a  $\beta_2$  subunit truncated prior to these phosphorylation sites displayed normal PKA modulation of  $\text{Ca}_v1.2$ , thus ruling out involvement of any putative C-terminal phosphorylation sites in mediating  $\beta$ -adrenergic regulation of  $\text{Ca}_v1.2$  [50].

As a heteromultimeric complex,  $\text{Ca}_v1.2$  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  $\text{Ca}_v\beta_2$  and this association decreases after PKA phosphorylation of both *ahnak* and the  $\beta$  subunit [51]. While it was initially proposed that phosphorylation and subsequent release of *ahnak* from  $\beta_2$  allowed for greater binding of  $\beta_2$  to  $\alpha_{1C}$  leading to enhanced  $\text{Ca}^{2+}$  current during adrenergic activation, later studies showed that *ahnak*-deficient cardiomyocytes displayed preserved adrenergic modulation of  $\text{Ca}_v1.2$  [52].

### Additional phosphorylation sites

After phosphorylation of Ser1928 was shown to be non-essential for  $\beta$ -adrenergic regulation of  $\text{Ca}_v1.2$  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  $\text{Ca}_v1.2$  channels in mice [54]. The transgenic and endogenous  $\text{Ca}_v1.2$  currents are distinguishable by application of nisoldipine, a  $\text{Ca}^{2+}$  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  $\alpha_{1C}$ , designated pseudo-WT (pWT  $\alpha_{1C}$ ). The resultant DHP-resistant  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  channels at the surface was unchanged, yet basal  $\text{Ca}^{2+}$  currents were substantially reduced [56,57]. Hofmann and colleagues subsequently created S1700A/T1704A knock-in mice and concluded that isoproterenol stimulated  $\text{Ca}^{2+}$  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  $\beta$ -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  $\alpha_{1C}$  site as essential for  $\beta$ -adrenergic modulation of  $\text{Ca}_v1.2$  led us to hypothesize that a combination of sites in  $\alpha_{1C}$  is required. After identifying conserved PKA consensus sequences in  $\alpha_{1C}$  of five species (mouse, rat, rabbit, guinea pig and human), we generated  $\alpha_{1C}$  transgenic mice ("22-mutant  $\alpha_{1C}$ ") 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  $\alpha_{1C}$  might be different amongst

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  $\text{Ca}^{2+}$  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  $\text{Ca}_V1.2$  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  $\text{Ca}^{2+}$  current [59]. These results indicate that  $\beta$ -adrenergic stimulation of  $\text{Ca}_V1.2$  does not involve direct phosphorylation of  $\alpha_{1C}$  or  $\beta_2$  subunits.

### 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  $\text{Ca}_V1.2$  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  $\sim 20$  nm of the  $\text{Ca}^{2+}$  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

inhibit all high-voltage-activated  $\text{Ca}^{2+}$  channels [64,65], was decreased from the neighborhood of  $\text{Ca}_V1.2$ .

Rad-knockout mice display an increased maximum  $\text{Ca}^{2+}$  current, and the  $\text{Ca}^{2+}$  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  $\text{Ca}_V1.2$  since adenoviral-induced overexpression of Rad [68] and Rem [69] in cultured cardiomyocytes ablated  $\text{Ca}_V1.2$  and attenuated adrenergic regulation. The proximity labeling experiments suggested, however, that Rad was the missing link.

The robust heterologous reconstitution of PKA regulation of  $\text{Ca}_V1.2$  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  $\text{Ba}^{2+}$  rather than  $\text{Ca}^{2+}$  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  $\text{Ca}_V1.2$  subunits. We reasoned that excess Rad could eliminate  $\text{Ca}^{2+}$  current.

In HEK293T cells transfected with only  $\alpha_{1C}$  plus  $\beta_{2B}$ , superfusion of forskolin over 1–3 minutes had no impact on  $\text{Ba}^{2+}$  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  $\text{Ca}_V1.2$  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

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  $Ca_V1.2$  via  $Ca_V\beta$ -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  $Ca_V1.2$  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  $Ca_V1.2$  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  $Ca_V\beta$ , 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  $Ca^{2+}$  channels, such as  $Ca_V2.2$  and  $Ca_V1.3$ , 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  $Ca^{2+}$  currents for patients with heart failure for instance. Future projects will also explore the role of Rad phosphorylation using phospho-mutant knock-in mice, and how the neighborhood of the  $Ca^{2+}$  channels change in disease states.

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The authors declare no competing interests.

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## References

- [1] Cannon WB. Bodily changes in pain, hunger, fear, and rage: an account of recent researches into the function of emotional excitement. New York, NY: D Appleton & Company; 1915.
- [2] Dixon RA, Kobilka BK, Strader DJ, et al. Cloning of the gene and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin. *Nature*. 1986;321:75–79.
- [3] Lacerda AE, Kim HS, Ruth P, et al. Normalization of current kinetics by interaction between the alpha 1 and beta subunits of the skeletal muscle dihydropyridine-sensitive  $Ca^{2+}$  channel. *Nature*. 1991;352(6335):527–530.
- [4] Fabiato A, Fabiato F. Excitation-contraction coupling of isolated cardiac fibers with disrupted or closed sarcolemmas: calcium-dependent cyclic and tonic contractions. *Circ Res*. 1972;31(3):293–307.
- [5] Fabiato F, Fabiato A. Excitation-contraction coupling and regulation of contractility studied on isolated adult myocardial cells. *Acta Cardiol*. 1972;27(2):243–248.
- [6] Bers DM. Cardiac excitation-contraction coupling. *Nature*. 2002;415(6868):198–205.
- [7] Marx SO, Reiken S, Hisamatsu Y, et al. PKA phosphorylation dissociates FKBP12.6 from the calcium release

- channel (ryanodine receptor): defective regulation in failing hearts. *Cell*. 2000;101(4):365–376.
- [8] Rao V, Cheng Y, Lindert S, et al. PKA phosphorylation of cardiac troponin i modulates activation and relaxation kinetics of ventricular myofibrils. *Biophys J*. 2014;107(5):1196–1204.
- [9] Kirchberber MA, Tada M, Katz AM. Phospholamban: a regulatory protein of the cardiac sarcoplasmic reticulum. *Recent Adv Stud Cardiac Struct Metab*. 1975;5:103–115.
- [10] Reuter H. The dependence of slow inward current in Purkinje fibres on the extracellular calcium-concentration. *J Physiol*. 1967;192:479–492.
- [11] Vassort G, Rougier O, Garnier D, et al. Effects of adrenaline on membrane inward currents during the cardiac action potential. *Pflugers Arch*. 1969;309:70–81.
- [12] Kamp TJ, Hell JW. Regulation of cardiac L-type calcium channels by protein kinase A and protein kinase C. *Circ Res*. 2000;87:1095–1102.
- [13] Tsien RW. Calcium channels in excitable cell membranes. *Annu Rev Physiol*. 1983;45(1):341–358.
- [14] Trautwein W, Hescheler J. Regulation of cardiac L-type calcium current by phosphorylation and G proteins. *Annu Rev Physiol*. 1990;52(1):257–274.
- [15] Wang X, Tsien RW. Suspect that modulates the heart-beat is ensnared. *Nature*. 2020;577(7792):624–626.
- [16] Weiss S, Oz S, Benmocha A, et al. Regulation of cardiac L-type Ca(2)(+) channel CaV1.2 via the beta-adrenergic-cAMP-protein kinase A pathway: old dogmas, advances, and new uncertainties. *Circ Res*. 2013;113:617–631.
- [17] Kameyama A, Nakayama T. Calcium efflux through cardiac calcium channels reconstituted into liposomes—flux measurement with fura-2. *Biochem Biophys Res Commun*. 1988;154(3):1067–1074.
- [18] Lory P, Nargeot J. Cyclic AMP-dependent modulation of cardiac Ca channels expressed in *Xenopus laevis* oocytes. *Biochem Biophys Res Commun*. 1992;182(3):1059–1065.
- [19] Sculptoreanu A, Rotman E, Takahashi M, et al. Voltage-dependent potentiation of the activity of cardiac L-type calcium channel alpha 1 subunits due to phosphorylation by cAMP-dependent protein kinase. *Proc Natl Acad Sci U S A*. 1993;90(21):10135–10139.
- [20] Nunoki K, Florio V, Catterall WA. Activation of purified calcium channels by stoichiometric protein phosphorylation. *Proc Natl Acad Sci U S A*. 1989;86(17):6816–6820.
- [21] Chang CF, Gutierrez LM, Mundina-Weilenmann C, et al. Dihydropyridine-sensitive calcium channels from skeletal muscle. II. Functional effects of differential phosphorylation of channel subunits. *J Biol Chem*. 1991;266(25):16395–16400.
- [22] Yoshida A, Takahashi M, Nishimura S, et al. Cyclic AMP-dependent phosphorylation and regulation of the cardiac dihydropyridine-sensitive Ca channel. *FEBS Lett*. 1992;309(3):343–349.
- [23] Gao T, Yatani A, Dell'Acqua ML, et al. cAMP-dependent regulation of cardiac L-type Ca<sup>2+</sup> channels requires membrane targeting of PKA and phosphorylation of channel subunits. *Neuron*. 1997;19(1):185–196.
- [24] Gray PC, Johnson BD, Westenbroek RE, et al. Primary structure and function of an A kinase anchoring protein associated with calcium channels. *Neuron*. 1998;20(5):1017–1026.
- [25] Gray PC, Tibbs VC, Catterall WA, et al. Identification of a 15-kDa cAMP-dependent protein kinase-anchoring protein associated with skeletal muscle L-type calcium channels. *J Biol Chem*. 1997;272(10):6297–6302.
- [26] Fraser ID, Tavalin SJ, Lester LB, et al. A novel lipid-anchored A-kinase Anchoring Protein facilitates cAMP-responsive membrane events. *Embo J*. 1998;17:2261–2272.
- [27] Singer-Lahat D, Lotan I, Biel M, et al. Cardiac calcium channels expressed in *Xenopus* oocytes are modulated by dephosphorylation but not by cAMP-dependent phosphorylation. *Recept Channels*. 1994;2(3):215–226.
- [28] Perez-Reyes E, Yuan W, Wei X, et al. Regulation of the cloned L-type cardiac calcium channel by cyclic-AMP-dependent protein kinase. *FEBS Lett*. 1994;342(2):119–123.
- [29] Zong X, Schreieck J, Mehrke G, et al. On the regulation of the expressed L-type calcium channel by cAMP-dependent phosphorylation. *Pflugers Arch*. 1995;430(3):340–347.
- [30] Mikala G, Klockner U, Varadi M, et al. cAMP-dependent phosphorylation sites and macroscopic activity of recombinant cardiac L-type calcium channels. *Mol Cell Biochem*. 1998;185(1/2):95–109.
- [31] De Jongh KS, Merrick DK, Catterall WA. Subunits of purified calcium channels: a 212-kDa form of alpha 1 and partial amino acid sequence of a phosphorylation site of an independent beta subunit. *Proc Natl Acad Sci U S A*. 1989;86(21):8585–8589.
- [32] De Jongh KS, Warner C, Colvin AA, et al. Characterization of the two size forms of the alpha 1 subunit of skeletal muscle L-type calcium channels. *Proc Natl Acad Sci U S A*. 1991;88(23):10778–10782.
- [33] Hell JW, Yokoyama CT, Wong ST, et al. Differential phosphorylation of two size forms of the neuronal class C L-type calcium channel alpha 1 subunit. *J Biol Chem*. 1993;268(26):19451–19457.
- [34] Hell JW, Westenbroek RE, Breeze LJ, et al. N-methyl-D-aspartate receptor-induced proteolytic conversion of postsynaptic class C L-type calcium channels in hippocampal neurons. *Proc Natl Acad Sci U S A*. 1996;93(8):3362–3367.
- [35] De Jongh KS, Murphy BJ, Colvin AA, et al. Specific phosphorylation of a site in the full-length form of the alpha 1 subunit of the cardiac L-type calcium channel by adenosine 3',5'-cyclic monophosphate-dependent protein kinase. *Biochemistry*. 1996;35:10392–10402.
- [36] Wei X, Neely A, Lacerda AE, et al. Modification of Ca<sup>2+</sup> channel activity by deletions at the carboxyl terminus of the

- cardiac alpha 1 subunit. *J Biol Chem.* 1994;269(3):1635–1640.
- [37] Fuller MD, Emrick MA, Sadilek M, et al. Molecular mechanism of calcium channel regulation in the fight-or-flight response. *Sci Signal.* 2010;3(141):ra70.
- [38] Katchman A, Yang L, Zakharov SI, et al. Proteolytic cleavage and PKA phosphorylation of  $\alpha 1C$  subunit are not required for adrenergic regulation of Ca V 1.2 in the heart. *Proc Natl Acad Sci U S A.* 2017;114(34):9194–9199.
- [39] Mikami A, Imoto K, Tanabe T, et al. Primary structure and functional expression of the cardiac dihydropyridine-sensitive calcium channel. *Nature.* 1989;340(6230):230–233.
- [40] Leach RN, Brickley K, Norman RI. Cyclic AMP-dependent protein kinase phosphorylates residues in the C-terminal domain of the cardiac L-type calcium channel alpha1 subunit. *Biochim Biophys Acta.* 1996;1281(2):205–212.
- [41] Takahashi E, Fukuda K, Miyoshi S, et al. Leukemia inhibitory factor activates cardiac L-Type Ca<sup>2+</sup> channels via phosphorylation of serine 1829 in the rabbit Cav1.2 subunit. *Circ Res.* 2004;94:1242–1248.
- [42] Erxleben C, Gomez-Alegria C, Darden T, et al. Modulation of cardiac Ca(V)1.2 channels by dihydropyridine and phosphatase inhibitor requires Ser-1142 in the domain III pore loop. *Proc Natl Acad Sci U S A.* 2003;100(5):2929–2934.
- [43] Mitterdorfer J, Froschmayr M, Grabner M, et al. Identification of PK-A phosphorylation sites in the carboxyl terminus of L-type calcium channel  $\alpha 1$  subunits †. *Biochemistry.* 1996;35(29):9400–9406.
- [44] Haase H, Bartel S, Karczewski P, et al. In-vivo phosphorylation of the cardiac L-type calcium channel beta-subunit in response to catecholamines. *Mol Cell Biochem.* 1996;163–164(1):99–106.
- [45] Bunemann M, Gerhardstein BL, Gao T, et al. Functional regulation of L-type calcium channels via protein kinase a-mediated phosphorylation of the  $\beta 2$  subunit. *J Biol Chem.* 1999;274(48):33851–33854.
- [46] Gerhardstein BL, Puri TS, Chien AJ, et al. Identification of the sites phosphorylated by cyclic AMP-dependent protein kinase on the  $\beta 2$  subunit of L-type voltage-dependent calcium channels †. *Biochemistry.* 1999;38(32):10361–10370.
- [47] Ganesan AN, Maack C, Johns DC, et al.  $\beta$ -Adrenergic stimulation of L-type Ca<sup>2+</sup> channels in cardiac myocytes requires the distal carboxyl terminus of  $\alpha 1C$  but not serine 1928. *Circ Res.* 2006;98(2):e11–8.
- [48] Lemke T, Welling A, Christel CJ, et al. Unchanged  $\beta$ -Adrenergic stimulation of cardiac L-type calcium channels in Ca v 1.2 phosphorylation site S1928A mutant mice. *J Biol Chem.* 2008;283(50):34738–34744.
- [49] Miriyala J, Nguyen T, Yue DT, et al. Role of Ca V  $\beta$  subunits, and lack of functional reserve, in protein kinase a modulation of cardiac Ca V 1.2 channels. *Circ Res.* 2008;102(7):e54–64.
- [50] Brandmayr J, Poomvanicha M, Domes K, et al. Deletion of the C-terminal phosphorylation sites in the cardiac  $\beta$ -subunit does not affect the basic  $\beta$ -adrenergic response of the heart and the Cav 1.2 channel. *J Biol Chem.* 2012;287(27):22584–22592.
- [51] Haase H, Alvarez J, Petzhold D, et al. Ahnak is critical for cardiac Ca(V)1.2 calcium channel function and its beta-adrenergic regulation. *Faseb J.* 2005;19:1969–1977.
- [52] Pankonien I, Alvarez JL, Doller A, et al. Ahnak1 is a tuneable modulator of cardiac Ca(v)1.2 calcium channel activity. *J Muscle Res Cell Motil.* 2011;32(4–5):281–290.
- [53] Emrick MA, Sadilek M, Konoki K, et al. Beta-adrenergic-regulated phosphorylation of the skeletal muscle Ca(V)1.1 channel in the fight-or-flight response. *Proc Natl Acad Sci U S A.* 2010;107:18712–18717.
- [54] Yang L, Katchman A, Samad T, et al. beta-adrenergic regulation of the L-type Ca<sup>2+</sup> channel does not require phosphorylation of alpha1C Ser1700. *Circ Res.* 2013;113:871–880.
- [55] Muth JN, Yamaguchi H, Mikala G, et al. Cardiac-specific overexpression of the alpha(1) subunit of the L-type voltage-dependent Ca(2+) channel in transgenic mice. Loss of isoproterenol-induced contraction. *J Biol Chem.* 1999;274:21503–21506.
- [56] Fu Y, Westenbroek RE, Scheuer T, et al. Phosphorylation sites required for regulation of cardiac calcium channels in the fight-or-flight response. *Proc Natl Acad Sci U S A.* 2013;110(48):19621–19626.
- [57] Yang L, Dai DF, Yuan C, et al. Loss of beta-adrenergic-stimulated phosphorylation of CaV1.2 channels on Ser1700 leads to heart failure. *Proc Natl Acad Sci U S A.* 2016;113:E7976–E85.
- [58] Poomvanicha M, Matthes J, Domes K, et al. Beta-adrenergic regulation of the heart expressing the Ser1700A/Thr1704A mutated Cav1.2 channel. *J Mol Cell Cardiol.* 2017;111:10–16.
- [59] Liu G, Papa A, Katchman AN, et al. Mechanism of adrenergic CaV1.2 stimulation revealed by proximity proteomics. *Nature.* 2020;577(7792):695–700.
- [60] Yang L, Katchman A, Kushner J, et al. Cardiac CaV1.2 channels require beta subunits for beta-adrenergic-mediated modulation but not trafficking. *J Clin Invest.* 2019;129:647–658.
- [61] Rhee HW, Zou P, Udeshi ND, et al. Proteomic mapping of mitochondria in living cells via spatially restricted enzymatic tagging. *Science.* 2013;339(6125):1328–1331.
- [62] Hung V, Zou P, Rhee HW, et al. Proteomic mapping of the human mitochondrial intermembrane space in live cells via ratiometric APEX tagging. *Mol Cell.* 2014;55(2):332–341.
- [63] Paek J, Kalocsay M, Staus DP, et al. Multidimensional tracking of GPCR signaling via peroxidase-catalyzed proximity labeling. *Cell.* 2017;169(2):338–49 e11.
- [64] Beguin P, Nagashima K, Gonoï T, et al. Regulation of Ca<sup>2+</sup> channel expression at the cell surface by the small G-protein kir/Gem. *Nature.* 2001;411(6838):701–706.



- [65] Finlin BS, Crump SM, Satin J, et al. Regulation of voltage-gated calcium channel activity by the Rem and Rad GTPases. *Proc Natl Acad Sci U S A*. 2003;100(24):14469–14474.
- [66] Manning JR, Yin G, Kaminski CN, et al. Rad GTPase deletion increases L-type calcium channel current leading to increased cardiac contraction. *J Am Heart Assoc*. 2013;2(6):e000459.
- [67] Levitan BM, Manning JR, Withers CN, et al. Rad-deletion phenocopies tonic sympathetic stimulation of the heart. *J Cardiovasc Transl Res*. 2016;9(5–6):432–444.
- [68] Wang G, Zhu X, Xie W, et al. Rad as a novel regulator of excitation-contraction coupling and beta-adrenergic signaling in heart. *Circ Res*. 2010;106(2):317–327.
- [69] Xu X, Marx SO, Colecraft HM. Molecular mechanisms, and selective pharmacological rescue, of Rem-inhibited CaV1.2 channels in heart. *Circ Res*. 2010;107(5):620–630.
- [70] Moyers JS, Zhu J, Kahn CR. Effects of phosphorylation on function of the Rad GTPase. *Biochem J*. 1998;333(Pt 3):609–614.
- [71] Yang T, Puckerin A, Colecraft HM. Distinct RGK GTPases differentially use alpha1- and auxiliary beta-binding-dependent mechanisms to inhibit CaV1.2/CaV2.2 channels. *PLoS One*. 2012;7:e37079.
- [72] Puckerin AA, Chang DD, Shuja Z, et al. Engineering selectivity into RGK GTPase inhibition of voltage-dependent calcium channels. *Proc Natl Acad Sci U S A*. 2018;115(47):12051–12056.
- [73] Beguin P, Ng YJ, Krause C, et al. RGK small GTP-binding proteins interact with the nucleotide kinase domain of Ca<sup>2+</sup>-channel  $\beta$ -subunits via an uncommon effector binding domain. *J Biol Chem*. 2007;282(15):11509–11520.
- [74] Katz AM. Role of phosphorylation of the sarcoplasmic reticulum in the cardiac response to catecholamines. *Eur Heart J*. 1980;1(suppl 1):A:29–33.