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Adrenergic Regulation of Calcium Channels in the Heart

Arianne Papa[1], **Jared Kushner**[2], **Steven O. Marx**[2,3]

[1]Department of Physiology and Cellular Biophysics, Vagelos College of Physicians and Surgeons, Columbia University, New York, NY, USA

[2]Division of Cardiology, Department of Medicine, Vagelos College of Physicians and Surgeons, Columbia University, New York, NY, USA

[3]Department of Molecular Pharmacology and Therapeutics, Vagelos College of Physicians and Surgeons, Columbia University, New York, NY, USA

Abstract

Each heartbeat is initiated by the action potential, an electrical signal that depolarizes the plasma membrane and activates a cycle of calcium influx via voltage-gated calcium channels, calcium release via ryanodine receptors, and calcium reuptake and efflux via calcium-ATPase pumps and sodium-calcium exchangers. Agonists of the sympathetic nervous system bind to adrenergic receptors in cardiomyocytes, which, via cascading signal transduction pathways and protein kinase A (PKA), increase the heart rate (chronotropy), the strength of myocardial contraction (inotropy), and the rate of myocardial relaxation (lusitropy). These effects correlate with increased intracellular concentration of calcium, which is required for the augmentation of cardiomyocyte contraction. Despite extensive investigations, the molecular mechanisms underlying sympathetic nervous system regulation of calcium influx in cardiomyocytes have remained elusive over the last 40 years. Recent studies have uncovered the mechanisms underlying this fundamental biologic process, namely that PKA phosphorylates a calcium channel inhibitor, Rad, thereby releasing inhibition and increasing calcium influx. Here, we describe an updated model for how signals from adrenergic agonists are transduced to stimulate calcium influx and contractility in the heart.

Keywords

calcium channel; sympathetic nervous system; phosphorylation; heart; excitation-contraction coupling

INTRODUCTION

Sidney Ringer, in the late nineteenth century, recognized that calcium (Ca^{2+}) was essential for cardiac contraction. While perfusing isolated frog hearts, removal of Ca^{2+} from the perfusion buffer stopped the heart from contracting (1), demonstrating that external Ca^{2+}

sm460@cumc.columbia.edu .

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is required. Cardiac excitation-contraction coupling occurs when initiation of the cardiac action potential activates voltage-gated L-type Ca^{2+} ($Ca_V1.2$) channels, which reside in the transverse tubules (T-tubules) (2–4). The Ca^{2+} influx via $Ca_V1.2$ triggers opening of ryanodine receptor 2 (RyR2), leading to Ca^{2+} release from the sarcoplasmic reticulum (SR). Calcium then binds troponin C, enabling myofilament cross-linking and contraction. Both voltage-gated Ca^{2+} channels and RyR2 channels close, and relaxation of the heart ensues as Ca^{2+} is removed from the cytosol via the sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase (SERCA), modulated by phospholamban (PLB), and the Na^+-Ca^{2+} exchanger (NCX) (Figure 1).

T-tubules: transverse tubules

RyR2: ryanodine receptor 2

SR: sarcoplasmic reticulum

SERCA: sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase

PLB: phospholamban

NCX: Na^+-Ca^{2+} exchanger

Cardiomyocyte contraction is augmented by β-adrenergic signaling and protein kinase A (PKA), the activation of which substantially increases the force of contraction, sarcomere shortening, and the rate of relaxation. PKA regulates several targets, including $Ca_V1.2$ channels, RyR2, and PLB, leading to increased Ca^{2+} entry, increased Ca^{2+} release from the SR, and increased Ca^{2+} reuptake by SERCA, respectively (5–10). Despite decades of investigations, the mechanisms responsible for adrenergic stimulation of Ca^{2+} channels in the heart were not well understood. Initially, the focus was on phosphorylation sites on the $Ca_V1.2$ pore-forming α_{1C} subunit, which consists of four homologous transmembrane domains of six transmembrane segments and cytoplasmic N and C termini, and the channel's β subunit, which interacts with an 18-residue sequence in the pore-forming subunit intracellular linker between domains I and II, termed the α-interacting domain (AID) (11–13). In this review, we offer an overview of the mechanisms responsible for adrenergic regulation of Ca^{2+} handling in the heart and focus on the investigations identifying the signaling responsible for PKA-mediated stimulation of Ca^{2+} influx in the heart.

PKA: protein kinase A

AID: α-interacting domain

SYMPATHETIC NERVOUS SYSTEM REGULATION OF CARDIAC FUNCTION

In 1895, George Oliver and Edward Albert Sharpey-Schäfer (14) showed that administering extracts of suprarenal or adrenal gland to anesthetized dogs augments both cardiac function and blood pressure and increases skeletal muscle perfusion at the expense of blood flow to nonessential vascular beds. By 1915, Walter Cannon (15, p. 108) described how the sympathetic nervous system rapidly coordinates the body's adaptation to pain or excitement "…to meet by extra action the urgent demands of struggle or escape," which he would later describe as the "fight or flight" response. In a series of experiments with collaborators, and drawing on the work of Max Lewandosky and T.R. Elliot, Cannon reproduced Oliver's and Sharpey-Schäfer's findings through electrical stimulation of the spine or splanchnic nerves, through purified adrenin and through washout release of tied-off adrenal veins (15, 16). He also found that both medical students before exams and felines subjected to either conscious confinement or anesthetized splanchnic nerve stimulation develop elevated blood sugar, adding to his theory of a multi-organ stress response that prioritizes the needs of the metabolically active immediately essential organs—the heart, lungs, brain, and skeletal muscle—at the expense of "the vegetative organs of the interior, which serve the routine needs of the body" (15, p. 108).

Ulf von Euler would win his share of a Nobel Prize for work identifying norepinephrine as the primary neurotransmitter of the sympathetic nervous system (16). Puzzled over the differences in potency of various synthetic and natural catecholamines on striated muscle as well as on vascular and airway constriction and relaxation, Raymond Ahlquist proposed the existence of separate excitatory and inhibitory α- and β-adrenergic receptors (17). This theory was lent credence in 1958, when C.E. Powell and I.H. Slater (18) published the physiologic effects of the first β-selective adrenergic-blocking drug, showing that it did not affect epinephrine- or norepinephrine-induced increases in blood pressure but did block isoproterenol-induced hypotension in cats. They additionally showed blockade of epinephrine-induced uterine relaxation and airway relaxation in different model systems. Neil Moran and Marjorie Perkins (17) blunted cardiac inotropic and chronotropic responses to catecholamines with a selective β-blocker in vagotomized and sympathectomized dogs, confirming the primacy of the β-adrenergic receptor in the cardiac fight or flight response. James Black would go on to win the Nobel Prize for his discovery of the first clinically useful beta-selective blocker, now known as propranolol (19). Other essential work that advanced the field and earned Nobel recognition includes Brian Kobilka's and Robert Lefkowitz's identification of the sequence and structure of the β2-adrenergic receptor as well as Earl Sutherland's work identifying adenylyl cyclase activation and cAMP generation as key effectors of β-adrenergic stimulation (20, 21).

TARGETS OF THE SYMPATHETIC NERVOUS SYSTEM IN THE HEART

The sympathetic nervous system modulates the heart rate (chronotropy), the force of contraction (inotropy), and the rate of relaxation (lusitropy). The principal targets of PKA

in ventricular and atrial cardiomyocytes are the L-type Ca^{2+} channels (principally $Ca_V1.2$), RyR2, and PLB, leading to increased Ca^{2+} entry, increased Ca^{2+} release from the SR, and increased Ca^{2+} reuptake by SERCA (5–10), respectively. This increased Ca^{2+} entry triggers yet more RyR2 openings, and because of greater Ca^{2+} reuptake and loading of the SR, increased fractional Ca^{2+} release (4, 22, 23). PKA also phosphorylates troponin I (TnI) and the cardiac myosin binding protein C (cMyBP-C), which reduces myofilament Ca^{2+} affinity, thereby promoting dissociation of Ca^{2+} from the myofilaments during diastole (24–26). PKA phosphorylation of cMyBP-C also accelerates cross-bridge detachment rates. The lusitropic effect of β-adrenergic stimulation, however, is predominantly mediated by the phosphorylation of PLB and the accelerated rate of SR Ca^{2+} reuptake (4).

The heart rate is principally controlled by the sinus node in the right atrium. Hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels and L-type Ca^{2+} channels have been proposed to comprise the key mechanisms underlying the effects of catecholamines on heart rate (27). An increase in cAMP caused by adrenergic stimulation augments the open probability (P_o) and shifts the activation curve of HCN channels, thereby increasing the rate of diastolic depolarization. Adrenergic agonists also control chronotropy by activating PKA that activates $Ca_V1.3$ channels (28–30) and RyR channels (31, 32) via phosphorylation.

As the heart rate increases, the ventricular action potential duration shortens, thereby increasing the diastolic filling time. The shortening of the action potential duration is detected as a reduction in the QT interval on an electrocardiogram. An important target for PKA-dependent phosphorylation in the regulation of the human cardiac action potential is the slowly activating delayed rectifier KCNQ1 channel (I_{KS}) (Figure 1). PKA regulation of I_{Ks} requires a macromolecular complex consisting of the A-kinase anchoring protein (AKAP), also known as yotiao, which recruits PKA and protein phosphatase 1 (PP1) to the channel, and PKA phosphorylation of serine 27 (Ser27) on the N terminus (33).

AKAP: A-kinase anchoring protein

ADRENERGIC REGULATION OF VOLTAGE-GATED Ca^{2+} CHANNELS IN THE HEART

The mechanisms underlying β-adrenergic activation of Ca^{2+} influx in both atrial and ventricular cardiomyocytes have been studied for decades, with seminal studies demonstrating that activation of PKA is required (34–37). β-Adrenergic agonists increase Ca^{2+} current by 2–3 fold, principally by increasing the P_o of the channel, shifting from inactive and low P_o mode (modes 0 and 1, respectively) to mode 2, which is marked by high P_o, with long and frequent openings (5, 34–37). Adrenergic agonists may also increase the mobilization of a subsarcolemmal pool of $Ca_V1.2$-cargo-carrying endosomes, leading to increased T-tubule sarcolemmal $Ca_V1.2$ abundance (38).

How PKA controls Ca^{2+} influx in the heart has been controversial. Moreover, heterologous expression systems proved to be unreliable in recapitulating the PKA-mediated activation

of Ca^{2+} channels, making it challenging to establish mechanisms. Studies have focused on several possible approaches to establish regulatory mechanisms: (*a*) identifying PKA regulatory sites on the principal Ca^{2+} channel subunits in the heart, α_{1C} and β_2 (Figure 2a); (*b*) identifying a role for proteolytic cleavage of the C terminus in regulating PKA activation of $Ca_V1.2$; and (*c*) identifying a role for AKAPs in modulating adrenergic responsiveness. We review the major studies exploring these regulatory processes.

PHOSPHORYLATION OF α_{1C} AND β_{2B}

Full-length α_{1C} contains a residue phosphorylated by PKA, Ser1928, that is absent from channels with cleavage of the distal 30-kDa C terminus (39–43). The phosphorylation of Ser1928 was demonstrated in dissociated rat cardiomyocytes exposed to β-adrenergic agonists using a phospho-epitope-specific antibody (44). Some studies showed that phosphorylation of Ser1928 was required for PKA-mediated upregulation of a heterologously expressed Ca^{2+} current (45, 46), although a subsequent study reported PKA-induced activation of Ca^{2+} channels with an α_{1C} subunit truncated at residue 1905 (47). Several phosphorylation sites on the C terminus of the β subunit have also been identified (47, 48). The inability to reliably reconstitute PKA regulation using heterologous expression studies led the O'Rourke laboratory (49) to use adenovirus to express in cardiomyocytes a dihydropyridine (DHP)-resistant mutant α_{1C} subunit harboring alanine substitution of the Ser1928 residue. DHP resistance enabled the pharmacological discrimination of endogenous DHP-sensitive $Ca_V1.2$ channels from the virally transduced DHP-resistant Ca^{2+} current. They found that phosphorylation of Ser1928 was not required for β-adrenergic stimulation of $Ca_V1.2$. Additionally, using adenoviral overexpression of mutant β_2 subunits in cardiomyocytes, they demonstrated that phosphorylation of Ser478 and Ser479 of the β_2 subunit was not required. Similarly, using adenoviral overexpression of β_2 subunits, the Colecraft group (50) demonstrated that phosphorylation of Ser459 of the β_2 subunit was also not required for β-adrenergic stimulation of Ca^{2+} currents in cardiomyocytes.

DHP: dihydropyridine

Experiments using knockin mice provided more definitive evidence that phosphorylation of neither Ser1928 on the pore-forming α_{1C} subunit nor multiple sites on the C terminus of the auxiliary β_2 subunit were necessary for β-adrenergic stimulation of Ca^{2+} currents in cardiomyocytes. Alanine substitution of Ser1928 had no effect on basal or β-adrenergic agonist stimulation of Ca^{2+} current in isolated ventricular cardiomyocytes (51). Insertion of a stop codon designed to delete the variable C terminus of the β_2 subunit, which included all known PKA phosphorylation sites in β, did not prevent β-adrenergic regulation of $Ca_V1.2$ (52). Furthermore, mice with both alanine substitution of α_{1C} Ser1928 and deletion of the C-terminal residues of the β_2 subunit did not attenuate β-adrenergic stimulation of the $Ca_V1.2$ current (52), suggesting that regulation was not redundant at least for these residues. Thereafter, the Catterall group (53) identified two previously unrecognized phosphorylation sites in the C terminus of the skeletal muscle $Ca_V1.1$ channel by mass spectrometry (MS). These putative phosphorylation sites were conserved in $Ca_V1.2$ α_{1C} subunits at Ser1700 and threonine 1704 (Thr1704) (53). Ser1700 was predicted to be a substrate for PKA and

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), whereas Thr1704 was predicted to be a substrate of casein kinase II.

MS: mass spectrometry

TESTING COMBINATIONS OF PHOSPHORYLATION SITES IN α_{1C} AND β_{2B} SUBUNITS

Based on the lack of progress in identifying regulatory mechanisms, we felt that a combination of phosphorylation sites on the α_{1C} and β_2 subunits could explain the findings described above (Figure 2a). Overexpression of α_{1C} or β subunits can attenuate the adrenergic stimulation of the Ca^{2+} channels in the heart (50, 54–56) and can induce cardiac dysfunction or apoptosis (57–60). Therefore, we developed an approach of using a doxycycline-inducible, tissue-specific, transgenic mouse–expressing FLAG-epitope-tagged, DHP-resistant α_{1C}, which preserves hormonal regulation of $Ca_V1.2$ by limiting $Ca_V1.2$ overexpression (61) (Figure 2b). Similar to the work of O'Rourke and colleagues (49), we were able to distinguish between native DHP-sensitive $Ca_V1.2$ channels and transgenic DHP-resistant $Ca_V1.2$ using nisoldipine, a DHP antagonist. We have also used a similar approach to express mutant tetrodotoxin-sensitive or lidocaine-resistant $Na_V1.5$ channels in the heart (62–64). Initially, we generated two transgenic mice with inducible cardiomyocyte-specific expression: (*a*) mice with N-terminal 3X FLAG-epitope–tagged, DHP-resistant α_{1C}, designated pseudowild-type (pWT) α_{1C}; and (*b*) mice with alanine substitutions of Ser1700 and Thr1704 as well as deletion of the presumed proteolytic cleavage site. We found that isoproterenol and forskolin regulation of the DHP-resistant channels in isolated pWT and S1700A/T1704A mice cardiomyocytes were equivalent, implying that phosphorylation of these two residues is not essential for β-adrenergic stimulation of Ca^{2+} currents (61).

Subsequently, S1700A and combined S1700A/T1704A knockin mice were generated by the Catterall group (65, 66) and were reported to have reduced basal and β-adrenergic-induced activation of Ca^{2+} currents. They based their conclusion, however, on an unconventional metric: the difference in absolute current amplitude rather than the fold increase after isoproterenol. Their metric is valid if the surface density of Ca^{2+} channels was unchanged, yet basal Ca^{2+} currents were substantially reduced (65, 66). Hofmann and colleagues (67) independently created S1700A/T1704A knockin mice, concluding that isoproterenol stimulated Ca^{2+} current in the control and mutant S1700A/T1704A cardiomyocytes to the same extent. Furthermore, Hofmann recalculated Catterall's data and showed that in both groups' knockin mice, the β-adrenergic stimulation for WT and mutant channels was equivalent. This confirmed our initial findings (61), which we have further substantiated with additional transgenic mice (68–70).

The use of DHP-resistant transgenic mice is an ideal approach to test the hypothesis that more than one phosphorylation site on α_{1C} is required for adrenergic regulation. We identified conserved PKA consensus sequences, using bioinformatic methods, in the α_{1C} subunit of five species: mouse, rat, rabbit, guinea pig, and human (Figure 2c). We then generated transgenic mice in which 17 conserved consensus PKA phosphorylation

sites that were not previously studied and 5 conserved PKA/CaMKII phosphorylation sites known to be nonessential (including Ser1700 and Thr1704) were mutated to alanine (42, 49, 51, 52). Surprisingly, none were necessary (70). Could the functionally relevant PKA targets in α_{1C} be different among these five species? To test this possibility, we generated transgenic mice with alanine mutations at additional potential PKA sites in the mouse channel that were not conserved in other species, with a total of 51 alanine substitutions at 35 sites (Figure 2c). DHP-resistant 35-α mutant Ca^{2+} channels were stimulated by isoproterenol or forskolin (Figure 2e), indicating that phosphorylation of α_{1C} is not essential for adrenergic stimulation of $Ca_V1.2$ (68). The simplest explanation for this finding is that the β subunit contains previously unappreciated PKA phosphorylation sites (52). Similar to the approach used for testing putative phosphorylation sites in the α_{1C} subunit, we generated transgenic mice expressing mutant human β_{2B} subunits in which 37 alanine substitutions were made within 28 consensus PKA phosphorylation sites (68) (Figure 2d). Because there is no pharmacological approach to select for channels with mutant β subunits, we relied on the overexpression of mutant β subunits to competitively replace WT β_2 subunits in the $Ca_V1.2$ complex, which was confirmed using coimmunoprecipitation studies. $Ca_V1.2$ channels with the mutant β subunit displayed a normal isoproterenol- or forskolin-induced increase in peak Ca^{2+} current (Figure 2f) and a hyperpolarizing shift in the V_{50} of activation. Furthermore, mice expressing the 35-α mutant and the 28-β mutant were crossed, and the DHP-resistant channels in these double-mutant (α_{1C} and β_2) progeny also displayed normal adrenergic regulation (68) (Figure 2g). These studies rule out a role for all consensus PKA phosphorylation sites in α_{1C} and show that phospho-regulatory sites on α_{1C} and β_{2B} are not redundant and do not each fractionally contribute to the stimulatory effect of β-adrenergic agonists.

ROLE OF β SUBUNIT IN ADRENERGIC REGULATION OF $Ca_V1.2$

Global or cardiac-specific deletion of the dominant *Cacnb2* gene is embryonic lethal due to abnormal heart development (71). Similarly, in cells heterologously expressing $Ca_V1.2$ channels, β is obligatory for α_{1C} trafficking to the plasma membrane and for normalizing channel activation and inactivation gating properties (72–75). Unexpectedly, cardiomyocyte-specific, conditional deletion of the *Cacnb2* gene in adult mice caused only a modest 29% reduction in Ca^{2+} current, with no obvious cardiac impairment (76). There were two possible interpretations for this unexpected result. First, in adult myocytes, β binding to α_{1C} is not absolutely required for $Ca_V1.2$ surface expression. Alternatively, the remaining ~4% of β_2 expression and the relatively low level of β_3 expression could be sufficient for the trafficking and function of Ca^{2+} channels in the adult heart.

Although PKA phosphorylation of Ser or Thr residues in the β subunit is not required for adrenergic activation of $Ca_V1.2$ (68, 69), we suspected that β subunit binding to α_{1C} may be required. We created transgenic mice with mutations in the AID of the α_1-subunit I-II loop, thereby preventing the high-affinity binding of β and α_{1C} subunits (11–13, 77). When expressed in cardiomyocytes, the FLAG-tagged AID-mutant α_{1C} did not bind the β subunit. In contrast to heterologously expressed AID-mutant α_{1C} channels, but consistent with the results obtained from the conditional β_2 knockout mice (76), β-less Ca^{2+} channels in cardiomyocytes were capable of trafficking to the dyadic membrane (69). AID-mutant α_{1C}

channels exhibited rare sojourns to the high-activity mode and had a higher propensity for blank and low activity sweeps (78). Furthermore, the β-less Ca^{2+} channels were completely unresponsive to isoproterenol (69). Taken together, these findings suggest that β subunits are required for Ca^{2+} channels to enter a high P_o state even though phosphorylation of β is not required.

The DHP-resistant AID-mutant transgenic mice also offered insights into the role of β-adrenergic stimulation of $Ca_V1.2$ in the fight or flight response (69): (*a*) In isolated AID-mutant α_{1C} cardiomyocytes, isoproterenol, in the presence of nisoldipine, increased fractional shortening of myocytes by only 25% compared to 100% in pWT α_{1C} cardiomyocytes; and (*b*) at the organ level, 200 nM isoproterenol increased cardiac contractility by 3.3 fold, whereas in hearts isolated from the AID-mutant mice, isoproterenol increased cardiac contractility by only 1.2 fold (69). This finding was predicted from modeling studies of rabbit (79) and mouse ventricular myocytes (80–82), both of which showed that specific removal of PKA stimulation of $Ca_V1.2$ decreased both the Ca^{2+} transient and force below basal levels. In contrast, acute removal of RyR2 phosphorylation or PLB phosphorylation had much smaller or negligible effects.

ROLE OF C-TERMINAL PROTEOLYTIC CLEAVAGE OF α_{1C} IN ADRENERGIC REGULATION OF $Ca_V1.2$

Posttranslational proteolytic cleavage of α_{1C} yields a distal C-terminal truncated α_{1C} of ~210 kDa (42, 45, 83–85). The fraction of cleaved α_{1C} subunits in cardiomyocytes is unresolved, with estimates ranging from none to full truncation, perhaps related to species, age, and experimental conditions (86). With optimized rapid extraction conditions, such as high concentrations of the calpain inhibitor, EGTA, and all solutions and instruments precooled to 0°C, immunoblotting showed that ~50% of the detectable α_{1C} is in its long form in heart extracts (87). In skeletal muscle, the site of cleavage was determined by MS to be at Ala1664, which corresponds to Ala1800 in cardiac α_{1C}. The distal C-terminal fragment is presumed to remain coupled by interactions with the proximal fragment, tonically inhibiting it (42, 84, 85, 88, 89). The distal C terminus is also partially localized to the nucleus where it may modify gene expression in neurons, smooth muscle, and cardiomyocytes (90–92).

Heterologous expression of an α_{1C} cDNA with deletion of the distal C terminus demonstrated an increased current amplitude and P_o and a hyperpolarizing shift in activation, which are similar to what is observed after adrenergic stimulation of $Ca_V1.2$ in the heart. Thus, it was speculated that PKA regulation of $Ca_V1.2$ requires the proteolytic cleavage of the distal C terminus and subsequent phosphorylation-dependent release of the inhibition imparted by the distal C terminus (53, 93, 94). Furthermore, Dascal's group (95) demonstrated a small cAMP-/PKA-dependent increase in Ca^{2+} current in truncated α_{1C} subunits but not full-length α_{1C} subunits when heterologously expressed in *Xenopus* oocytes. Taken together, cleavage is proposed to set the basal $Ca_V1.2$ activity, which is then augmented by adrenergic stimulation (42, 53, 84, 85, 89, 96–98). One caveat is that a truncated α_{1C} subunit is never expressed without its distal C terminus in vivo. Furthermore,

knockin mice expressing a truncated α_{1C} at either Gly1796 or Asp1904 die at birth due to low membrane expression of $Ca_V1.2$ (98, 99), which is inconsistent with the studies performed using heterologous expression.

The functional relevance of proteolytic cleavage of α_{1C} has not been demonstrated in cardiomyocytes. Indirect evidence, consisting of MS analysis of skeletal muscle α_{1S} proteolytic peptides and sequence alignments of α_{1S} and α_{1C}, was the basis for the hypothesis that Ala1800 within the ^{1798}NNANN motif is the α_{1C} proteolytic site (85). The persistence of α_{1C} cleavage in cardiomyocytes after deletion of the ^{1798}NNANN motif could result from the presence of a nearby similar motif, ^{1794}NANI^{1797}. Thus, we created a transgenic mouse with cardiomyocyte expression of α_{1C} with deletion of ^{1794}NANINNANN^{1802}, along with deletion of a nearby PEST sequence (^{1769}DTESP) (70). Deletion of these sites, but not the PEST sequence alone, yielded a channel that was resistant to proteolytic cleavage. Further, we found that C-terminal proteolytic cleavage of α_{1C} is not required for β-adrenergic stimulation of $Ca_V1.2$ in the heart. Although these experiments clearly rule out the necessity of C-terminal proteolytic cleavage for adrenergic agonist stimulation of Ca^{2+} channels in the heart, the methodology cannot address whether the C-terminal fragment is required for regulating transcription in the heart (91, 92).

ROLE OF A-KINASE-ANCHORING PROTEINS IN ADRENERGIC REGULATION OF $Ca_V1.2$

AKAPs may determine the specificity and the speed by which targets, such as ion channels, respond to sympathetic nervous system stimulation (100). During adrenergic stimulation, Ca^{2+} current amplitude increases at only half the rate of rising cAMP levels (101), implying that phosphorylation is rate limiting and that disrupting localization of PKA should be evident by the reduced rate and amplitude of stimulation. The importance of AKAPs in the adrenergic regulation of $Ca_V1.2$ is supported by considerable experimental evidence. Three groups have independently shown that β-adrenergic modulation of Ca^{2+} currents in neonatal rat, adult rat, and adult mouse cardiomyocytes is markedly blunted by the intracellular dialysis, via a patch pipette, of peptides designed to competitively disrupt the binding of PKA to an AKAP (45, 102–104). Although considerable evidence initially pointed to important roles for Akap5 and Akap7 (45, 53, 89, 104–107), mice in which Akap5, Akap7, or both are deleted retained full β-adrenergic upregulation of $Ca_V1.2$ channels in ventricular myocytes in response to isoproterenol (108, 109). Thus, it is likely that an AKAP other than Akap5 and Akap7 is required for β-adrenergic regulation of $Ca_V1.2$, or that several different AKAPs can permit PKA localization to the channel.

Cypher/Zasp, a member of the PDZ–LIM domain family that directly complexes with Z-line-associated proteins such as α-actinin-2, was proposed as an AKAP that regulates $Ca_V1.2$ function (110). In Cypher/Zasp null mice, the cell surface density of $Ca_V1.2$ channels and the basal Ca^{2+} current were significantly reduced despite a large increase in total $Ca_V1.2$ protein, indicating that the assembly of $Ca_V1.2$, insertion into the cell surface, and stability of $Ca_V1.2$ on the cell surface may be impaired. Isoproterenol stimulated Ca^{2+} currents by nearly twofold in Cypher/Zasp null mice, which was decreased by ~30%

compared to control hearts (see 110, figure 3b). Taken together, these results suggest that Cypher/Zasp may contribute to β-adrenergic regulation, but there is likely redundancy.

USING PROXIMITY PROTEOMICS IN THE HEART TO ELUCIDATE ADRENERGIC SIGNALING

An alternative hypothesis to explain adrenergic regulation of $Ca_V1.2$ is that PKA phosphorylates an additional protein in the Ca^{2+} channel complex instead of direct phosphorylation of α_{1C} or β. The PKA target could be either a channel activator, recruited to the $Ca_V1.2$ complex, or a channel inhibitor, released from either α_{1C} or β subunits.

Important interacting proteins may associate transiently and as such are unlikely to be detected by coimmunoprecipitation. Moreover, standard methodologies to detect protein–protein interactions have limitations and do not always represent the protein interactions within the native intracellular milieu. Even among directly interacting proteins, many may have dissociation constants >10–100 μM and fast off-rate constants (111). We developed an in vivo platform to identify the interactome of $Ca_V1.2$ by adapting the ascorbate peroxidase 2 (APEX2) methodology (112–115). Unlike other proteome network maps in cardiomyocytes, this method enabled the identification of interacting and bystander proteins within ~20 nm in living cells (68). APEX2 is ideally suited for relatively rapid dynamic changes in subcellular neighborhoods because the labeling time is relatively short compared to biotin ligases such as TurboID or BioID (114–121).

APEX2: ascorbate peroxidase 2

We created transgenic mice with inducible, cardiomyocyte-specific expression of DHP-resistant α_{1C} or β_{2B} with APEX2 and a V5 epitope conjugated to the N termini (Figure 3a). Fusing APEX2 to α_{1C} and β_{2B} did not affect β-adrenergic agonist stimulation of $Ca_V1.2$ current (68) or the subcellular localization of $Ca_V1.2$ channels in the T-tubules (Figure 3b). Incubating isolated ventricular cardiomyocytes with biotin-phenol followed by exposure to H_2O_2 induced robust biotinylation of proteins in a striated z-disk pattern, which is consistent with localization of $Ca_V1.2$ channels at T-tubules (Figure 3b). Biotinylated proteins were affinity purified in denaturing conditions using streptavidin. Western blotting and tandem mass tag synchronous precursor selection triple-stage mass spectrometry (TMT SPS MS^3) demonstrated the enrichment of known interacting proteins, such as calmodulin and junctophilin, as well as bystander proteins, such as RyR and NCX. Using proximity labeling in both isolated cardiomyocytes and Langendorff-perfused hearts and TMT SPS MS^3 (Figure 3c), we found that isoproterenol induced a change in the extent of biotinylation of several proteins. Increased biotinylation of the PKA catalytic subunit likely indicated recruitment to the $Ca_V1.2$ channel neighborhood, whereas decreased biotinylation of Rad (Ras associated with diabetes) was indicative of reduced accessibility to labeling, likely due to less Rad near $Ca_V1.2$ channels (Figure 3d,e). In contrast, the amount of Rad did not change in cardiomyocytes isolated from nontransgenic mice (68).

TMT SPS MS^3: tandem mass tag synchronous precursor selection triple-stage mass spectrometry

Rad: Ras associated with diabetes

RAD IS THE PKA TARGET

Rad is a member of the RGK (Rad, Rem, Rem2, Gem/Kir) Ras family of proteins. In a seminal study, Gem/Kir was discovered in a yeast two-hybrid screen of MIN6 cells as an interacting protein of $Ca_V\beta_3$ (122). All RGK proteins are intracellular inhibitors of high-voltage-activated Ca^{2+} channels (123–126). Sequence analysis and electrophysiological studies suggest that the interaction between RGK proteins and Ca^{2+} channels has been strictly conserved, originating prior to the deuterostome/protostome split (127). Rad, originally discovered as a protein overexpressed in skeletal muscle of patients with diabetes, is expressed in heart, placenta, lung, and skeletal muscle (128). Mice with deletion of Rad, either globally or specifically in heart, displayed increased basal Ca^{2+} currents with activation at lower voltages and reduced β-adrenergic stimulation of Ca^{2+} channels in isolated cardiomyocytes (129–131). Conditional Rad knockout mice also have elevated heart rates at baseline and during sleep (132).

RGK: Rad, Rem, Rem2, Gem/Kir

In patients with end-stage heart failure undergoing transplantation, Rad mRNA and protein levels were substantially reduced compared to controls (133). Could this be a natural compensatory mechanism to enhance Ca^{2+} entry and increase contractility, as was observed in Rad knockout mice (129–131, 134)? A reduction in Rad could also account, at least in part, for decreased adrenergic reserve. Furthermore, Rad may play an important role in cardiac hypertrophy. Within 24 h after transverse aortic constriction in rats, Rad protein levels decreased, which persisted for at least 14 days (133). In human embryonic stem cell (H9 cell line)-derived cardiomyocytes, deletion of Rad caused a hypertrophic phenotype, which was attenuated by blocking the increased Ca^{2+} current with verapamil (135).

The robust heterologous reconstitution of PKA regulation of $Ca_V1.2$ currents has been long pursued (136) but was unachievable with expression of only α_{1C} and β subunits (Figure 4a). We found that Rad was the missing ingredient (68). Applying forskolin to HEK293T cells expressing WT α_{1C} + β_{2B} + Rad increased the maximal conductance (G_{max}) (Figure 4b) and shifted the V_{50} for activation, similar to our observations in cardiomyocytes. Previous studies identified a single PKA phosphorylation site on the C terminus of Rad (137). Using MS, we identified an additional three phosphorylated residues in Rad after stimulation with forskolin (68). Alanine substitutions of these four Ser, two on the N terminus and two on the C terminus of Rad (4SA-mutant), prevented the forskolin-induced increase in G_{max} and V_{50} shift. Phosphorylation of two Ser residues in the C-terminal polybasic membrane region of Rad is crucial to PKA regulation of $Ca_v1.2$. Alanine substitutions at Ser272 and Ser300

(2SA-mutant) prevented both the forskolin-induced increase in G_{max} (Figure 4c) and V_{50} shift (68). Similar to cardiomyocytes, the forskolin-induced stimulation of Ca^{2+} channels was not dependent on phosphorylation of either the α_{1C} or β_{2B} subunits in HEK cells expressing Rad. The phosphorylation sites in Rad are conserved across species and across other members of the RGK GTPase family (68). Thus, at baseline, Rad, inhibits $Ca_V1.2$; on adrenergic activation, PKA phosphorylates Rad, which releases this inhibition. Disinhibition equals activation.

ADRENERGIC $CA_V1.2$ ACTIVATION VIA RAD PHOSPHORYLATION CONVERGES AT THE α_{1C} I-II LOOP

Rad can inhibit $Ca_V1.2$ via binding to either the α_{1C} or β subunits (138). We demonstrated that PKA phosphorylation of Rad markedly reduces Rad binding to the β subunit. Moreover, eliminating Rad binding to β, via mutation of the interaction site on either β or Rad, prevented both Rad inhibition and the adrenergic regulation of Ca^{2+} channels (68). The requirement of the β subunit is consistent with the prior findings that adrenergic regulation of $Ca_V1.2$ requires the interaction of α_{1C} and β subunits (69).

To explore how distal conformational changes involving Rad interaction with the $Ca_V\beta$ subunit and phosphorylation-dependent signaling are ultimately conveyed to the channel pore-domain, we generated transgenic mice with expression of $Ca_V1.2$ α_{1C} subunits with flexibility-inducing polyglycine substitutions in the I-II loop (GGG-α_{1C}) (78). These mutations have been previously shown to disrupt coupling between the pore-domain and the AID of the α_{1C} subunit (77, 139, 140). Introducing three glycine residues that disrupt a rigid IS6-AID helix reduced basal P_o despite intact binding of $Ca_V\beta$ to the α_{1C} I-II loop and eliminated β-adrenergic agonist stimulation of $Ca_V1.2$ current. Thus, we speculate that (*a*) $Ca_V\beta$ binding to α_{1C} stabilizes an increased P_o gating mode by a mechanism that requires an intact rigid linker between the β subunit binding site in the I-II loop and the channel pore; and (*b*) the release of Rad-mediated inhibition of Ca^{2+} channel activity by β-adrenergic agonists requires phosphorylation of the C terminus of Rad, which leads to decreased binding of Rad to the β subunit. With the dissociation of Rad, the β subunit can stabilize a high P_o gating mode.

RGK GTPase REGULATION OF $Ca_V1.3$ AND $Ca_V2.2$

The family of RGK GTPases is known to inhibit other voltage-gated Ca^{2+} channels. We speculated that phosphorylation of RGK GTPases could be a common mechanism to regulate voltage-gated Ca^{2+} channels that bind β subunits. $Ca_V1.3$ channels contribute to pacemaker activity in the sinus node and atrial cells (141) and are expressed in adrenal chromaffin cells (142). Similar to $Ca_V1.2$ channels, expression and PKA phosphorylation of Rad are also required for PKA-dependent activation of heterologously expressed $Ca_V1.3$ channels (68) (Figure 4d). $Ca_V2.2$ channels are expressed in presynaptic terminals in the brain. Coexpression of Rad or Rem enabled PKA-dependent activation of $Ca_V2.2$ channels in HEK cells (68). Thus, it is apparent that this mechanism of regulation of voltage-gated Ca^{2+} channels is modular.

CONCLUSIONS AND FUTURE DIRECTIONS

Despite the importance of adrenergic regulation of cardiac function, elucidating the underlying mechanisms for β-adrenergic stimulation of Ca^{2+} influx has been difficult. Similar to the inhibition of SERCA by PLB and the release of inhibition by PKA and CaMKII phosphorylation of PLB, the adrenergic regulation of Ca^{2+} channels involves a phosphorylation-dependent disinhibition mediated by RGK GTPases (Figure 4e,f). Phosphorylation of α_{1C} or β_{2B} subunits is not required in the heart (68). The relevant C-terminal phosphorylation sites of Rad are conserved through evolution and in all RGK proteins, implying the importance of this signaling pathway in other tissues. We speculate that this mechanism of regulation is advantageous by permitting tissue-specific modulation because $Ca_V1.2$ is expressed ubiquitously, whereas Rad is not, and the extent of adrenergic regulation varies substantially in different organs, with the heart being the greatest. Although phosphorylation of Ser1928 in the C terminus of α_{1C} is not required for adrenergic regulation of $Ca_V1.2$ in cardiomyocytes (49, 51, 68, 70) or for adrenergic agonist stimulation of heterologously expressed $Ca_V1.2$ channels in HEK cells (68) and *Xenopus* oocytes (143), it has been shown to modulate Ca^{2+} currents in hippocampal neurons and vascular smooth muscle (106, 144, 145). The mechanisms that impart a stimulatory effect on Ser1928 phosphorylation in hippocampal neurons and vascular smooth muscle, but not cardiomyocytes, are not known. The experimental conditions may be important, as Ser1928-dependent stimulation in hippocampal neurons was recorded in the presence of Bay K 8644, an activator of $Ca_V1.2$ (145). As opposed to the physiological role for β-adrenergic agonist stimulation of Ca^{2+} currents in the heart, β-adrenergic agonists promote vasorelaxation in the vasculature, dependent on activation of K^+ channel–induced hyperpolarization and a subsequent reduction in Ca^{2+} influx in vascular smooth muscle. In vascular smooth muscle, the role of Ser1928 phosphorylation may be linked to hyperglycemia-mediated activation of purinergic receptors, which promotes Ca^{2+} influx and increased vasoreactivity (106, 146).

Identification of the mechanisms responsible for adrenergic regulation of $Ca_V1.2$ and $Ca_V1.3$ enables substantial additional investigations. The next steps will be to create genetically altered mice with mutations of the phosphorylation sites of Rad or the interacting sites of β_{2B} or Rad. Conditions such as heart failure or hypertrophy alter the global expression of Rad in animals and humans (133), but we do not yet know whether heart failure causes changes in the $Ca_V1.2$ macromolecular complex or whether signaling that affects Rad phosphorylation is altered. Harnessing the mechanism by which the sympathetic nervous system regulates Ca^{2+} influx could be an innovative approach for the treatment of heart failure and arrhythmias.

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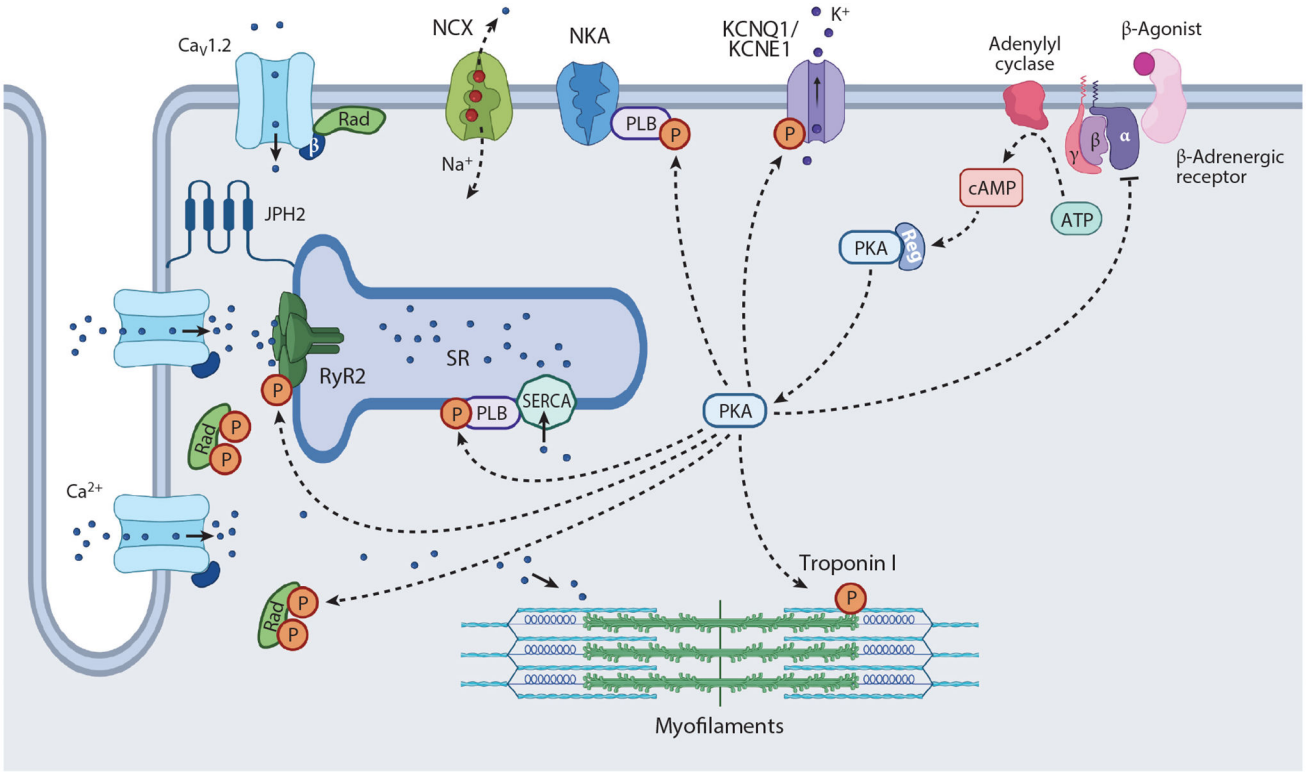
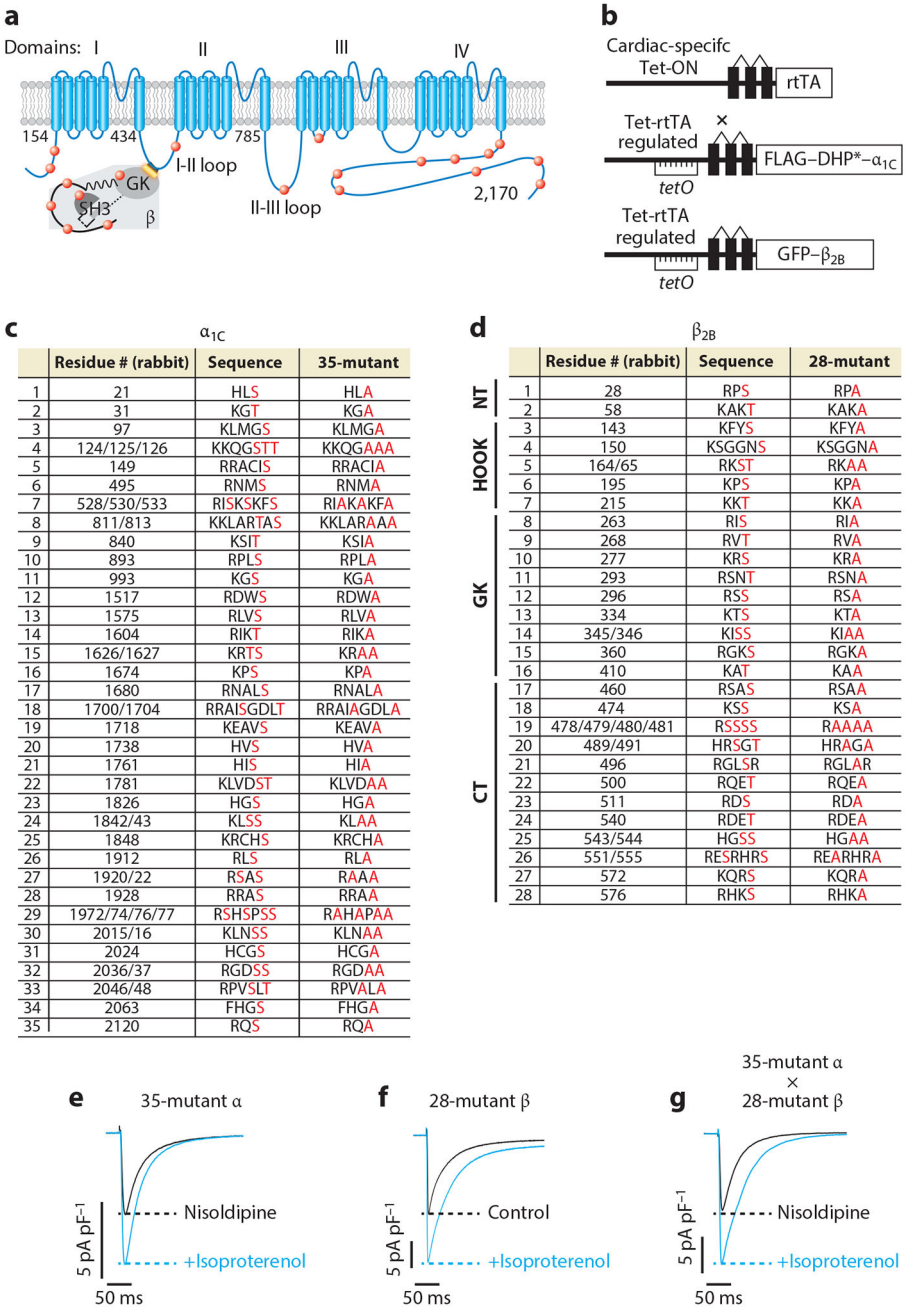


Figure 1.

Schematic depicting β-adrenergic agonist stimulation in cardiomyocytes. Depolarization of membrane potential activates L-type Ca^{2+} channels, principally $Ca_V1.2$, in transverse tubules, which triggers RyR2 to open. Ca^{2+} stored in the SR is released, which binds troponin C, leading to myofilament cross-bridging. Thereupon, Ca^{2+} influx and Ca^{2+} release from the SR are terminated, and Ca^{2+} is either pumped back into the SR by the SERCA or transported out of the cell by the NCX. β-Adrenergic agonist binds to β-adrenergic receptor, which increases cAMP generation and activates PKA. PKA phosphorylates Rad, PLB, and RyR2, which causes increased Ca^{2+} influx, SR Ca^{2+} release, and SR Ca^{2+} reuptake, thereby augmenting inotropy and lusitropy. Phosphorylation of troponin I and KCNQ1 also contribute to enhanced lusitropy, the latter through increases in the I_{KS}. Abbreviations: I_{KS}, slowly activating delayed rectifier KCNQ1 channel; JPH2, junctophilin 2; NCX, Na^+-Ca^{2+} exchanger; NKA, Na^+-K^+-ATPase; P, phosphorylation; PKA, protein kinase A; PLB, phospholamban; Rad, Ras associated with diabetes; Reg, regulatory; RyR2, ryanodine receptor 2; SERCA, sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum. Figure adapted from image created with BioRender.com.



	Residue # (rabbit)	Sequence	35-mutant
1	21	HLS	HLA
2	31	KGT	KGA
3	97	KLMGS	KLMGA
4	124/125/126	KKQGSTT	KKQGAAA
5	149	RRACIS	RRACIA
6	495	RNMS	RNMA
7	528/530/533	RISKSKFS	RIAKAKFA
8	811/813	KKLARTAS	KKLARAAA
9	840	KSIT	KSIA
10	893	RPLS	RPLA
11	993	KGS	KGA
12	1517	RDWS	RDWA
13	1575	RLVS	RLVA
14	1604	RIKT	RIKA
15	1626/1627	KRTS	KRAA
16	1674	KPS	KPA
17	1680	RNALS	RNALA
18	1700/1704	RRAISGDLT	RRAIAGDLA
19	1718	KEAVS	KEAVA
20	1738	HVS	HVA
21	1761	HIS	HIA
22	1781	KLVDST	KLVDAA
23	1826	HGS	HGA
24	1842/43	KLSS	KLAA
25	1848	KRCHS	KRCHA
26	1912	RLS	RLA
27	1920/22	RSAS	RAAA
28	1928	RRAS	RRAA
29	1972/74/76/77	RSHSPSS	RAHAPAA
30	2015/16	KLNSS	KLNAA
31	2024	HCGS	HCGA
32	2036/37	RGDSS	RGDAA
33	2046/48	RPVSLT	RPVALA
34	2063	FHGS	FHGA
35	2120	RQS	RQA

		Residue # (rabbit)	Sequence	28-mutant
NT	1	28	RPS	RPA
	2	58	KAKT	KAKA
	3	143	KFYS	KFYA
HOOK	4	150	KSGGNS	KSGGNA
	5	164/65	RKST	RKAA
	6	195	KPS	KPA
	7	215	KKT	KKA
GK	8	263	RIS	RIA
	9	268	RVT	RVA
	10	277	KRS	KRA
	11	293	RSNT	RSNA
	12	296	RSS	RSA
	13	334	KTS	KTA
	14	345/346	KISS	KIAA
	15	360	RGKS	RGKA
	16	410	KAT	KAA
CT	17	460	RSAS	RSAA
	18	474	KSS	KSA
	19	478/479/480/481	RSSSS	RAAAA
	20	489/491	HRSGT	HRAGA
	21	496	RGLSR	RGLAR
	22	500	RQET	RQEA
	23	511	RDS	RDA
	24	540	RDET	RDEA
	25	543/544	HGSS	HGAA
	26	551/555	RESRHRS	REARHRA
	27	572	KQRS	KQRA
	28	576	RHKS	RHKA

Figure 2.

β-Adrenergic regulation of $Ca_V1.2$ does not require protein kinase A (PKA) phosphorylation of α_{1C} and β subunits. (*a*) Schematic of rabbit cardiac α_{1C} and β subunits. Red dots indicate some of the putative PKA phosphorylation sites. (*b*) Schematic of the transgene system (147) used for the creation of transgenic mice. Reverse tetracycline-controlled transactivator (rtTA) expression is driven by an α-myosin heavy chain (αMHC) promoter. The cDNAs for FLAG-DHP-resistant (DHP*) α_{1C} or green fluorescent protein (GFP)-β_{2B} were ligated behind *tetO* sequences. (*c*) The 35 putative PKA phosphorylation sites in rabbit α_{1C}. The 51 residues in red, denoting predicted phosphorylation sites or within the

immediate region of the predicted phosphorylation site, were replaced with alanine in the 35-mutant α_{1C} transgenic mice. (*d*) The 37 residues within 28 sites are either predicted phosphorylation sites or within the immediate vicinity of predicted phosphorylation sites and were mutated to alanine in the 28-mutant β_{2B} transgenic mice. The sites are distributed in the N-terminal (NT), HOOK, guanylate kinase–like (GK), and C-terminal (CT) domains of β_{2B}. (*e–g*) Exemplar whole-cell $Ca_V1.2$ currents of 35-mutant α cardiomyocytes, 28-mutant β transgenic mice cardiomyocytes, and 35-mutant α X 28-mutant β transgenic mice cardiomyocytes. Figure adapted from Reference 68.

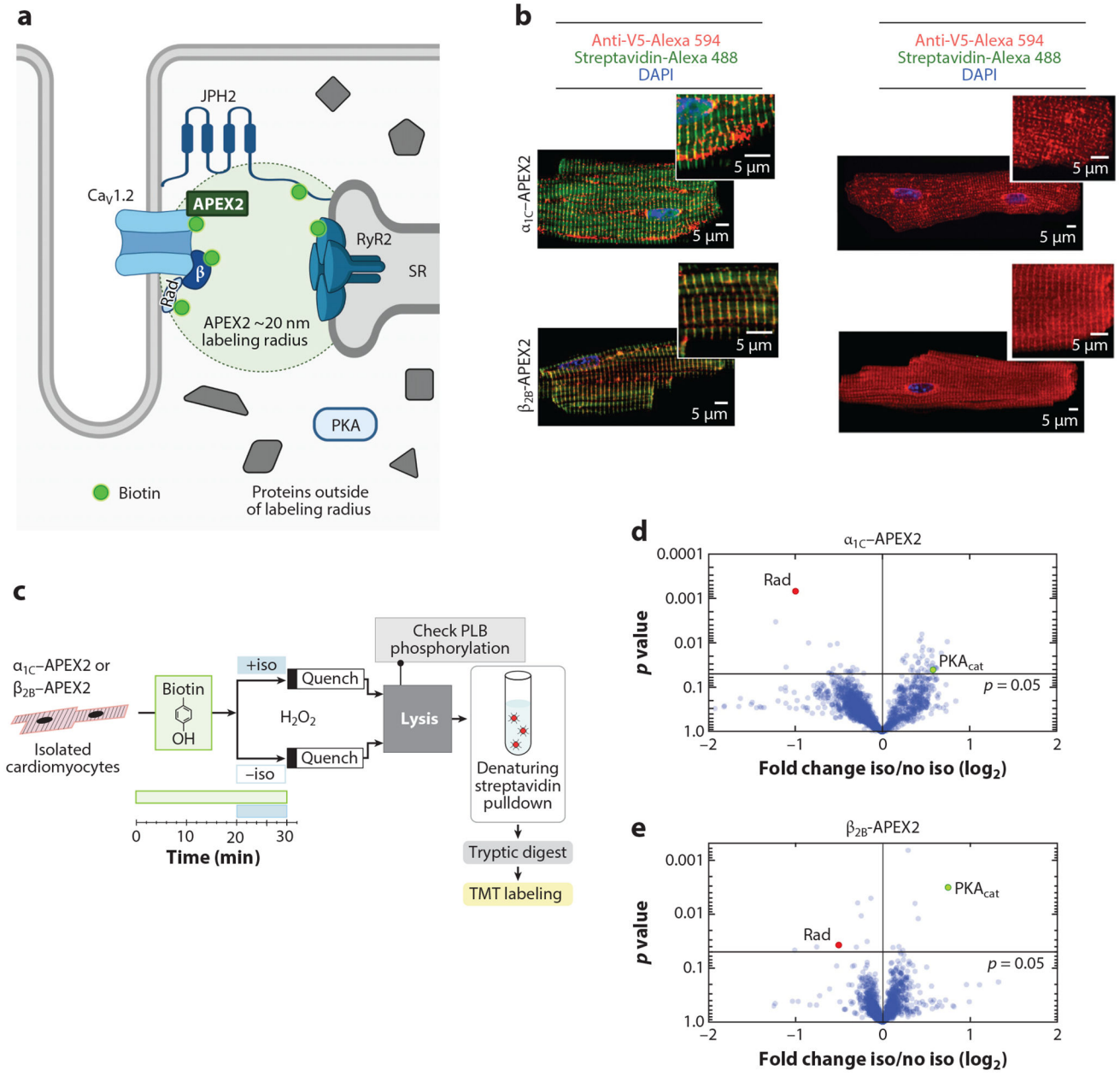


Figure 3.

Proximity labeling using APEX2 to identify the mechanism underlying adrenergic agonist-induced augmentation of Ca^{2+} current. (*a*) Schematic depicting localization of APEX2-conjugated $Ca_V1.2$ channels in the dyadic space of cardiomyocytes. Panel *a* adapted from image created with BioRender.com. (*b*) Immunofluorescence of cardiomyocytes isolated from $α_{1C}$-APEX2- and $β_{2B}$-APEX2-expressing mice exposed to biotin-phenol and H_2O_2 or no H_2O_2. Nuclear labeling with DAPI stain. (*c*) Schematic of workflow for isolated cardiomyocytes. (*d–e*) Volcano plots of fold-change for relative protein quantification by tandem mass tag mass spectrometry of $α_{1C}$-APEX2 and $β_{2B}$-APEX2 samples. Non-adjusted unpaired two-tailed *t*-test. Rad (*red dots*) is reduced and PKA catalytic subunit (PKA_{cat}; *green dots*) is increased. Panels *b–e* adapted from Reference 68. Abbreviations: APEX2,

ascorbate peroxidase 2; JPH2, junctophilin 2; PLB, phospholamban; Rad, Ras associated with diabetes; RyR2, ryanodine receptor 2; SR, sarcoplasmic reticulum; TMT, tandem mass tag.

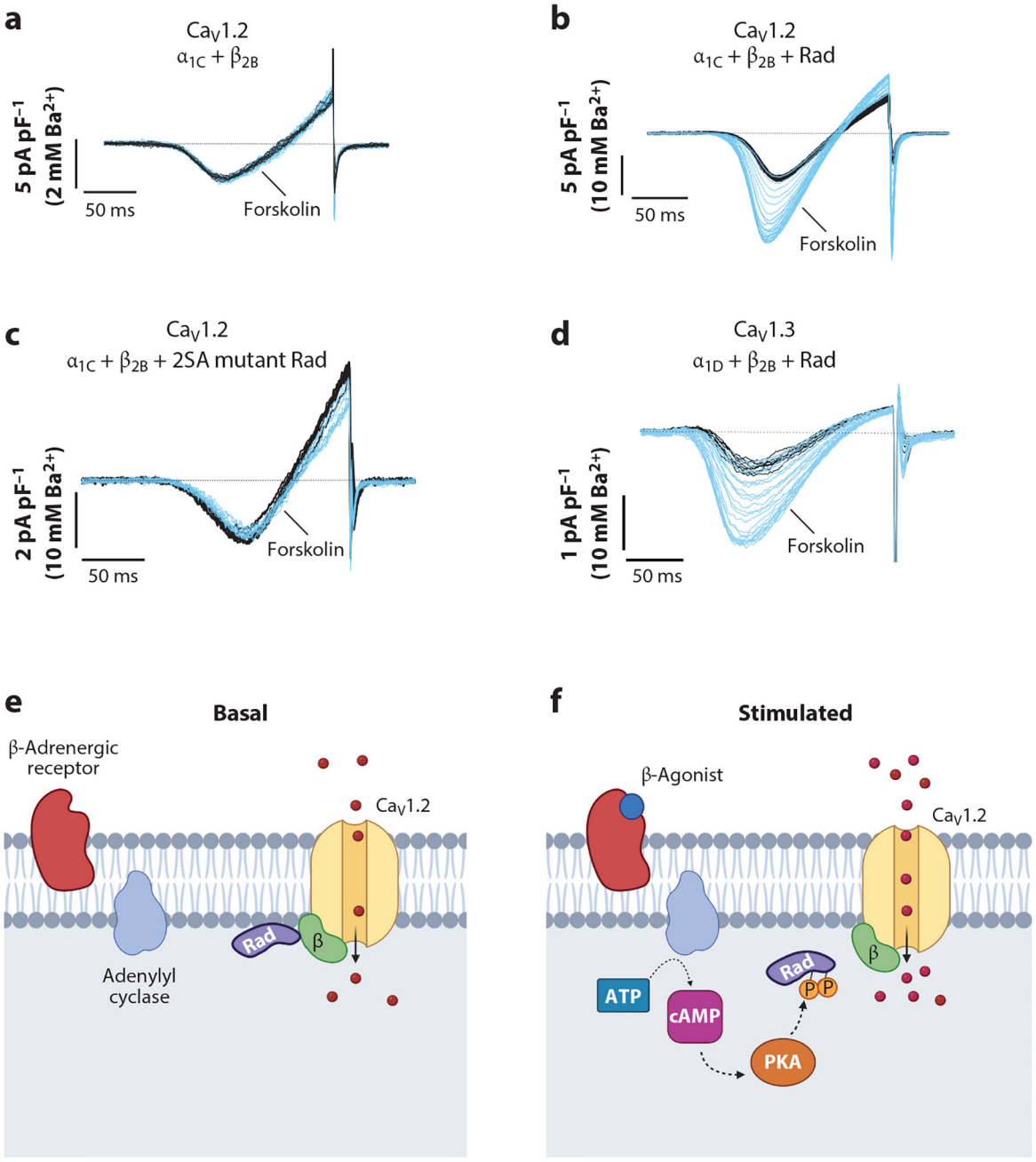


Figure 4.

Phosphorylation of Rad is required for cAMP-PKA activation of voltage-gated Ca^{2+} channels. (*a–c*) Ba^{2+} current elicited by voltage ramp every 10 s, with black traces obtained before and blue traces obtained after forskolin. The α_{1C}, β_{2B}, and WT and mutant Rad were heterologously expressed in HEK293T cells. (*d*) $Ca_V1.3$ α_{1D}, β_{2B}, and Rad were heterologously expressed in HEK293T cells. Panels *a–d* adapted from Reference 68. (*e–f*) Proposed models of β-adrenergic regulation of $Ca_V1.2$ channels. PKA phosphorylates several residues on Rad, causing dissociation of Rad from the $Ca_V1.2$ complex and therefore increased Ca^{2+} influx. Panels *e* and *f* adapted from image created with BioRender.com.

Abbreviations: P, phosphorylation; PKA, protein kinase A; Rad, Ras associated with diabetes; WT, wild-type.