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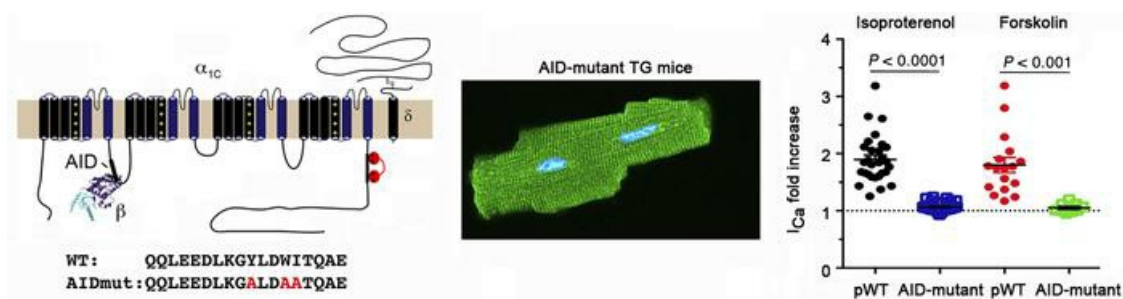
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Cardiac Ca_v1.2 channels require β subunits for β-adrenergic-mediated modulation but not trafficking

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Ca²⁺ channel β-subunit interactions with pore-forming α-subunits are long-thought to be obligatory for channel trafficking to the cell surface and for tuning of basal biophysical properties in many tissues. Unexpectedly, we demonstrate that transgenic expression of mutant α_{1C} subunits lacking capacity to bind Ca_vβ can traffic to the sarcolemma in adult cardiomyocytes in vivo and sustain normal excitation-contraction coupling. However, these β-less Ca²⁺ channels cannot be stimulated by β-adrenergic pathway agonists, and thus adrenergic augmentation of contractility is markedly impaired in isolated cardiomyocytes and in hearts. Similarly, viral-mediated expression of a β-subunit-sequestering peptide sharply curtailed β-adrenergic stimulation of WT Ca²⁺ channels, identifying an approach to specifically modulate β-adrenergic regulation of cardiac contractility. Our data demonstrate that β subunits are required for β-adrenergic regulation of Ca_v1.2 channels and positive inotropy in the heart, but are dispensable for Ca_v1.2 trafficking to the adult cardiomyocyte cell surface, and for basal function and excitation-contraction coupling.

Introduction

In heart cells, Ca²⁺ influx via Ca_v1.2 channels mediates excitation-contraction (E-C) coupling, controls action potential duration, and regulates gene expression. Ca_v1.2 channels are multi-subunit proteins composed minimally of a pore-forming α_{1C} and regulatory β and α_{2δ} subunits (1–4). In adult ventricular cardiomyocytes, most Ca_v1.2 channels localize to transverse tubules where they lie in close proximity (~12 nm) and apposed to ryanodine receptors (RyR2) at dyadic junctions (5). Dysregulation of Ca_v1.2 activity, surface density, or subcellular localization in cardiomyocytes can result in cardiac arrhythmias, heart failure, and sudden death.

Reconstitution experiments concluded that binding to β subunits is indispensable for α_{1C} trafficking to the cell surface (6–14). The physiological relevance of this finding was initially supported by β₂ knockout mice, which were embryonic lethal, likely secondary to a decreased L-type Ca²⁺ current (15). An initial idea that β binding to the α_{1C}-interaction domain (AID) of the α₁-subunit I-II loop shielded an ER retention signal in the I-II loop to allow forward trafficking of the channel proved inadequate in subsequent experiments (9, 16–18). Surprisingly, cardiomyocyte-specific, conditional deletion of the *Cacnb2* gene in adult mice reduced β₂ protein by 96% but caused only a modest 29% reduction in Ca²⁺ current, with no obvious cardiac impairment (19). Interpretation

of this result is ambiguous, however, as it is complicated by the remnant (~4%) β₂ expression as well as the presence other Ca_vβ isoforms expressed in adult cardiomyocytes (13). Moreover, a contrasting viewpoint was provided by a study in which shRNA-mediated knockdown of β₂ in adult rat myocytes substantially diminished Ca²⁺ current (20).

To definitively address the controversies regarding the role of β subunits in mediating trafficking and regulation of Ca²⁺ channels in the heart, we created transgenic mice lines with 3 mutations in the AID, which renders the pore-forming α_{1C} subunit incapable of binding β subunits. With this new model, we definitively demonstrate in vivo that β subunit binding to α_{1C} is not required for trafficking and that the basal function of β-less Ca²⁺ channels is only minimally altered.

Instead, we found that the β subunit is obligatory for transducing β-adrenergic signals to cardiac Ca_v1.2 channels. Cardiac Ca_v1.2 channels are prominently upregulated by β-adrenergic agonists via activation of protein kinase A (PKA) (21, 22) as part of the fundamental flight-or-fight response, yet the detailed mechanisms by which PKA activates Ca_v1.2 remain unknown despite several decades of investigation. Recently, we reported that alanine substitution of all consensus, conserved PKA phosphorylation sites (> 22 serines/ threonines) in the α_{1C} subunit did not affect adrenergic regulation of Ca_v1.2 in vivo (23). Prior studies also ruled out a contribution for the β subunit, as substitution or elimination of potential PKA phosphorylation sites did not perturb β-adrenergic regulation (24–27), although other consensus PKA sites are present in the N-terminal regions of the protein. We found that β subunit binding to α_{1C}, but not PKA phosphorylation of β, is absolutely essential for the augmentation of Ca²⁺ current and cardiac contractile response to β-adrenergic PKA stimulation. These findings identify the key regulatory mechanisms impacting β-adrenergic regulation of Ca²⁺ influx and contractility in the heart.

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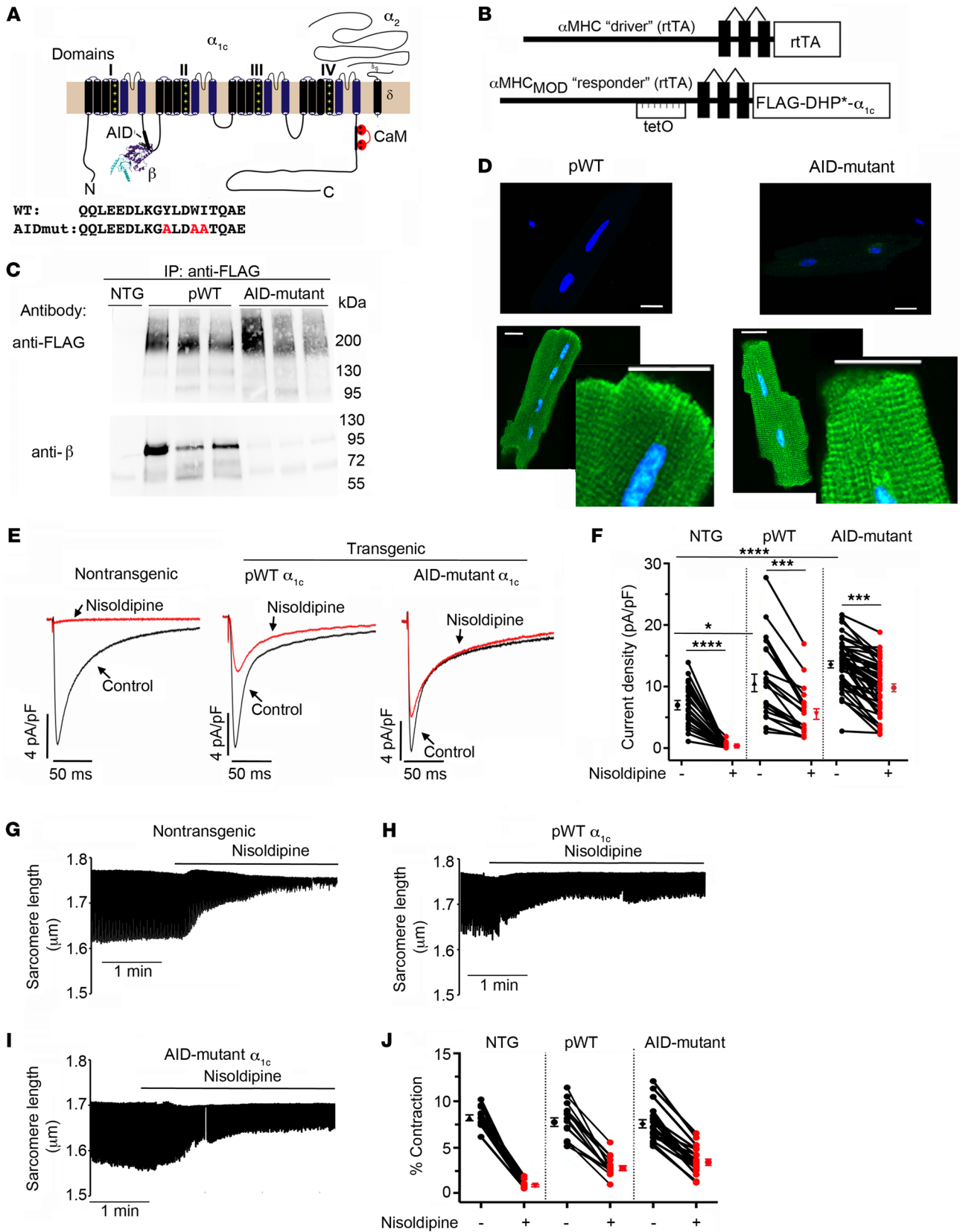


Figure 1. AID-mutant α_{1C} channels trafficking and function in cardiomyocytes. (A) Schematic of rabbit cardiac α_{1C} subunit topology showing β -subunit binding to α -interacting domain (AID) motif in I-II loop. WT and mutant-AID motif in the I-II loop of α_{1C} . (B) Schematic representation of the binary transgene system. The α MHC_{MOD} construct is a modified α MHC promoter containing the *tet*-operon for regulated expression of FLAG-tagged DHP-resistant (DHP*) α_{1C} . (C) Anti-FLAG (upper) and anti- β immunoblots (lower) of anti-FLAG antibody immunoprecipitation of cardiac homogenates of nontransgenic (NTG), pWT α_{1C} , and AID-mutant α_{1C} mice. Representative of 3 experiments. (D) Immunostaining of pWT and AID-mutant α_{1C} cardiomyocytes. Anti-FLAG and FITC-conjugated secondary antibodies, and nuclear labeling with Hoechst stain. Negative control omitted anti-FLAG antibody. Images obtained with confocal microscopy at $\times 40$. Scale bars: 20 μ m. (E) Exemplar whole-cell $Ca_v1.2$ currents recorded from freshly dissociated cardiomyocytes of NTG, pWT, and AID-mutant α_{1C} transgenic mice. Pulses from -60 mV to 0 mV before (black traces) and 3 minutes after (red traces) administration of 300 nM nisoldipine. (F) Scatter plot showing current densities before and after administration of 300 nM nisoldipine. Mean \pm SEM. * $P < 0.05$ NTG versus transgenic pWT α_{1C} , **** $P < 0.0001$ NTG versus transgenic AID-mutant α_{1C} and also NTG pre- versus post-nisoldipine, *** $P < 0.001$ pWT or AID-mutant α_{1C} pre- versus post-nisoldipine. One-way ANOVA and Dunnett's multiple comparison test. NTG, $n = 8$ cardiomyocytes from 5 mice; pWT, $n = 21$ cardiomyocytes from 7 mice; AID-mutant, $n = 45$ cardiomyocytes from 9 mice. (G-I) Representative time courses of changes in sarcomere length after superfusion of 300 nM nisoldipine-containing solution for cardiomyocytes isolated from NTG mice (G) and pWT (H) and AID-mutant transgenic α_{1C} mice. Cardiomyocytes were field-stimulated at 1 Hz. (J) Scatter plot showing percentage of contraction of sarcomere length in the absence and presence of nisoldipine for cardiomyocytes isolated from NTG mice and pWT and AID-mutant α_{1C} transgenic mice. NTG, $n = 12$ cells from 3 mice; pWT, $n = 16$ cells from 3 mice; AID-mutant, $n = 18$ cells from 3 mice.

Results

β -less $Ca_v1.2$ channels traffic to membrane in adult cardiomyocytes. Alanine substitutions of 3 conserved residues—Y467, W470, and I471—in rabbit α_{1C} AID (Figure 1A) increases the K_D of β subunit binding from 5 nM to greater than 6 M (28–31). β_2 subunits failed to coprecipitate with the AID-mutant α_{1C} when coexpressed with AID-mutant α_{1C} in tsA201 cells (Supplemental Figure 1A; supplemental material available online with this article; <https://doi.org/10.1172/JCI123878DS1>) confirming the critical importance of this region for β binding. We then created transgenic mice with cardiac-specific and doxycycline-inducible expression of N-terminal 3X-FLAG-tagged dihydropyridine-resistant (DHP-resistant) (T1066Y/Q1070M) (32, 33) AID-mutant rabbit α_{1C} (Figure 1B). Controls were provided by transgenic FLAG-tagged DHP-resistant α_{1C} subunits with WT AIDs, termed pseudo-WT (pWT) α_{1C} . Coimmunoprecipitation experiments from transgenic mice hearts confirmed that pWT α_{1C} associates with endogenous β subunit, but AID-mutant α_{1C} does not (Figure 1C). The anti- β antibody recognizes all $Ca_v\beta$ subunits, thus ruling out compensation from other β subunits in heart and thus confirming that the AID motif is essential to mediate the high-affinity binding between α_{1C} and β_2 in cardiomyocytes.

We assessed the impact of loss of β binding on AID-mutant α_{1C} subcellular localization and functional expression in cardiomyocytes using 3 complementary approaches. First, immunofluorescence experiments using anti-FLAG antibody on fixed cardiomyocytes indicated that both transgenic pWT α_{1C} and AID-mutant α_{1C} channels displayed a similar striated pattern consistent with surface membrane distribution and localization in

transverse tubules (Figure 1D). Second, we exploited the T1066Y/Q1070M mutations that impart relative DHP-resistance (32, 33) to block Ca^{2+} currents from endogenous DHP-sensitive $Ca_v1.2$ with nisoldipine and isolate Ca^{2+} current from transgenic pWT α_{1C} or AID-mutant α_{1C} channels. Compared with cardiomyocytes isolated from NTG control mice, cardiomyocytes isolated from both pWT and AID-mutant α_{1C} transgenic mice had increased peak Ca^{2+} currents, and substantial peak Ca^{2+} currents remaining after exposure to nisoldipine (Figure 1, E and F). Third, field-stimulated contraction of cardiomyocytes isolated from transgenic AID-mutant α_{1C} mice persisted in the presence of 300 nM nisoldipine (Figure 1, I and J), similar to the contraction of cardiomyocytes isolated from transgenic pWT α_{1C} mice in the presence of nisoldipine. Contraction of cardiomyocytes isolated from NTG was markedly inhibited by nisoldipine (Figure 1, G and H). Overall, these results demonstrate that transgenic β -less AID-mutant α_{1C} channels traffic to the sarcolemma and trigger E-C coupling in cardiomyocytes. This is in stark contrast to the necessary role of β binding for surface trafficking and function of $Ca_v1.2$ channels reconstituted in heterologous cells (Supplemental Figure 1, A and B), or expressed in hippocampal neurons (34).

We also considered that endogenous WT α_{1C} channels could couple with AID-mutant α_{1C} channels to facilitate trafficking of β -less channels to the surface membranes in cardiomyocytes, which could be the basis for the observed differences between cardiomyocytes and heterologous expression systems. To determine whether coupling-induced trafficking could occur, we coexpressed either DHP-resistant pWT α_{1C} or DHP-resistant AID-mutant α_{1C} with both WT α_{1C} and β_2 subunits in tsA201. In the presence of nisoldipine, which inhibits the WT α_{1C} channels, tsA201 cells expressing the AID-mutant α_{1C} channels had no remaining Ca^{2+} current (Supplemental Figure 1C, right), whereas cells expressing the DHP-resistant pWT α_{1C} had remaining current (Supplemental Figure 1C, left), implying that at least in tsA201 cells, β -less channels were unable to “hitchhike” to the membrane with WT channels.

PKA modulation of $Ca_v1.2$ channels is dependent on α_{1C} - β interactions. In heterologous expression studies, β subunits not only enable α_{1C} surface trafficking, but also can differentially induce, depending on β subunit isoform, a hyperpolarizing shift in the voltage dependence of $Ca_v1.2$ activation and increase the channel open probability (P_o) (12, 27). We assessed the biophysical properties of the transgenic β -less AID-mutant α_{1C} channels compared with transgenic pWT Ca^{2+} channels. Surprisingly, normalized current-voltage (I - V) relationships of nisoldipine-resistant transgenic pWT and AID-mutant α_{1C} channels were remarkably similar (Figure 2A). The midpoint potentials, derived from a Boltzmann function, for steady-state activation demonstrated a small, non-significant 1-mV hyperpolarizing shift for the AID-mutant channels compared with control pWT channels (Figure 2B), whereas the slope factors for the 2 channel types were not different (Figure 2C). Furthermore, the inactivation kinetics of nisoldipine-resistant Ca^{2+} currents were not significantly different at any test potential between cardiomyocytes isolated from pWT and AID-mutant α_{1C} , respectively (Figure 2D). Therefore, in adult cardiomyocytes, $Ca_v1.2$ channels comprised of transgenic β -less α_{1C} have similar voltage dependence of activation and inactivation kinetics as transgenic pWT $Ca_v1.2$ channels.

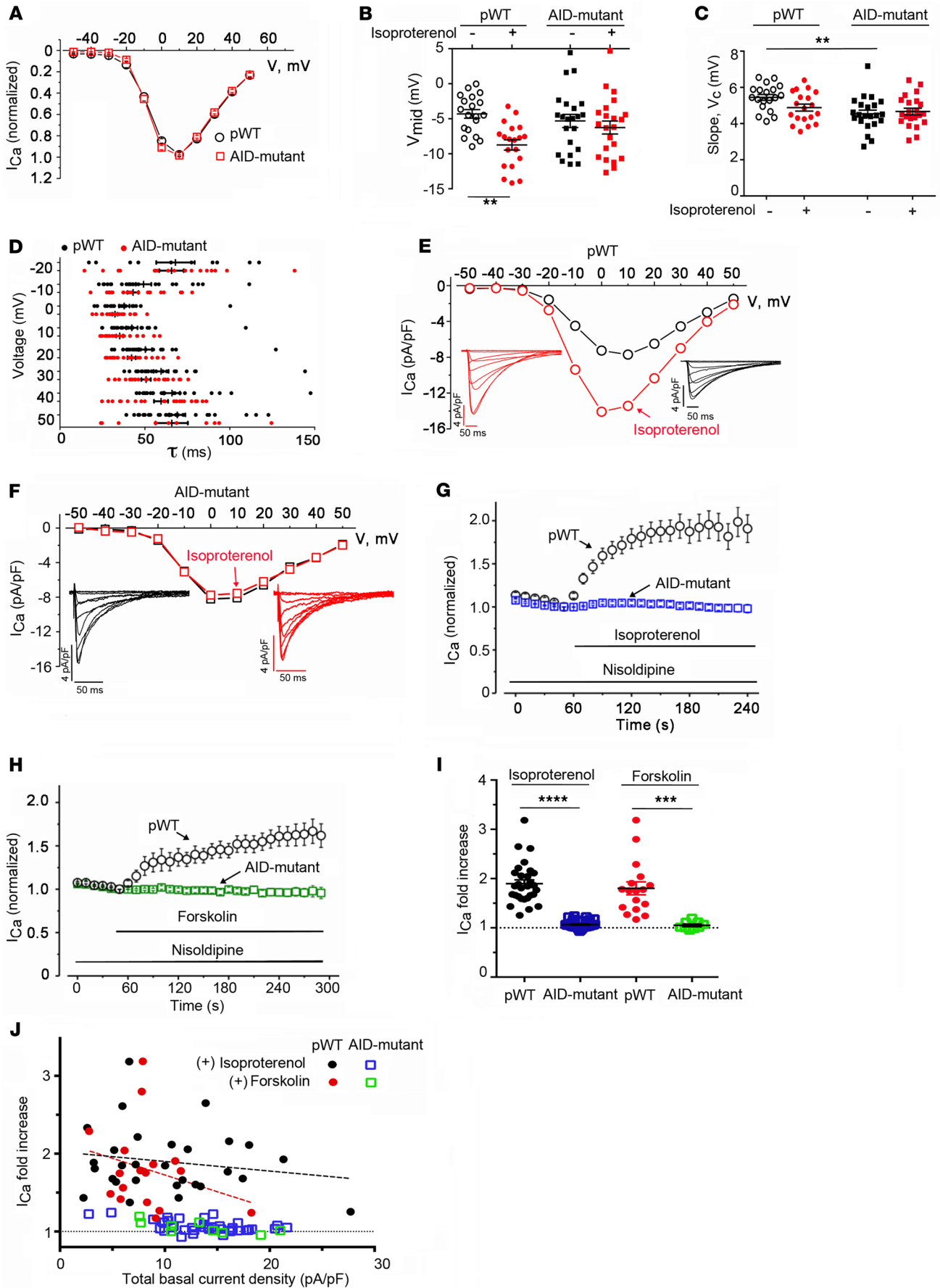


Figure 2. AID-mutant $\text{Ca}_v1.2$ channels lack β -adrenergic regulation. (A) Normalized $\text{Ca}_v1.2$ current-voltage relationships for transgenic pWT and AID-mutant α_{1c} cardiomyocytes in the presence of nisoldipine ($n = 19$ cardiomyocytes from 3 pWT α_{1c} transgenic mice; $n = 18$ cardiomyocytes from 6 AID-mutant α_{1c} transgenic mice). **(B and C)** Bar graphs of Boltzmann function parameters V_{mid} and slope ($V_{1/2}$). $^{**}P < 0.01$, ANOVA and Sidak's multiple comparison test; $n = 19$ cardiomyocytes from 3 pWT α_{1c} transgenic mice; $n = 18$ cardiomyocytes from 6 AID-mutant α_{1c} transgenic mice. **(D)** Summary of time constants of inactivation at the indicated potentials obtained from a single exponential fit ($n = 24$ pWT α_{1c} cardiomyocytes from 4 mice and $n = 24$ AID-mutant α_{1c} cardiomyocytes from 4 mice). $P > 0.05$ pWT versus AID-mutant for all voltages using Sidak's multiple comparison test. **(E and F)** Exemplar nisoldipine-resistant current-voltage relationships of transgenic pWT α_{1c} **(E)** and AID-mutant α_{1c} **(F)** acquired in the absence (black trace) and presence of 200 nM isoproterenol (red trace). **(G)** Diary plot of normalized nisoldipine-resistant I_{Ca} amplitude at 0 mV (normalized to 1 at 50 seconds prior to isoproterenol) of pWT and AID-mutant α_{1c} cardiomyocytes. Cells exposed to 300 nM nisoldipine followed by 200 nM isoproterenol in the continued presence of nisoldipine. pWT, $n = 30$ cardiomyocytes from 5 mice; AID-mutant, $n = 45$ cardiomyocytes from 7 mice. $P < 0.0001$ by 1-way ANOVA/multiple comparison at all time points 30 seconds after isoproterenol. **(H)** Diary plot of normalized nisoldipine-resistant I_{Ca} amplitude at +10 mV (normalized to 1 at 50 seconds, prior to forskolin) of pWT and AID-mutant α_{1c} cardiomyocytes. Cells exposed to 300 nM nisoldipine followed by 10 μM forskolin in the continued presence of nisoldipine. pWT: $n = 15$ cardiomyocytes from 2 mice; AID-mutant: $n = 20$ cardiomyocytes from 6 mice. $P < 0.0001$ by 1-way ANOVA/multiple comparison at all time points 30 seconds after forskolin. **(I)** Bar graph of isoproterenol- or forskolin-induced fold increase in nisoldipine-resistant I_{Ca} . Mean \pm SEM. $^{***}P < 0.001$; $^{****}P < 0.0001$ by t test. **(J)** Graph of isoproterenol- and forskolin-induced increase in nisoldipine-resistant current stratified by total basal current density before nisoldipine for pWT α_{1c} and AID-mutant α_{1c} transgenic mice. Lines fitted by linear regression for pWT cells for isoproterenol (black) and forskolin (red). For isoproterenol, pWT α_{1c} , $n = 29$ cardiomyocytes; AID-mutant α_{1c} , $n = 45$ cardiomyocytes. For forskolin, pWT α_{1c} , $n = 17$ cardiomyocytes; AID-mutant α_{1c} , $n = 9$ cardiomyocytes.

We next determined the sensitivity of $\text{Ca}_v1.2$ channels containing either transgenic pWT α_{1c} or AID-mutant α_{1c} to PKA modulation. In cardiomyocytes isolated from mice expressing transgenic pWT α_{1c} , 200 nM isoproterenol increased the nisoldipine-insensitive current by a mean of 1.9-fold \pm 0.1-fold (Figure 2, E–J), and shifted the V_{mid} in the hyperpolarizing direction by a mean of 4.4 mV (Figure 2B). Similarly, forskolin, which directly activates adenylyl cyclase, thereby bypassing β -adrenergic receptors, increased transgenic pWT α_{1c} Ca^{2+} currents by 1.8-fold \pm 0.1-fold (Figure 2, H–J). In sharp contrast, Ca^{2+} currents through transgenic AID-mutant α_{1c} $\text{Ca}_v1.2$ channels were insensitive to either isoproterenol (Figure 2, B, F, G, I, and J) or forskolin (Figure 2, H–J). In cardiomyocytes, there is an inverse relationship between total peak current and isoproterenol-induced or forskolin-induced fold increase in Ca^{2+} current (27). In cardiomyocytes isolated from transgenic pWT α_{1c} mice, we observed an inverse relationship between basal current density and isoproterenol- or forskolin-induced increase in Ca^{2+} current (Figure 2J). For the transgenic AID-mutant β -less channels, however, activation of PKA by either forskolin or isoproterenol had no effect on Ca^{2+} current, regardless of basal Ca^{2+} current density (Figure 2J).

To address whether the YWI/AAA mutations themselves produced an intrinsic insensitivity of the channel to PKA modulation, we sought to engender conditions under which there would be a predominance of β -less endogenous $\text{Ca}_v1.2$ channels

in isolated cardiomyocytes. We achieved this by using adenovirus to overexpress a YFP-tagged 18-residue AID peptide derived from α_{1c} I-II loop (or a mutant YWI/AAA peptide as a control) in cultured adult guinea pig ventricular cardiomyocytes. We reasoned that this intervention would serve as a sponge for endogenous β subunits, leaving a majority of endogenous $\text{Ca}_v1.2$ channels devoid of β . In control cells expressing either GFP or YFP-tagged mutant (YWI/AAA) AID peptide incapable of binding β , 1 μM forskolin resulted in a robust 4- to 5-fold increase in whole-cell current amplitude (Figure 3, A, C, D, and F–H). By contrast, this response was sharply curtailed in cardiomyocytes overexpressing YFP-AID peptide (Figure 3, B, E, G, and H). Hence, β -less WT α_{1c} channels also demonstrate a marked insensitivity to PKA modulation.

We also considered 2 trivial explanations that could potentially account for the insensitivity of AID-mutant α_{1c} to PKA stimulation: (a) these channels were already phosphorylated by PKA under basal conditions, or (b) the β -adrenergic signaling pathway was compromised in cardiomyocytes from AID-mutant α_{1c} transgenic mice. To address whether transgenic AID-mutant α_{1c} channels were basally PKA phosphorylated, we used a cell-permeable cAMP-PKA inhibitor (Rp-8-Br-cAMPS), which functions by occupying cAMP binding sites thereby preventing activation of PKA holoenzyme. Rp-8-Br-cAMPS reverses isoproterenol-mediated upregulation of endogenous $\text{Ca}_v1.2$ by approximately 96% (23). In transgenic AID-mutant mice cardiomyocytes, Rp-8-Br-cAMPS did not inhibit nisoldipine-resistant basal current (Supplemental Figure 2A), ruling out the idea that AID-mutant α_{1c} channels were basally PKA phosphorylated. The integrity of the β -adrenergic pathway in transgenic AID-mutant mice cardiomyocytes was assessed by probing whether isoproterenol application led to phosphorylation of phospholamban, a well-known PKA target in the heart (35). Western blotting indicated that phospholamban was appropriately phosphorylated at Ser¹⁶ in response to isoproterenol (Supplemental Figure 2B), confirming that the β -adrenergic signaling pathway was intact in AID-mutant transgenic mice cardiomyocytes.

β -adrenergic regulation of $\text{Ca}_v1.2$ does not require PKA phosphorylation of β subunits. The simplest explanation for the necessary role of α_{1c} - β interaction in PKA modulation of $\text{Ca}_v1.2$ is that the β -subunit contains phosphorylation site(s) that are vital to this regulation. Indeed, 2 phosphorylation sites on β_2 C-terminus (Ser⁵¹² and Ser⁵⁷⁰) were previously identified and proposed to play a role in PKA modulation of $\text{Ca}_v1.2$ (36). However, a knock-in mouse expressing a β_2 subunit truncated after Pro⁵⁰¹ displayed normal PKA modulation of $\text{Ca}_v1.2$, thus ruling out involvement of any putative C-terminal phosphorylation sites (24). Nevertheless, it remained possible that previously unappreciated phosphorylation sites N-terminal to Pro⁵⁰¹ could mediate the increased $\text{Ca}_v1.2$ channel activity in response to activated PKA. Using both manual sequence analyses and several web-based PKA phosphorylation prediction tools (37–41), we identified 18 conserved consensus PKA phosphorylation sites in the N-terminus, SH3, and GK domains of human β_{2b} (residues labeled red in Supplemental Figure 3). We mutated all 18 Ser/Thr residues to Ala in human β_{2b} , and generated transgenic mice with inducible cardiomyocyte-specific expression of either GFP-tagged WT or 18-mutant β_{2b} subunits using the same bitransgenic system as in

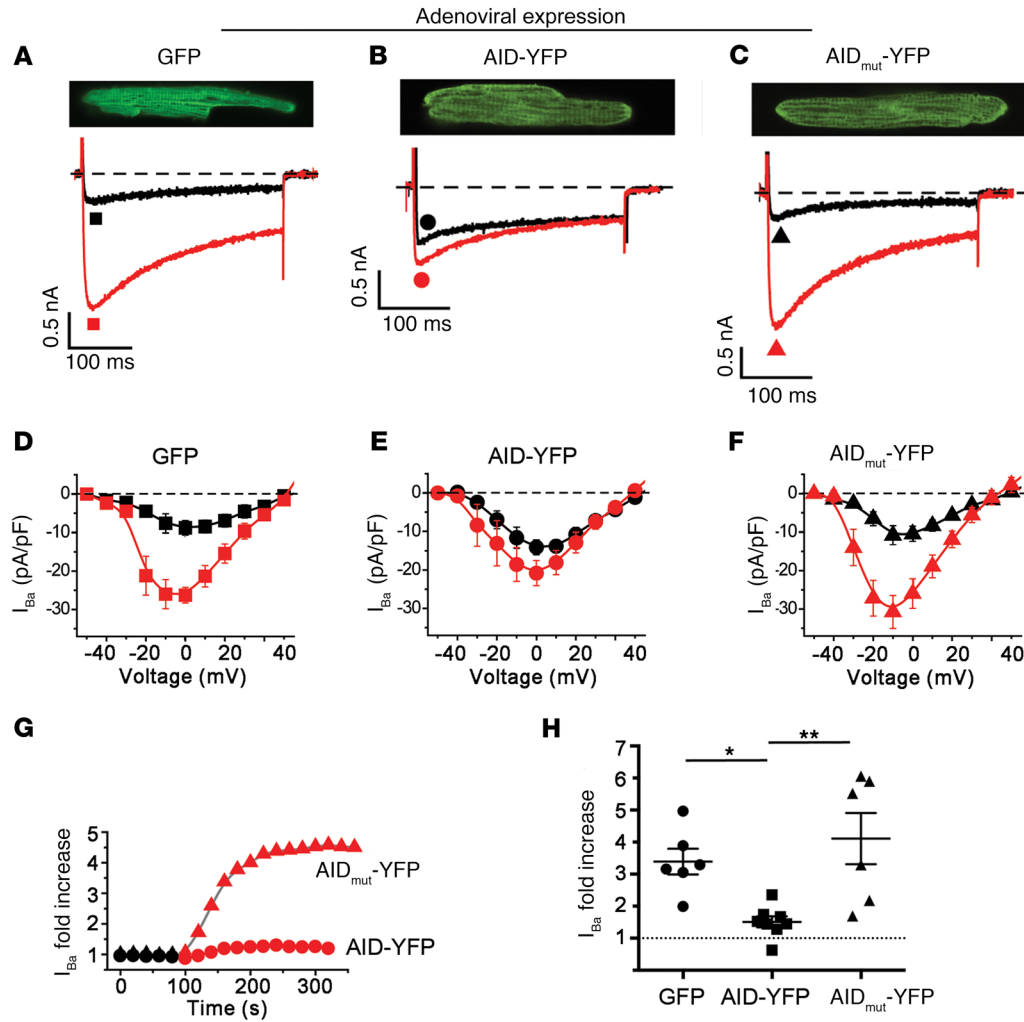


Figure 3. β -less WT endogenous $\text{Ca}_v1.2$ channels are not stimulated by PKA. (A–C) Adenovirus-induced GFP, AID-YFP, and AID-mutant YFP expression in cultured guinea pig ventricular myocytes. Top: exemplar confocal images from guinea pig cardiomyocytes expressing GFP, AID-YFP peptide, or AID-mutant YFP peptide. Bottom: exemplar whole-cell Ba^{2+} currents from GFP and YFP-expressing guinea pig ventricular cardiomyocytes before (black trace) and after (red trace) application of $1 \mu\text{M}$ forskolin. (D–F) Current-voltage relationships from GFP, AID-YFP, and AID-mutant YFP-expressing cardiomyocytes before (black) and after (red) superfusion of $1 \mu\text{M}$ forskolin. (G) Representative diary plot showing time course of forskolin-induced increase in $\text{Ca}_v1.2$ current. (H) Forskolin-induced increase in $\text{Ca}_v1.2$ current. * $P < 0.05$, ** $P < 0.01$ by 1-way ANOVA and Tukey's multiple comparison test.

Figure 1B. The WT and mutant β_{2b} transgenic mice were fed doxycycline for 1 week, thus ensuring high levels of expression of the GFP-tagged β_2 subunits (Figure 4A). We exploited the larger size of GFP-tagged β_2 subunits compared with endogenous β to determine relative expression of transgenic and native β_2 subunits (Figure 4B). Western blot indicated that in cardiomyocytes from transgenic mice, both GFP β_2 and GFP-mutant β_2 were markedly overexpressed ($\sim 9:1$) compared with endogenous β_2 (Figure 4C). Isoproterenol increased peak $\text{Ca}_v1.2$ current by a mean of $1.5\text{-fold} \pm 0.1\text{-fold}$ in GFP-WT β_2 -expressing cells and $1.6\text{-fold} \pm 0.1\text{-fold}$ in GFP-mutant β_2 -expressing cells, respectively, similar to nontransgenic mice (Figure 4, D–G). For both GFP-WT and GFP-mutant β_{2b} Ca^{2+} channels, isoproterenol shifted the V_{mid} of steady-state activation by -7.0 mV and -7.5 mV, respectively. These data indicate that, although the α_{1C} - β_2 interaction is necessary for β -adrenergic regulation of $\text{Ca}_v1.2$, direct PKA phosphorylation of β_2 is not involved.

β -adrenergic regulation of cardiac contractility requires PKA regulation of $\text{Ca}_v1.2$. We next exploited the findings that transgenic β -less AID-mutant α_{1C} channels are insensitive to PKA modulation to probe the specific role of $\text{Ca}_v1.2$ modulation in the positive inotropic effect of β -adrenergic agonists in both isolated cardiomyocytes and in the whole heart. In transgenic pWT α_{1C} cardiomyocytes, with endogenous $\text{Ca}_v1.2$ channels silenced with nisoldipine, isoproterenol produced a robust 100% increase in fractional shortening (Figure 5, A and C). By contrast, this response was severely diminished in cardiomyocytes expressing transgenic β -less AID-mutant α_{1C} channels in which isoproterenol produced a relatively meager 25% increase in fractional shortening (Figure 5, B and C). Consistent with the effects of isoproterenol on phospholamban phosphorylation (Supplemental Figure 2B), isoproterenol enhanced relaxation in cardiomyocytes isolated from both pWT and AID-mutant α_{1C} transgenic mice (Figure 5D).

We then assessed the role of $\text{Ca}_v1.2$ modulation in β -adrenergic agonist-induced positive inotropy at the whole-organ level

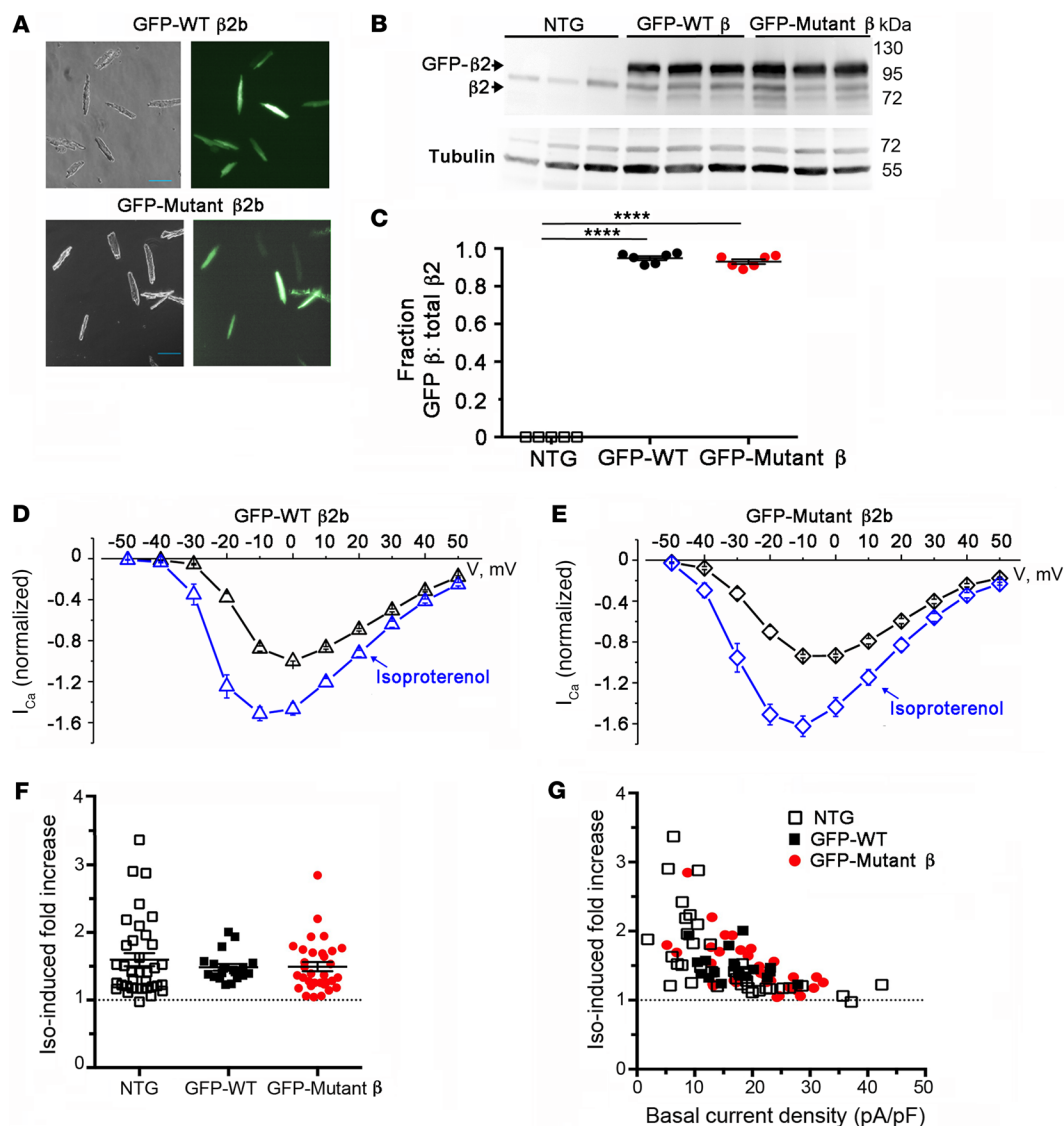


Figure 4. PKA phosphorylation of $\text{Ca}_v\beta$ is not required for β -adrenergic regulation of $\text{Ca}_v1.2$. (A) Bright-field and GFP image of WT and mutant β_{2b} -expressing cardiomyocytes. Scale bars: 100 μm . (B) Immunoblots using anti- β_2 antibody (upper) and anti-tubulin antibody of homogenates from the hearts of nontransgenic (NTG) and doxycycline-fed GFP-WT β_2 and GFP-mutant β_2 -expressing mice. (C) Graph of densitometry of fraction of GFP- β /total β . Mean \pm SEM; $n = 6$ mice for NTG, WT, and mutant β_2 . **** $P < 0.0001$ compared with nontransgenic by 1-way ANOVA and Dunnett's multiple comparison test. (D and E) Normalized current-voltage relationships of GFP-WT β_2 and GFP-mutant β_2 cardiomyocytes acquired before and after superfusion of 200 nM isoproterenol. Isoproterenol shifted the V_{mid} of steady-state activation of GFP-WT β_2 and GFP-mutant β_2 cardiomyocytes by -7.0 mV ($P < 0.0001$, t test, $n = 15$) and -7.5 mV ($P < 0.001$, t test, $n = 30$), respectively. (F) Column scatter plot depicting the fold increase in peak current caused by isoproterenol. Mean \pm SEM; $n = 36$ cardiomyocytes from 5 NTG mice; $n = 19$ cardiomyocytes from 4 GFP-WT β_{2b} mice; $n = 32$ cardiomyocytes from 5 mutant β_{2b} mice. $P = 0.55$ by 1-way ANOVA. (G) Graphs of isoproterenol-induced increase in current stratified by total basal current density for cardiomyocytes isolated from NTG mice, GFP-WT β_{2b} mice, and GFP-mutant β_{2b} transgenic mice.

by inserting a pressure-transduced balloon into the left ventricle of Langendorff-perfused transgenic mice hearts. This approach enabled measurement of cardiac contractility independent of vascular or systemic effects. Hearts were paced at 400 beats per minute to remove the potentially confounding effect of heart rate variability on contractility (42). After baseline measurements, 300 nM nisoldipine was infused into the coronary arteries via the aorta to suppress endogenous $\text{Ca}_v1.2$ channel currents. In hearts from nontransgenic mice, nisoldipine markedly reduced basal cardiac contractility due to the block of endogenous $\text{Ca}_v1.2$ channels (Figure 5E). In pWT α_{1C} hearts, infusion of nisoldipine yielded a comparatively weaker effect on basal con-

tractility owing to the expression of DHP-resistant Ca^{2+} channels (Figure 5F); a further infusion of 200 nM isoproterenol strongly increased cardiac contractility by 3.3-fold (Figure 5, G and I). By contrast, using the same experimental paradigm in hearts from β -less AID-mutant transgenic mice, the response to isoproterenol was nearly abolished, yielding an average increase in cardiac contractility of only 1.2-fold (Figure 5, H and I).

Discussion

Much of our current understanding regarding mechanisms underlying $\text{Ca}_v1.2$ trafficking and modulation derives from studies on recombinant channels reconstituted in heterologous cells. These

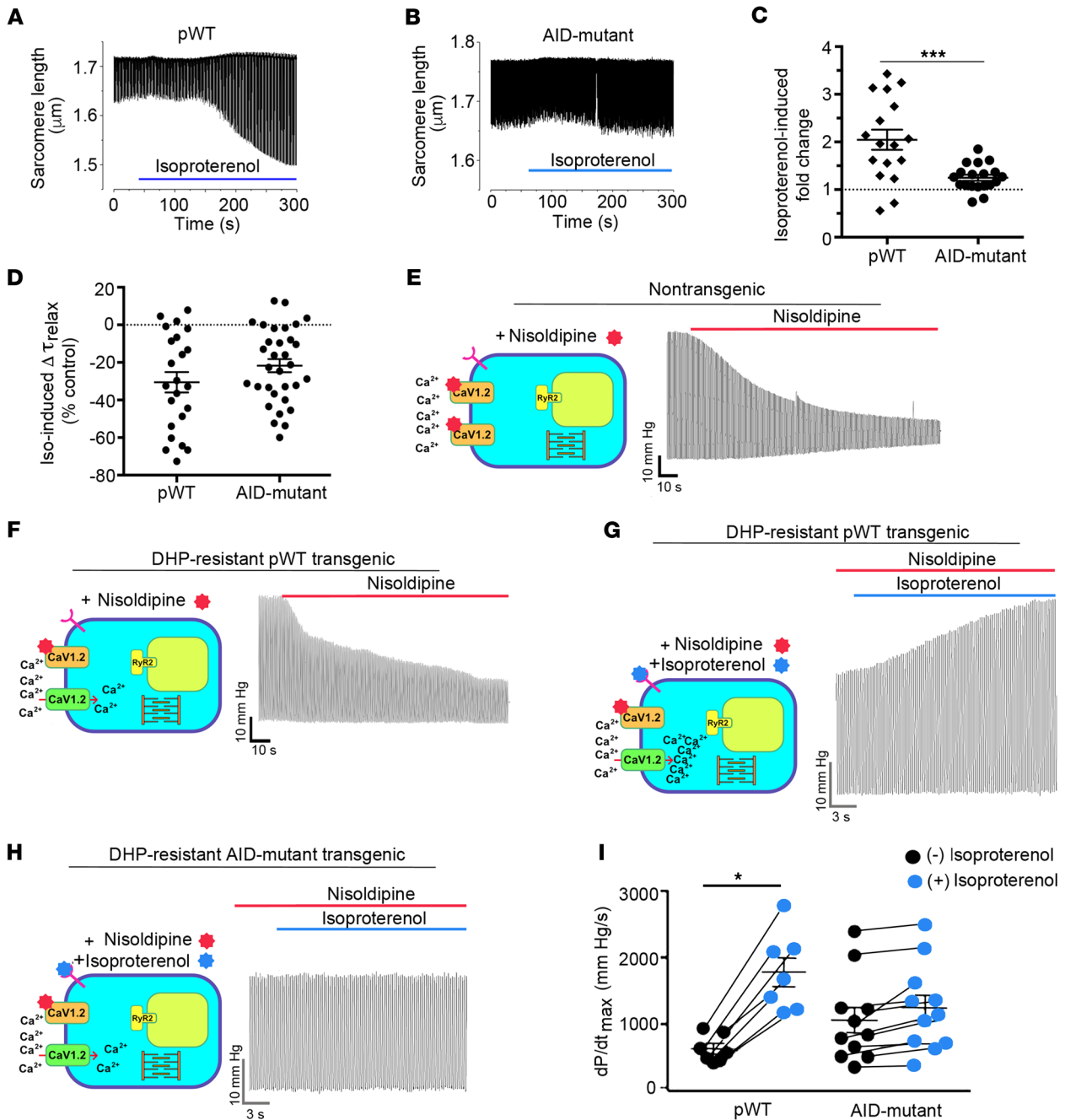


Figure 5. Attenuated β -adrenergic-stimulated inotropy in AID-mutant α_{1c} transgenic mice. (A and B) Cells with robust shortening induced by 1 Hz electrical stimulation in the presence of 300 nM nisoldipine were used. Isoproterenol (200 nM) was superfused with 300 nM nisoldipine. (C) Plot of isoproterenol-induced fold change in sarcomere length compared with before isoproterenol. Mean \pm SEM; $n = 17$ for pWT α_{1c} cardiomyocytes and $n = 19$ cardiomyocytes for AID-mutant α_{1c} . *** $P < 0.001$ by t test. (D) Plot of isoproterenol-induced percentage of change in $\tau_{relaxation}$ of sarcomere length compared with before isoproterenol. Mean \pm SEM; $n = 23$ cardiomyocytes from 3 mice and $n = 32$ cardiomyocytes from 3 mice. $P = 0.16$ by t test. (E and F) Representative traces depicted effect of perfusion of 300 nM nisoldipine on left ventricular contraction in isolated Langendorff-perfused hearts resected from NTG mice and pWT α_{1c} transgenic mice. (G and H) Representative traces of nisoldipine-resistant LV pressure before and during isoproterenol infusion, in hearts resected from pWT α_{1c} and AID-mutant α_{1c} transgenic mice. (I) Quantitative summary of dP/dt_{max} before and during isoproterenol infusion. $n = 7$ pWT α_{1c} transgenic mice; $n = 11$ AID-mutant α_{1c} transgenic mice. * $P < 0.05$ by t test.

cells lack the complex cytoarchitecture and intracellular milieu of adult cardiomyocytes. Recently, we developed an approach that utilizes transgenic mice expressing doxycycline-inducible, cardiac-specific, DHP-resistant α_{1c} . Compared with knockin mice models (43, 44), this approach is both cost-effective and rapid, and

perhaps more importantly, enables us to induce brief expression of mutant channels in adults, permitting the comparison of WT and mutant α_{1c} structure-function mechanisms in the absence of developmental abnormalities and heart failure. The titration of the level of $Ca_v1.2$ expression is important, as the magnitude of β -adrener-

gic stimulation of $\text{Ca}_v1.2$ is reduced with increased basal current density (27, 45–49). Stratifying the magnitude of β -adrenergic-mediated upregulation of $\text{Ca}_v1.2$ current by total basal current density attenuates this confounding variable (Figure 2J and Figure 4G).

Overall, we show that in cardiomyocytes, the AID motif is required for the high-affinity interaction between α_{1C} and β subunits, and that β -less $\text{Ca}_v1.2$ channels traffic to the dyad and produce currents that mediate normal E-C coupling. The AID-mutant β -less Ca^{2+} currents were completely refractory to PKA activation. These findings, combined with our recent studies (23), fundamentally recast our views on mechanisms underlying $\text{Ca}_v1.2$ trafficking and PKA modulation in cardiomyocytes as they show that (a) it is possible for β -less channels to traffic to the cell surface, (b) β_2 binding to α_{1C} is indispensable for PKA modulation of $\text{Ca}_v1.2$, and that β -adrenergic regulation of $\text{Ca}_v1.2$ can be specifically attenuated by sequestering β subunits, and (c) conserved consensus PKA phosphorylation sites in α_{1C} (23) and β_{2b} are not required for β -adrenergic regulation of $\text{Ca}_v1.2$ in the heart. Further, we directly show that β -adrenergic modulation of $\text{Ca}_v1.2$ is critical for sympathetic augmentation of cardiac inotropy, which is essential for the fight-or-flight response.

When coexpressed with α_1 subunits in heterologous expression systems such as *Xenopus* oocytes or human embryonic kidney (HEK) cells, β subunits markedly augment current density by increasing membrane targeting and altering electrophysiological properties (6–8). In the adult heart, however, Ca^{2+} channels can traffic to the surface membrane without binding to β . How β -less α_{1C} channels traffic to the dyad in cardiomyocytes but not in a less complex system such as HEK cells is not yet clear. Although low-affinity interactions between heterologously expressed β subunit GK and SH3 domains and the $\text{Ca}_v2.1$ α subunit in oocytes have been described (50), these potential interactions do not appear to be sufficient to rescue the trafficking of AID-mutant $\text{Ca}_v1.2$ channels in tsA-201 cells. Moreover, conditional knockout of *Cacnb2* in adult cardiomyocytes caused only a 29% reduction in current density (19).

Regardless of the mechanisms enabling trafficking to the cell surface, β -less $\text{Ca}_v1.2$ channels are functionally normal under basal conditions in adult cardiomyocytes. However, the β -less channels cannot be regulated by adrenergic-PKA stimulation, although the β subunit does not appear to be the functional target of PKA. To differentiate between the lack of β binding as opposed to the mutations in the AID as causative of the defect in β -adrenergic regulation of $\text{Ca}_v1.2$, we used the complementary approach of expressing using adenovirus, YFP-AID- and YFP-mutant AID-containing peptides in cultured adult guinea pig ventricular myocytes. The response to forskolin was markedly reduced by preventing β subunits from interacting with endogenous WT α_{1C} , implying that lack of β binding to α_{1C} is sufficient to prevent β -adrenergic regulation of $\text{Ca}_v1.2$ in the heart. Our studies cannot address where and when β subunits first interact with α_{1C} subunits in the heart.

Identifying the functional PKA target is more complicated. It is likely not solely α_{1C} , based on our prior studies eliminating all conserved consensus PKA phosphorylation sites in the α_{1C} subunit (23). Likewise, it is not solely β , based on eliminating all conserved PKA phosphorylation sites in β_2 (Figure 4). Thus, our findings suggest that either there is redundancy between α_{1C} and β subunits,

such that PKA phosphorylation of either subunit is sufficient to mediate adrenergic regulation of Ca^{2+} channels in the heart, or that PKA phosphorylation of the core $\text{Ca}_v1.2$ subunits, α_{1C} and β , are not necessary for β -adrenergic regulation of the Ca^{2+} influx in the heart. This can be addressed by cross-breeding the transgenic mice harboring Ala substitutions of all PKA consensus sites in α_{1C} and β_{2b} . Although PKA phosphorylation of β is not required, β subunits, via binding to the I-II loop, could regulate pore opening and voltage-sensor movement. The domain I S6-AID linker forms a continuous helix that may act as a rigid rod through which β subunits modulate channel gating (51).

The loss of β -adrenergic activation of $\text{Ca}_v1.2$ correlated with a markedly attenuated β -adrenergic contractile response. Originally proposed by Fabiato, $\text{Ca}_v1.2$ current has 2 distinct roles in E-C coupling: triggering the release of Ca^{2+} from the sarcoplasmic reticulum (SR) and loading the cell (and SR) with Ca^{2+} (52). The loss of adrenergic regulation of $\text{Ca}_v1.2$ could affect both triggering of RyR2 and the loading of SR with Ca^{2+} , thereby attenuating the adrenergically driven inotropic response. We believe that our findings are the first to demonstrate experimentally the vital role of β -adrenergic stimulation of $\text{Ca}_v1.2$ in shaping the flight-or-flight response in the heart, and validate a recently proposed mathematical model predicting that the loss of β -adrenergic stimulation of $\text{Ca}_v1.2$ would markedly limit Ca^{2+} transients and contraction (53). PKA and Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) phosphorylation of RyR2 also enhances the open probability of the RyR2 Ca^{2+} release channels in the SR by enhancing their sensitivity to cytosolic (54) and synchronizing SR Ca^{2+} release (55–57). It remains controversial, however, as to whether increasing the open probability of RyR2 is critically important for inotropic responses in the heart (58–60). We demonstrate that without augmented $\text{Ca}_v1.2$ current to load the cell with additional Ca^{2+} and/or enhance RyR opening via Ca^{2+} -induced Ca^{2+} release, β -adrenergic agonist-induced phosphorylation of RyR2 and phospholamban does not result in substantial β -adrenergic augmentation of cardiac contractility.

In summary, we have found that Ca^{2+} channel β -subunit binding to the pore-forming α_{1C} subunit is not required for trafficking and function of the Ca^{2+} channel in the heart. The loss of α_{1C} - β_2 binding causes marked attenuation of β -adrenergic-induced stimulation of $\text{Ca}_v1.2$ and inotropy. Thus, we identify a new function for β subunits in the heart: as an essential component of the PKA-mediated augmentation of $\text{Ca}_v1.2$ and increased cardiac contractility that occurs during the physiological fight-or-flight response.

Methods

Reagents. Nisoldipine and Rp-8-Br-cAMPS were purchased from Santa Cruz Biotechnology. All other chemicals were acquired from MilliporeSigma.

Animals. The α_{1C} transgenic constructs were generated by fusing rabbit *Cacna1c* cDNA (accession X15539) to the modified murine α -myosin heavy chain (MHC) tetracycline-inducible promoter (“responder” line) vector (gift of Jeffrey Robbins and Jeffrey Molken- tin, University of Cincinnati, Cincinnati, OH) (61, 62). The α_{1C} subunit was engineered to be both DHP insensitive with the substitutions T1066Y and Q1070M (32, 33) and tagged with a 3X-FLAG epitope. We made alanine substitutions of 3 conserved residues, Y467, W470, and I471, in the AID domain of rabbit α_{1C} (Figure 1A). Two distinct

AID-mutant α_{1c} were created and studied. The results obtained from each of these lines were equivalent and therefore the data were pooled. The β_{2b} transgenic constructs were generated by ligating a N-terminal GFP-tagged human *CACNB2b* cDNA (accession AAG01473) to the tetracycline-inducible vector. These mice were bred with cardiac-specific (α MHC), doxycycline-regulated, codon-optimized reverse transcriptional transactivator (rtTA) mice (obtained via the Mutant Mouse Resource and Research Center [MMRRC]) (63) to generate double-transgenic mice. The α_{1c} transgenic animals received 0.2 g/kg doxycycline-impregnated food (Bio Serv catalog S3888) for 1–2 days and the GFP- β_{2b} transgenic mice received the doxycycline-impregnated food for 1 week to maximize expression.

Generation of adenoviral vectors and infection of guinea pig ventricular cardiomyocytes. Replication-deficient adenoviral vectors expressing AID-YFP and AID-mutant YFP were generated using the AdEasy Adenoviral Vector System (Agilent Technologies) according to the manufacturer's instructions. Briefly, sequences for AID-YFP and AID-mutant YFP were PCR-amplified and cloned into pShuttle-CMV vector. After linearization with PmeI, shuttle vectors were electroporated into BJ5183 cells containing pAdEasy-1 viral plasmid. Positive recombinants were amplified, linearized with Pac I, and transfected into AD-293 cells using the calcium phosphate precipitation method. Transfected cells were monitored for development of adenoviral plaques, after which the cells were freeze-thawed and the lysate used to infect a 10-cm dish of 90% confluent HEK293 cells. Viral expansion and purification were carried out as previously described (64).

Adult guinea pig ventricular myocytes were isolated by enzymatic digestion using a Langendorff perfusion apparatus, and cultured as previously described (27). Animal treatment and use were in accordance with a protocol approved by the Columbia University Institutional Animal Care and Use Committee. Heart cells were infected 2–3 hours after plating with 5–20 μ l adenoviral vector stock ($\approx 10^{11}$ – 10^{12} viral particles/ml).

Immunoprecipitation, immunoblots, and immunofluorescence. Cardiac lysates from 6- to 12-week-old doxycycline-fed transgenic mice were prepared from either whole hearts or isolated ventricular cardiomyocytes (65). Immunoprecipitations were performed in modified RIPA buffer consisting of 50 mM Tris HCl; pH 7.4, 150 mM NaCl, Triton X-100 (0.25%), 10 mM EDTA, 10 mM EGTA, 10 μ M Calpain inhibitor I, 10 μ M Calpain inhibitor II, and Complete Mini tablets (1 per 7 ml), using anti-FLAG antibody (MilliporeSigma) overnight. Immune complexes were collected using protein A (Amersham) for 2 hours, followed by extensive washing. Proteins were size-fractionated, transferred to nitrocellulose membranes, and probed with anti-FLAG antibody (MilliporeSigma), anti-tubulin antibody (Santa Cruz Biotechnology), and custom anti- α_{1c} and anti- β_2 antibodies (65). Detection was performed with a charge-coupled device camera (Carestream Imaging), and ImageQuant software was used for quantification. Isolated cardiomyocytes were fixed for 15 minutes in 4% paraformaldehyde, and indirect immunofluorescence was performed using a 1:200 rabbit anti-FLAG antibody and 1:200 FITC-labeled goat-anti-rabbit antibody (MilliporeSigma). Images were acquired using a confocal microscope.

Cellular electrophysiology. Membrane currents from isolated mouse ventricular cardiomyocytes (66) were measured by the whole-cell patch-clamp method using a MultiClamp 700B amplifier and pCLAMP 10 software (Molecular Devices) as described (65). The

pipette solution contained 40 mM CsCl, 90 mM Cs gluconate, 10 mM BAPTA, 1 mM MgCl₂, 4 mM Mg-ATP, 2 mM CaCl₂, and 10 mM HEPES, adjusted to pH 7.2 with CsOH. After the isolated cardiomyocytes were adequately buffered with 10 mM BAPTA in the internal solution, the isolated cardiomyocytes were superfused with 140 mM TEA-Cl, 1.8 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 10 mM HEPES, adjusted to pH 7.4 with CsOH. For experiments in tsA-201 cells, TEA-Cl was reduced to 130 mM, and 10 mM BaCl₂ was used instead of CaCl₂. Pipette series resistances were usually less than 1 M Ω after 60% compensation. Leak currents and capacitance transients were subtracted by a P/4 protocol. Voltages were corrected for the liquid junction potential of –10 mV. To measure Ca²⁺ peak currents, the cell membrane potential was held at –50 mV and stepped to +10 mV for 350 ms every 10 seconds. To evaluate the I-V relationship for Ca²⁺ currents, the same protocol was repeated with steps between –50 mV to +50 mV in 10-mV increments. All experiments were performed at room temperature, 22°C \pm 1°C. The parameters of voltage-dependent activation were obtained using a modified Boltzmann distribution: $I(V) = G_{\max} * (V - E_{\text{rev}}) / [1 + \exp((V_{\text{mid}} - V) / V_c)]$, where $I(V)$ is peak current, G_{\max} is maximal conductance, E_{rev} is reversal potential, V_{mid} is the midpoint, and V_c is the slope factor.

Whole-cell recordings of virally infected cultured guinea pig ventricular myocytes were conducted at room temperature as previously described (27, 67). Patch pipettes typically had 1–2 M Ω series resistance when filled with internal solution containing 150 mM cesium-methanesulfonate, 10 mM EGTA, 5 mM CsCl, 1 mM MgCl₂, 10 mM HEPES, and 4 mM MgATP (pH 7.3). Cells were perfused with normal Tyrode external solution during formation of gigaohm seal. After successful break-in to the whole-cell configuration, the perfusing medium was switched to an external recording solution containing 155 mM N-methyl-D-glucamine-aspartate, 10 mM 4-aminopyridine, 1 mM MgCl₂, 5 mM BaCl₂, and 10 mM HEPES (pH 7.4). Currents were sampled at 50 KHz and filtered at 5 KHz, and leak and capacitive currents were subtracted using a P/8 protocol.

Fractional shortening of isolated cardiomyocytes. Freshly isolated myocytes were superfused with a Tyrode's solution containing 1.0 mM CaCl₂ and 300 nM nisoldipine. Myocytes were field stimulated at 1 Hz. Percent contraction of sarcomere length was measured using the SarcLen module (Ionoptix) and calculated as the difference of shortest sarcomere length during a contraction subtracted from the relaxed sarcomere length, divided by the relaxed sarcomere length, all averaged over at least 8 contractions.

Ex vivo cardiac contractility. The cannulated hearts were retrogradely perfused on a Langendorff system with a modified Krebs solution (118.5 mM NaCl, 25 mM NaHCO₃, 4.7 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 11 mM glucose, 1.8 mM Ca²⁺). Left ventricular (LV) pressure was measured using a balloon catheter connected to an APT-300 pressure transducer, which was connected to a PowerLab digitizer (ADInstruments). Hearts were paced at 400 beats per minute using electrodes connected to a pacing stimulator system. After initial assessment of cardiac contractility, 300 nM nisoldipine was perfused to silence endogenous Ca²⁺ currents. The effects of nisoldipine on contractility were assessed after at least 3 minutes and on stabilization of LV pressures. Thereafter, 200 nM isoproterenol was perfused with 300 nM nisoldipine for at least 3 minutes. Peak LV pressure during the 3-minute period was used for the assessment of β -adrenergic agonist stimulation.

Statistics. Results are mean \pm SEM. For multiple group comparisons, 1-way ANOVA followed by multiple comparison testing was performed. For comparisons between 2 groups, an unpaired Student's *t* test was used. Statistical analyses were performed using Prism 6 (Graphpad Software). Differences were considered statistically significant at *P* values less than 0.05.

Data availability. The data and study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure.

Study approval. The Institutional Animal Care and Use Committee at Columbia University approved all animal experiments.

Author contributions

SOM, HMC, and GSP conceived the study. LY, A Katchman, JK, A Kushnir, SIZ, SV, SOM, HMC, and GSP determined the study methodology. LY, A Katchman, JK, A Kushnir, SIZ, BC, ZS, PS, GL, AP, DR,

SOM, and HMC carried out the study investigation. SOM, HMC, and GSP wrote the original draft of the manuscript. LY, A Katchman, JK, A Kushnir, SIZ, BC, SV, GL, AP, DR, GSP, HMC, and SOM reviewed and edited the manuscript. SOM, HMC, and GSP acquired funding for the study. SOM, HMC, and GSP contributed resources to the study.

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