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Cardiac Ca\textsubscript{\(V\)1.2} channels require \(\beta\) subunits for \(\beta\)-adrenergic–mediated modulation but not trafficking

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Ca\textsuperscript{2+} channel \(\beta\)-subunit interactions with pore-forming \(\alpha\)-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 \(\alpha\textsubscript{1C}\) subunits lacking capacity to bind Ca\textsubscript{\(\alpha\)},\(\beta\) can traffic to the sarcolemma in adult cardiomyocytes in vivo and sustain normal excitation-contraction coupling. However, these \(\beta\)-less Ca\textsuperscript{2+} channels cannot be stimulated by \(\beta\)-adrenergic pathway agonists, and thus adrenergic augmentation of contractility is markedly impaired in isolated cardiomyocytes and in hearts. Similarly, viral-mediated expression of a \(\beta\)-subunit–sequestering peptide sharply curtained \(\beta\)-adrenergic stimulation of WT Ca\textsuperscript{2+} channels, identifying an approach to specifically modulate \(\beta\)-adrenergic regulation of cardiac contractility. Our data demonstrate that \(\beta\) subunits are required for \(\beta\)-adrenergic regulation of Ca\textsubscript{\(V\)1.2} channels and positive inotropy in the heart, but are dispensable for Ca\textsubscript{\(V\)1.2} trafficking to the adult cardiomyocyte cell surface, and for basal function and excitation-contraction coupling.

Introduction

In heart cells, Ca\textsuperscript{2+} influx via Ca\textsubscript{\(V\)1.2} channels mediates excitation-contraction (E-C) coupling, controls action potential duration, and regulates gene expression. Ca\textsubscript{\(V\)1.2} channels are multi-subunit proteins composed minimally of a pore-forming \(\alpha\textsubscript{1C}\) and regulatory \(\beta\) and \(\alpha\textsubscript{0}\) subunits (1–4). In adult ventricular cardiomyocytes, most Ca\textsubscript{\(V\)1.2} 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\textsubscript{\(V\)1.2} activity, surface density, or subcellular localization in cardiomyocytes can result in cardiac arrhythmias, heart failure, and sudden death.

Reconstitution experiments concluded that binding to \(\beta\) subunits is indispensable for \(\alpha\textsubscript{1C}\) trafficking to the cell surface (6–14). The physiological relevance of this finding was initially supported by \(\beta\)-knockout mice, which were embryonic lethal, likely secondary to a decreased L-type Ca\textsuperscript{2+} current (15). An initial idea that \(\beta\) binding to the \(\alpha\)-interaction domain (AID) of the \(\alpha\)-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 \(\beta\) protein by 96% but caused only a modest 29% reduction in Ca\textsuperscript{2+} current, with no obvious cardiac impairment (19). Interpretation of this result is ambiguous, however, as it is complicated by the remnant (~4%) \(\beta\) expression as well as the presence other Ca\textsubscript{\(\alpha\)},\(\beta\) isoforms expressed in adult cardiomyocytes (13). Moreover, a contrasting viewpoint was provided by a study in which shRNA-mediated knockdown of \(\beta\) in adult rat myocytes substantially diminished Ca\textsuperscript{2+} current (20).

To definitively address the controversies regarding the role of \(\beta\) subunits in mediating trafficking and regulation of Ca\textsuperscript{2+} channels in the heart, we created transgenic mice lines with 3 mutations in the AID, which renders the pore-forming \(\alpha\)-subunit incapable of binding \(\beta\) subunits. With this new model, we definitively demonstrate in vivo that \(\beta\) subunit binding to \(\alpha\textsubscript{1C}\) is not required for trafficking and that the basal function of \(\beta\)-less Ca\textsuperscript{2+} channels is only minimally altered.

Instead, we found that the \(\beta\) subunit is obligatory for transducing \(\beta\)-adrenergic signals to cardiac Ca\textsubscript{\(V\)1.2} channels. Cardiac Ca\textsubscript{\(V\)1.2} channels are prominently upregulated by \(\beta\)-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\textsubscript{\(V\)1.2} 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 \(\alpha\textsubscript{1C}\) subunit did not affect adrenergic regulation of CaV1.2 in vivo (23). Prior studies also ruled out a contribution for the \(\beta\) subunit, as substitution or elimination of potential PKA phosphorylation sites did not perturb \(\beta\)-adrenergic regulation (24–27), although other consensus PKA sites are present in the N-terminal regions of the protein. We found that \(\beta\) subunit binding to \(\alpha\textsubscript{1C}\), but not PKA phosphorylation of \(\beta\), is absolutely essential for the augmentation of Ca\textsuperscript{2+} current and cardiac contractile response to \(\beta\)-adrenergic PKA stimulation. These findings identify the key regulatory mechanisms impacting \(\beta\)-adrenergic regulation of Ca\textsuperscript{2+} influx and contractility in the heart.
Figure 1. AID-mutant α_c channels trafficking and function in cardiomyocytes. (A) Schematic of rabbit cardiac α_c subunit topology showing β-subunit binding to α1C-interacting domain (AID) motif in I-II loop. WT and mutant-AID motif in the I-II loop of α_c. (B) Schematic representation of the binary transgene system. The αMHCD construct is a modified αMHCD promoter containing the tet-operon for regulated expression of FLAG-tagged DHP-resistant (DHP*) α_c. (C) Anti-FLAG (upper) and anti-β immunoblots (lower) of anti–FLAG antibody immunoprecipitation of α_c-subunit binding to α1C, for WT and AID-mutant α_c mice. Representative of 3 experiments. (D) Immunostaining of pWT and AID-mutant α_c 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 ×40. Scale bars: 20 μm. (E) Exemplar whole-cell CaV1.2 currents recorded from freshly dissociated cardiomyocytes of NTG, pWT, and AID-mutant α_c 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 ± SEM. ***P < 0.005 NTG versus transgenic pWT α_c. ****P < 0.0001 NTG versus transgenic AID-mutant α_c, and also NTG pre- versus post-nisoldipine. ***P < 0.001 pWT or AID-mutant α_c 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_c 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_c 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 CaV1.2 channels traffic to membrane in adult cardiomyocytes. Alanine substitutions of 3 conserved residues—Y467, W470, and I471—in rabbit α_c AID (Figure 1A) increases the K_0 of β subunit binding from 5 nM to greater than 6 M (28–31). β subunits failed to coprecipitate with the AID-mutant α_c when coexpressed with AID-mutant α_c 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_c (Figure 1B). Controls were provided by transgenic FLAG-tagged DHP-resistant α_c subunits with WT AIDs, termed pseudo-WT (pWT) α_c. Coimmunoprecipitation experiments from transgenic mice hearts confirmed that pWT α_c associates with endogenous β subunit, but AID-mutant α_c does not (Figure 1C). The α-c anti-β antibody recognizes all CaV1.2 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_c and β subunits in cardiomyocytes.

We assessed the impact of loss of β binding on AID-mutant α_c 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_c and AID-mutant α_c 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 CaV1.2 with nisoldipine and isolate Ca^{2+} current from transgenic pWT α_c or AID-mutant α_c channels. Compared with cardiomyocytes isolated from NTG control mice, cardiomyocytes isolated from both pWT and AID-mutant α_c 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_c 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_c 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_c 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 CaV1.2 channels reconstituted in heterologous cells (Supplemental Figure 1, A and B), or expressed in hippocampal neurons (34).

We also considered that endogenous WT α_c channels could couple with AID-mutant α_c 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_c or DHP-resistant AID-mutant α_c with both WT α_c and β subunits in tsA201. In the presence of nisoldipine, which inhibits the WT α_c channels, tsA201 cells expressing the AID-mutant α_c channels had no remaining Ca^{2+} current (Supplemental Figure 1C, right), whereas cells expressing the DHP-resistant pWT α_c 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 CaV1.2 channels is dependent on α_c-β interactions. In heterologous expression studies, β subunits not only enable α_c surface trafficking, but also can differentially induce, depending on β subunit isoform, a hyperpolarizing shift in the voltage dependence of CaV1.2 activation and increase the channel open probability (P) (12, 27). We assessed the biophysical properties of the transgenic β-less AID-mutant α_c channels compared with transgenic pWT CaV1.2 channels. Surprisingly, normalized current-voltage (I-V) relationships of nisoldipine-resistant transgenic pWT and AID-mutant α_c 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 transgenic CaV1.2 currents were not significantly different at any test potential between cardiomyocytes isolated from pWT and AID-mutant α_c channels (Figure 2D). Therefore, in adult cardiomyocytes, CaV1.2 channels comprised of transgenic β-less α_c have similar voltage dependence of activation and inactivation kinetics as transgenic pWT CaV1.2 channels.
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Figure 2. AID-mutant Ca1.2 channels lack β-adrenergic regulation. (A) Normalized Ca1.2 current-voltage relationships for transgenic pWT and AID-mutant α1c cardiomyocytes in the presence of nisoldipine (n = 19 cardiomyocytes from 3 pWT α1c transgenic mice; n = 18 cardiomyocytes from 6 AID-mutant α1c transgenic mice). (B and C) Bar graphs of Boltzmann function parameters Vm and slope (V1/2). **P < 0.01. ANOVA and Sidak’s multiple comparison test; n = 19 cardiomyocytes from 3 pWT α1c transgenic mice; n = 18 cardiomyocytes from 6 AID-mutant α1c transgenic mice. (D) Summary of time constants of inactivation at the indicated potentials obtained from a single exponential fit (n = 24 pWT α1c cardiomyocytes from 4 mice and n = 24 AID-mutant α1c 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 α1c (E) and AID-mutant α1c (F) acquired in the absence (black trace) and presence of 200 nM isoproterenol (red trace). (G) Diary plot of normalized nisoldipine-resistant Iα1c amplitude at 0 mV (normalized to 1 at 50 seconds prior to isoproterenol) of pWT and AID-mutant α1c 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 Iα1c amplitude at -10 mV (normalized to 1 at 50 seconds, prior to forskolin) of pWT and AID-mutant α1c 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 Iα1c. Mean ± 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 α1c and AID-mutant α1c transgenic mice. Lines fitted by linear regression for pWT cells for isoproterenol (black) and forskolin (red). For isoproterenol, pWT α1c, n = 23 cardiomyocytes; AID-mutant α1c, n = 45 cardiomyocytes. For forskolin, pWT α1c, n = 17 cardiomyocytes; AID-mutant α1c, n = 9 cardiomyocytes.

We next determined the sensitivity of Ca1.2 channels containing either transgenic pWT α1c or AID-mutant α1c to PKA modulation. In cardiomyocytes isolated from mice expressing transgenic pWT α1c, 200 nM isoproterenol increased the nisoldipine-insensitive current by a mean of 1.9-fold ± 0.1-fold (Figure 2, E–J), and shifted the Vm 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 α1c Ca2+ currents by 1.8-fold ± 0.1-fold (Figure 2, H–J). In sharp contrast, Ca2+ currents through transgenic AID-mutant α1c Ca1.2 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 Ca2+ current (27). In cardiomyocytes isolated from transgenic pWT α1c mice, we observed an inverse relationship between basal current density and isoproterenol- or forskolin-induced increase in Ca2+ current (Figure 2F). For the transgenic AID-mutant β-less channels, however, activation of PKA by either forskolin or isoproterenol had no effect on Ca2+ current, regardless of basal Ca2+ current density (Figure 2F).

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 Ca1.2 channels...
The WT and mutant β2 transgenic mice were fed doxycycline for 1 week, thus ensuring high levels of expression of the GFP-tagged β2 subunits (Figure 4A). We exploited the larger size of GFP-tagged β2 subunits compared with endogenous β2 to determine relative expression of transgenic and native β2 subunits (Figure 4B). Western blot indicated that in cardiomyocytes from transgenic mice, both GFP β2 and GFP-mutant β2 were markedly overexpressed (~9:1) compared with endogenous β2 (Figure 4C). Isoproterenol increased peak CaV1.2 current by a mean of 1.5-fold ± 0.1-fold in GFP-WT β2-expressing cells and 1.6-fold ± 0.1-fold in GFP-mutant β2-expressing cells, respectively, similar to nontransgenic mice (Figure 4, D–G). For both GFP-WT and GFP-mutant β2 CaV1.2 channels, isoproterenol shifted the V1/2 of steady-state activation by –7.0 mV and –7.5 mV, respectively. These data indicate that, although the α1C-β2 interaction is necessary for β-adrenergic regulation of CaV1.2, direct PKA phosphorylation of β2 is not involved.

**β-adrenergic regulation of cardiac contractility requires PKA regulation of CaV1.2.** We next exploited the findings that transgenic β-less AID-mutant α1C channels are insensitive to PKA modulation to probe the specific role of CaV1.2 modulation in the positive inotropic effect of β-adrenergic agonists in both isolated cardiomyocytes and in the whole heart. In transgenic pWT α1C cardiomyocytes, with endogenous CaV1.2 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 α1C 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 α1C transgenic mice (Figure 5D).
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 CaV1.2 channel currents. In hearts from nontransgenic mice, nisoldipine markedly reduced basal cardiac contractility due to the block of endogenous CaV1.2 channels (Figure 5E). In pWT α1C hearts, infusion of nisoldipine yielded a comparatively weaker effect on basal contractility owing to the expression of DHP-resistant Ca2+ 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 CaV1.2 trafficking and modulation derives from studies on recombinant channels reconstituted in heterologous cells. These
perhaps more importantly, enables us to induce brief expression of mutant channels in adults, permitting the comparison of WT and mutant α1C structure-function mechanisms in the absence of developmental abnormalities and heart failure. The titration of the level of CaV1.2 expression is important, as the magnitude of β-adrenergic stim-ulated inotropy in AID-mutant α1C 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 ± SEM; n = 17 for pWT α1C cardiomyocytes and n = 19 cardiomyocytes for AID-mutant α1C. ***P < 0.001 by t test. (D) Plot of isoproterenol-induced percentage of change in τ; mean ± 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 α1C transgenic mice. (G and H) Representative traces of nisoldipine-resistant LV pressure before and during isoproterenol infusion, in hearts resected from pWT α1C and AID-mutant α1C transgenic mice. (I) Quantitative summary of dP/dt max before and during isoproterenol infusion. n = 7 pWT α1C transgenic mice; n = 11 AID-mutant α1C transgenic mice. *P < 0.05 by t test.
gic stimulation of Ca\textsubscript{1,2} is reduced with increased basal current density (27, 45–49). Stratifying the magnitude of \( \beta \)-adrenergic-mediated upregulation of Ca\textsubscript{1,2} current by total basal current density attenuates this confounding variable (Figure 2) and Figure 4G).

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

When coexpressed with \( \alpha \) subunits in heterologous expression systems such as Xenopus oocytes or human embryonic kidney (HEK) cells, \( \beta \) subunits markedly augment current density by increasing membrane targeting and altering electrophysiological properties (6–8). In the adult heart, however, Ca\textsuperscript{2\textsuperscript{+}} channels can traffic to the surface membrane without binding to \( \beta \). How \( \beta \)-less \( \alpha \)ic 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 \( \beta \) subunit GK and SH3 domains and the Ca\textsubscript{2,1}2 channels in tsA-201 cells. Moreover, conditional knock-out of Cacnb2 in adult cardiomyocytes caused only a 29% reduction in current density (19).

Regardless of the mechanisms enabling trafficking to the cell surface, \( \beta \)-less Ca\textsubscript{1,2} channels are functionally normal under basal conditions in adult cardiomyocytes. However, the \( \beta \)-less channels cannot be regulated by adrenergic-PKA stimulation, although the \( \beta \) subunit does not appear to be the functional target of PKA. To differentiate between the lack of \( \beta \) binding as opposed to the mutations in the AID as causative of the defect in \( \beta \)-adrenergic regulation of Ca\textsubscript{1,2}, 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 \( \beta \) subunits from interacting with endogenous WT \( \alpha \)ic, implying that lack of \( \beta \) binding to \( \alpha \)ic is sufficient to prevent \( \beta \)-adrenergic regulation of Ca\textsubscript{1,2} in the heart. Our studies cannot address where and when \( \beta \) subunits first interact with \( \alpha \)ic subunits in the heart.

Identifying the functional PKA target is more complicated. It is likely not solely \( \alpha \)ic\textsuperscript{1C} based on our prior studies eliminating all conserved consensus PKA phosphorylation sites in the \( \alpha \)ic subunit (23). Likewise, it is not solely \( \beta \), based on eliminating all conserved PKA phosphorylation sites in \( \beta \)ic (Figure 4). Thus, our findings suggest that either there is redundancy between \( \alpha \)ic and \( \beta \) subunits, such that PKA phosphorylation of either subunit is sufficient to mediate adrenergic regulation of Ca\textsuperscript{2\textsuperscript{+}} in the heart, or that PKA phosphorylation of the core Ca\textsubscript{1,2} subunits, \( \alpha \)ic and \( \beta \), are not necessary for \( \beta \)-adrenergic regulation of the Ca\textsuperscript{2\textsuperscript{+}} influx in the heart. This can be addressed by cross-breeding the transgenic mice harboring Ala substitutions of all PKA consensus sites in \( \alpha \)ic and \( \beta \)ic. Although PKA phosphorylation of \( \beta \) is not required, \( \beta \) 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 \( \beta \) subunits modulate channel gating (51).

The loss of \( \beta \)-adrenergic activation of Ca\textsubscript{1,2} correlated with a markedly attenuated \( \beta \)-adrenergic contractile response. Originally proposed by Fabiato, Ca\textsubscript{1,2} current has 2 distinct roles in E-C coupling: triggering the release of Ca\textsuperscript{2\textsuperscript{+}} from the sarcoplasmic reticulum (SR) and loading the cell (and SR) with Ca\textsuperscript{2\textsuperscript{+}} (52). The loss of adrenergic regulation of Ca\textsubscript{1,2} could affect both triggering of RyR2 and the loading of SR with Ca\textsuperscript{2\textsuperscript{+}}, thereby attenuating the adrenergically driven inotropic response. We believe that our findings are the first to demonstrate experimentally the vital role of \( \beta \)-adrenergic stimulation of Ca\textsubscript{1,2} in shaping the flight-or-flight response in the heart, and validate a recently proposed mathematical model predicting that the loss of \( \beta \)-adrenergic stimulation of Ca\textsubscript{1,2} would markedly limit Ca\textsuperscript{2\textsuperscript{+}} transients and contraction (53). PKA and Ca\textsuperscript{2\textsuperscript{+}}/calmodulin-dependent protein kinase II (CaMKII) phosphorylation of RyR2 also enhances the open probability of the RyR2 Ca\textsuperscript{2\textsuperscript{+}} release channels in the SR by enhancing their sensitivity to cytosolic (54) and synchronizing SR Ca\textsuperscript{2\textsuperscript{+}} 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 Ca\textsubscript{1,2} current to load the cell with additional Ca\textsuperscript{2\textsuperscript{+}} and/or enhance RyR opening via Ca\textsuperscript{2\textsuperscript{+}}-induced Ca\textsuperscript{2\textsuperscript{+}} release, \( \beta \)-adrenergic agonist-induced phosphorylation of RyR2 and phospholamban does not result in substantial \( \beta \)-adrenergic augmentation of cardiac contractility.

In summary, we have found that Ca\textsuperscript{2\textsuperscript{+}} channel \( \beta \)-subunit binding to the pore-forming \( \alpha \)ic subunit is not required for trafficking and function of the Ca\textsuperscript{2\textsuperscript{+}} channel in the heart. The loss of \( \alpha \)ic\textsuperscript{1C} binding causes marked attenuation of \( \beta \)-adrenergic–induced stimulation of Ca\textsubscript{1,2} and inotropy. Thus, we identify a new function for \( \beta \) subunits in the heart: as an essential component of the PKA-mediated augmentation of Ca\textsubscript{1,2} 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 \( \alpha \)ic transgenic constructs were generated by fusing rabbit Cacna1c cDNA (accession X15539) to the modified murine \( \alpha \)-myosin heavy chain (MHC) tetracycline-inducible promoter (“responder” line) vector (gift of Jeffrey Robbins and Jeffrey Molkenstien, University of Cincinnati, Cincinnati, OH) (61, 62). The \( \alpha \)ic 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 \( \alpha \)ic (Figure 1A). Two distinct
AID-mutant αac were created and studied. The results obtained from each of these lines were equivalent and therefore the data were pooled. The β2a 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 cardi-ac-specific (αMHC), doxycycline-regulated, codon-optimized reverse transcriptional transactivator (rTATA) mice (obtained via the Mutant Mouse Resource and Research Center [MMRRC]) (63) to generate double-transgenic mice. The αac transgenic animals received 0.2 g/kg doxycycline-impregnated food (Bio Serv catalog S3888) for 2-1 days and the GFP-β2a 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 μl adenoviral vector stock (×1011-1012 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 μM Calpain inhibitor I, 10 μM 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-αac and anti-β2a 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 MΩ 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 ± 1°C. The parameters of voltage-dependent activation were obtained using a modified Boltzmann distribution: I(V) = Gmax * (V − Erev)/[(1 + exp (V − V1/2)/Vc)], where I(V) is peak current, Gmax is maximal conductance, Erev is reversal potential, V1/2 is the midpoint, and Vc 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 MΩ 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 gigahm 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-aminoypyridine, 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 Sarcomere 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 5-minute period was used for the assessment of β-adrenergic agonist stimulation.
Statistics. Results are mean ± 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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