β-Adrenergic Regulation of the L-type Ca\textsuperscript{2+} Channel Does Not Require Phosphorylation of α\textsubscript{1C} Ser\textsuperscript{1700}

Lin Yang,* Alexander Katchman,* Tahmina Samad, John P. Morrow, Richard L. Weinberg, Steven O. Marx

Rationale: Sympathetic nervous system triggered activation of protein kinase A, which phosphorylates several targets within cardiomyocytes, augments inotropy, chronotropy, and lusitropy. An important target of β-adrenergic stimulation is the sarcolemmal L-type Ca\textsuperscript{2+} channel, Ca\textsubscript{1.2}, which plays a key role in cardiac excitation–contraction coupling. The molecular mechanisms of β-adrenergic regulation of Ca\textsubscript{1.2} in cardiomyocytes, however, are incompletely known. Recently, it has been postulated that proteolytic cleavage at Ala\textsuperscript{1800} and protein kinase A phosphorylation of Ser\textsuperscript{1700} are required for β-adrenergic modulation of Ca\textsubscript{1.2}.

Objective: To assess the role of Ala\textsuperscript{1800} in the cleavage of α\textsubscript{1C} and the role of Ser\textsuperscript{1700} and Thr\textsuperscript{1704} in mediating the adrenergic regulation of Ca\textsubscript{1.2} in the heart.

Methods and Results: Using a transgenic approach that enables selective and inducible expression in mice of FLAG-epitope–tagged, dihydropyridine-resistant Ca\textsubscript{1.2} channels harboring mutations at key regulatory sites, we show that adrenergic regulation of Ca\textsubscript{1.2} current and fractional shortening of cardiomyocytes do not require phosphorylation of either Ser\textsuperscript{1700} or Thr\textsuperscript{1704} of the α\textsubscript{1C} subunit. The presence of Ala\textsuperscript{1800} and the 1798NNAN\textsuperscript{1801} motif in α\textsubscript{1C} is not required for proteolytic cleavage of the α\textsubscript{1C} C-terminus, and deletion of these residues did not perturb adrenergic modulation of Ca\textsubscript{1.2} current.

Conclusions: These results show that protein kinase A phosphorylation of α\textsubscript{1C} Ser\textsuperscript{1700} does not have a major role in the sympathetic stimulation of Ca\textsuperscript{2+} current and contraction in the adult murine heart. Moreover, this new transgenic approach enables functional and reproducible screening of α\textsubscript{1C} mutants in freshly isolated adult cardiomyocytes in a reliable, timely, cost-effective manner. (Circ Res. 2013;113:871-880.)

Key Words: adrenergic ■ calcium channels ■ excitation contraction coupling ■ ion channels ■ mice, transgenic ■ molecular electrophysiology ■ phosphorylation ■ sympathetic nervous system
Although heterologous expression of CaV1.2 channels has proven useful for investigating biophysical properties, it has not been as successful for exploring physiological modulation, especially as related to cardiomyocytes. Knock-in mice are considered the gold standard, but they are time-consuming and expensive to generate, and a phenotype of heart failure or death during the perinatal period may preclude studies at later stages of development.14,30

Although adenoviruses have been used to express CaV1.2 subunits in cardiomyocytes, creation of adenoviruses encoding $\alpha_{1C}$ is difficult because of the $\alpha_{1C}$ insert size and the cardiomyocytes need to be cultured for extended periods, potentially inducing dedifferentiation. Because overexpression of $\alpha_{1C}$ or $\beta$ subunits reduces the hormonal regulation of the channel 27,31–33 and can induce cardiac dysfunction or apoptosis,34–37 it is also important to limit the amount of overexpression. To circumvent these problems, which have limited progress in the field, we have developed an approach of using a doxycycline-inducible, tissue-specific, transgenic mouse expressing FLAG-epitope–tagged, DHP-resistant $\alpha_{1C}$. The approach preserves hormonal regulation of CaV1.2 by limiting CaV1.2 overexpression.

Prominent roles for proteolytic cleavage of $\alpha_{1C}$ at residue Ala$^{1800}$, and PKA phosphorylation of Ser$^{1700}$, in the C-terminus of $\alpha_{1C}$ (Figure 1A), in mediating $\beta$-adrenergic-induced enhancement of cardiac Ca$_{1,2}$ current have been proposed, on the basis of heterologous expression of Ca$_{1,2}$ subunits.8,14,15 In the absence of proteolytic cleavage at Ala$^{1800}$, PKA is unable to phosphorylate Ser$^{1700}$ and upregulate the activity of heterologously expressed Ca$_{1,2}$.14,15 Ala-substitution of the neighboring Thr$^{1704}$, a residue that may be phosphorylated by casein kinase II, reduced heterologously expressed basal Ca$_{1,2}$ channel activity in unstimulated tsA-201 cells, and when combined with Ala-substitution of Ser$^{1700}$, more effectively reduced forskolin-induced stimulation of Ca$_{1,2}$, compared with Ala-substitution of Ser$^{1700}$ alone. These concepts have not been tested in cardiomyocytes. We tested these predictions in native cardiomyocytes by creating a transgenic mouse expressing 3 mutations within $\alpha_{1C}$ (S1700A, T1704A, and $\Delta^{1798}NNAN^{1801}$).

**Methods**

**Reagents**

Nisoldipine (Santa Cruz) was dissolved daily in 30-mmol/L ethanol and was protected from light. All other chemicals were acquired from Sigma.

**Animals**

The pseudowild-type (pWT) $\alpha_{1C}$ and $\Delta^{NNAN}$-$\alpha_{1C}$ constructs were generated by fusing the rabbit CACNA1C cDNA (accession X15539) to the modified murine $\alpha$-myosin heavy chain (MHC), tetracycline-inducible promoter (responder line) vector (gift of Drs Robbins and Molkentin).38,39 A 3X FLAG-epitope was ligated in-frame to the N-terminus of $\alpha_{1C}$. The $\alpha_{1C}$ subunit was engineered to be DHP-insensitive with the substitutions T1066Y and Q1070M.40,41 Transgenic founder mice were identified with genomic DNA using polymerase chain reactions. These mice were bred with cardiac specific ($\alpha$MHC) doxycycline-regulated codon-optimized reverse transcriptional transactivator (rtTA) mice (obtained via Mutant Mouse Regional Resource Center and Dr Tom Cooper, Baylor College of Medicine)42 to generate double transgenic mice. We selected founder lines that did not express the transgenic $\alpha_{1C}$ in the absence of doxycycline.

**Nonstandard Abbreviations and Acronyms**

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<tr>
<td>DHP</td>
<td>dihydropyridine</td>
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<tr>
<td>MHC</td>
<td>myosin heavy chain</td>
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<tr>
<td>PKA</td>
<td>protein kinase A</td>
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<td>pWT</td>
<td>pseudowild-type</td>
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<td>rtTA</td>
<td>reverse transcriptional transactivator</td>
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<td>TG</td>
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**Figure 1.** Deletion of proteolytic cleavage site does not affect heterologously expressed Ca$_{1,2}$ channel expression or function. **A**, Schematic of cardiac $\alpha_{1C}$ subunit topology. The putative proteolytic cleavage site, $\Delta^{798}NNAN^{801}$, is identified. Red circles are putative PKA (Ser$^{1700}$) and casein kinase II (Thr$^{1704}$) phosphorylation sites. **B**, Highly conserved amino acid sequences surrounding putative proteolytic cleavage site, marked by asterisk symbol. **C**, Anti-$\alpha_{1C}$ antibody immunoblotting of extracts from wild-type (WT) $\alpha_{1C}$ and $\Delta^{NNAN}$-$\alpha_{1C}$–expressing tsA-201 cells. **D**, WT (black) and $\Delta^{NNAN}$-$\alpha_{1C}$ (red) current–voltage relationships and current traces (inset). Currents elicited by 400-ms test pulses between −60 mV and +60 mV from a holding potential of −70 mV.
To induce expression, animals received 0.2-g/kg doxycycline-impregnated food (Bio Serv Cat #S3888) for 1 to 5 days. The results presented were consistent across all founder lines and sex, and therefore, were pooled. The Institutional Animal Care and Use Committee at Columbia University approved all animal experiments.

**ImmunobLOTS and Immunofluorescence**

For immunobLOTS, cardiomyocytes were isolated from 8- to 12-week-old nontransgenic and doxycycline-fed transgenic mice. Cardiomyocytes were homogenized in a 1% Triton X-100 buffer containing (in mmol/L): 50 Tris-HCl (pH7.4) 150 NaCl, 10 ethylenediaminetetraacetic acid, 10 ethylene glycol tetraacetic acid, and protease inhibitors. The lysates were incubated on ice for 30 minutes, centrifuged at 14K rpm at 4°C for 10 minutes, and supernatants were collected. Proteins were size-fractionated on SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-FLAG (Sigma) or anti-αic antibodies. Detection was performed with a CCD camera (Carestream Imaging). Image quantification was performed using ImageQuant software. For immunofluorescence, isolated cardiomyocytes were fixed for 15 minutes in 4% paraformaldehyde. Indirect immunofluorescence was performed using a 1:200 rabbit anti-FLAG antibody (Sigma) and 1:200 fluorescein isothiocyanate–labeled goat–anti-rabbit antibody (Sigma). Images were acquired using a confocal microscope.

**Cellular Electrophysiology**

Lipofectamine 2000 (Life Technologies) was used to transfect tsA-201 cells, which were plated onto 12-mm glass coverslips. The experiments were performed 24 to 48 hours after transfection. The isolated cardiomyocytes and tsA-201 cells were superfused with (in mmol/L) 140 tetraethylammonium-Cl, 1.8 CaCl2, 1 MgCl2, 10 glucose, and ten 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, adjusted to pH 7.4 with CsOH. All experiments were performed at room temperature, 22±1°C. Membrane currents were measured by the whole-cell patch-clamp method using a MultiClamp 700B amplifier (Axon Instruments). The pipette solution contained (in mmol/L) 135 CsCl, 10 ethylene glycol tetraacetic acid, 1 MgCl2, 2 Mg-ATP, 2.0 CaCl2, and ten 4-(2-hydroxyethyl)-1-piperazinetetraacetic acid, adjusted to pH 7.2 with CsOH. Pipette series resistances were usually <1 MΩ after 60% compensation. Leak currents and capacitance transients were subtracted by a P/4 protocol. To measure Ca2+ peak currents, the cell membrane potential was held at −70 mV and stepped to +10 mV for 350 ms every 5 to 10 seconds. To evaluate the current–voltage relationship for Ca2+ currents, the same protocol was repeated with steps between −50 mV and +50 mV in 10 mV increments.

**Fractional Shortening**

Fresly isolated myocytes were superfused with a Tyrode’s solution containing 1.8 mmol/L CaCl2. Myocytes were field-stimulated at 1-Hz. In 1 series of experiments, nisoldipine (300 nmol/L) was superfused in the absence and presence of isoproterenol (200 nmol/L). In the second series of experiments, cardiomyocytes were placed in a Tyrode’s solution containing 300-nmol/L nisoldipine. A Tyrode’s solution containing 1.8-mmol/L CaCl2. Myocytes were superfused with a Tyrode’s solution containing 300-nmol/L nisoldipine as optimal for further experiments because nisoldipine (300 nmol/L) blocked >98% of heterologously expressed WT Ca1.2 current in tsA-201 cells, but only blocked 34.6±2.5% of DHP-insensitive αic (Online Figure I).

Seven pWT αic founder transgenic lines were originally generated. Two founder lines were lost because of mortality, possibly because of high levels of doxycycline-independent αic expression. Four founder lines, when crossed with αMHC-rtTA mice, demonstrated doxycycline-induced expression of αic, assessed by anti-FLAG antibody immunobLOTS (Figure 2A, upper; Online Figure II). One transgenic founder line, after crossing with αMHC-rtTA mice, did not demonstrate doxycycline-induced αic expression. Of 59 pWT αic bi-transgenic mice treated with doxycycline, 18 mice (31%) died within 5 days of doxycycline administration possibly because of high levels of doxycycline-dependent αic expression.

We also generated a transgenic mouse line expressing 3 mutations within αic: Ala-substitutions of Ser1700 (S1700A) and Thr1704 (T1704A), and deletion of the 1798NNAN1801 motif (ΔNNAN), in the background of a N-terminal 3X FLAG-epitope tag and DHP-resistance (ANNAN-S1700A-T1704A). Six ΔNNAN-S1700A-T1704A mutant transgenic founder lines were originally generated. Three founders, when crossed with αMHC-rtTA mice, demonstrated doxycycline-induced αic expression (Figure 2B, upper; Online Figure II). The other 3 founders, after crossing with αMHC-rtTA mice, had either no or low levels of doxycycline-induced expression. Of the 35 ΔNNAN-S1700A-T1704A mutant mice treated with doxycycline, 9 mice (26%) died within 5 days, possibly because of high levels of αic expression. The 41-pWT αic transgenic mice and the 26 ΔNNAN-S1700A-T1704A mutant transgenic mice form the basis of this study.

Confirming the expression of transgene, immunofluorescence staining of fixed cardiomyocytes from pWT and ΔNNAN-S1700A-T1704A mutant transgenic mice with an anti-FLAG antibody showed a membrane distribution of expressed αic subunits consistent with t-tubular localization (Figure 2D). No staining was detected in cardiomyocytes when the anti-FLAG antibody was omitted.

**Results**

**Generation of Inducible, Cardiac-Specific αic Transgenic Mice**

We deleted the highly conserved 1798NNAN1801 motif in αic (Figure 1B) and coexpressed the cDNA with the β2 subunit in tsA-201 cells. Deletion of this highly conserved region did not affect expression, trafficking to the surface, or the basal electrophysiological characteristics of Ca1.2 (Figure 1C–1D). Because proteolytic cleavage of αic does not occur when wild-type (WT) αic is expressed heterologously, the effect of deletion of the putative cleavage site on proteolysis of αic could not be assessed using this approach (Figure 1C).

We generated transgenic mice with inducible cardiomyocyte-specific expression of a N-terminal 3X FLAG-epitope–tagged, DHP-resistant αic, designated pWT αic, using a bitransgenic tetracycline-regulated system that permits robust expression only when both transgenes and doxycycline are present (Figure 2A). The αic subunit was engineered to be relatively DHP-insensitive with the substitutions T1066Y and Q1070M. The IC50 for nisoldipine block of heterologously expressed WT αic was 12 nmol/L, whereas the IC50 for pWT αic was 650 nmol/L (Online Figure I). We selected a concentration of 300 nmol/L nisoldipine as optimal for further experiments because nisoldipine (300 nmol/L) blocked >98% of heterologously expressed WT Ca1.2 current in tsA-201 cells, but only blocked 34.6±2.5% of DHP-insensitive αic (Online Figure I).

Statistical Analysis

Results are presented as mean±SEM. For multiple group comparisons, a 1-way ANOVA followed by Tukey, Sidek, or Dunnett post hoc tests were performed. For comparisons between 2 groups, an unpaired Student t test was used. Statistical analyses were performed using Graphpad Software. Differences were considered statistically significant at values of P<0.05.
Cardiomyocyte contraction requires Ca\textsuperscript{2+} influx via Ca\textsubscript{1,2}, which triggers sarcoplasmic reticulum Ca\textsuperscript{2+} release. Supersurf of nisoldipine inhibited the contraction of nontransgenic cardiomyocytes to electric field stimulation at 1-Hz (Figure 2E). In cardiomyocytes isolated from pWT α\textsubscript{1C} transgenic mice, the effect of nisoldipine was greatly diminished (Figure 2F). This indicates that the transgenic channels are correctly localized in the t-tubule and can initiate excitation–contraction coupling.

**Proteolytic Processing of Transgenic Channels**

Expression of cDNA encoding FLAG-tagged α\textsubscript{1C} in tsA-201 cells migrated as full-length α\textsubscript{1C} without evidence of proteolytic processing, detected by immunobLOTS using anti-FLAG and anti-α\textsubscript{1C} antibodies (Figures 1C and 2B). In cardiomyocytes isolated from nontransgenic mice (C57Bl/6), native α\textsubscript{1C} was detected as a full-length ≥240-kDa band and a cleaved ≥210-kDa band, using an anti-α\textsubscript{1C} antibody created against an internal epitope within the intracellular loop of domains II and III. Native α\textsubscript{1C} in nontransgenic mice cannot be detected using an anti-FLAG antibody (Figure 2B). Both the pWT α\textsubscript{1C} transgenic channels and the transgenic channels with a deletion of 1798NNAN\textsuperscript{1801} were proteolytically cleaved, detected using the anti-FLAG antibody (Figure 2B; Online Figure II). The ratios of cleaved to full-length pWT and ANNAN transgenic α\textsubscript{1C} were 62\%±4\% and 72\%±5\%, respectively, not significantly different than the 79\%±5\% cleavage of the native α\textsubscript{1C} (Figure 2C). Because deletion of the putative proteolytic cleavage site had no effect on the ratio of truncated to full-length α\textsubscript{1C} in cardiomyocytes, we can conclude that the NNAN motif and Ala\textsuperscript{1800} are not required for post-translational cleavage of α\textsubscript{1C}.

**Functional, Inducible Expression of pWT and Mutant DHP-Insensitive Transgenic α\textsubscript{1C} in Cardiomyocytes**

We measured Ca\textsubscript{1,2} currents in adult cardiomyocytes from nontransgenic and transgenic mice (Figure 3A–3E). The mean current density was significantly larger in the transgenic mice than in the nontransgenic mice (Figure 3A). The mean current density was significantly larger in the doxycycline-fed transgenic mice than in the nontransgenic mice (Figure 3B). This indicates that the transgenic channels are correctly localized in the t-tubule and can initiate excitation–contraction coupling.

Figure 2. Inducible, cardiac-specific FLAG-tagged α\textsubscript{1C}-expressing transgenic mice. A. Schematic representation of the binary transgene system. The αMHC-rtTA is the standard cardiac-specific reverse tetracycline-controlled transactivator system. The αMHC\textsubscript{αna} construct is a modified αMHC promoter containing the tet-uperon (tetO) for regulated expression of FLAG-tagged, DHP-resistant (DHP\textsuperscript{*}) α\textsubscript{1C}. B. Anti-FLAG antibody (upper) and anti-α\textsubscript{1C} antibody (lower) immunoblots showing FLAG-epitope–tagged α\textsubscript{1C} expression in tsA-201 cells transfected with FLAG-tagged α\textsubscript{1C} and expression in isolated cardiomyocytes from either pWT α\textsubscript{1C} or ANNAN-S1700A-T1704A transgenic mice. C. Bar graph of densitometries of cleaved α\textsubscript{1C} band divided by truncated-full-length α\textsubscript{1C} bands. n=4 NTG mice; n=10 pWT α\textsubscript{1C} mice; n=6 ANNAN-S1700A-T1704A mice. P<0.001 compared with nontransgenic. D. Immunostaining of pWT α\textsubscript{1C} and ANNAN-S1700A-1704A cardiomyocytes with or without (negative control) anti-FLAG antibody (Ab) and fluorescein isothiocyanate–conjugated secondary antibody, and nuclear labeling with Hoechst stain. Images obtained with confocal microscope at ×40 magnification. E and F. Time course of changes in sarcomere length after superfusion of nisoldipine (300 nmol/L) containing solution. Cardiomyocytes were field-stimulated at 1-Hz. αMHC indicates α-myosin heavy chain; DHP, dihydropyridine; NTG, nontransgenic; pWT, pseudowild-type; rtTA, reverse transcriptional transactivator; and TG, transgenic.
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mutant transgenic mice (n=82; P≤0.001 compared with nontransgenic). In other words, ≈30% of the peak current in the cardiomyocytes isolated from doxycycline-treated transgenic mice was insensitive to nisoldipine (Figure 3G).

The voltage dependence of CaV1.2 activation for endogenous, transgenic pWT and ΔNNAN-S1700A-T1704A α1C were equivalent (Figure 3D and 3E), implying that at least under basal conditions, the modulation of transgenic CaV1.2 channels by accessory proteins was similar to endogenous CaV1.2 channels.

Adrenergic Modulation of CaV1.2 in WT α1C Transgenic Mice

In cardiomyocytes isolated from nontransgenic mice, we measured the effects of the β-adrenergic agonist, isoproterenol, in the presence of nisoldipine. Isoproterenol (200 nmol/L) increased the small amount of residual CaV1.2 current by a mean of 2.5±0.2-fold (Figure 4A and 4F). Other groups have shown a similar response to isoproterenol stimulation in adult murine cardiomyocytes, with a range of 1.6- to 2.8-fold increase in basal currents.25,28,32,44,45

In the cardiomyocytes isolated from pWT α1C transgenic mice, isoproterenol increased the nisoldipine-insensitive peak current by a mean of 2.1±0.3-fold (Figure 5A; P=not significant compared with nontransgenic). In cardiomyocytes with peak CaV1.2 currents >15 pA/pF, in contrast, isoproterenol increased CaV1.2 currents by only 1.4±0.1-fold (Figure 5A; P<0.05). Across the broad range of basal current densities, the effect of isoproterenol on the nisoldipine-resistant current was inversely correlated with the basal total CaV1.2 current (Figure 5B). The diminished adrenergic modulation of the transgenic pWT CaV1.2 current compared with endogenous CaV1.2 is likely because of the increased basal CaV1.2 current density in the transgenic cardiomyocytes. Cardiomyocytes may have a limited number of permissive sites on the membrane where PKA-mediated upregulation of CaV1.2 current can occur and channels in excess of this limited number may be less responsive to β-adrenergic stimulation, thereby diluting the overall fold-increase in CaV1.2 currents.27

Phosphorylation of Ser1700 and Thr1704 Are Not Required for Isoproterenol- and Forskolin-Induced Stimulation of CaV1.2 Currents

Freshly isolated cardiomyocytes were isolated from doxycycline-treated ΔNNAN-S1700A-T1704A transgenic mice. In the presence of nisoldipine, isoproterenol increased peak CaV1.2 current by a mean of 1.7±0.1-fold, identical to the isoproterenol-induced augmentation of current in pWT α1C transgenic cardiomyocytes (P=not significant; pWT α1C versus ΔNNAN-S1700A-T1704A; Figure 4C, 4E, and 4F). In the presence
of nisoldipine, forskolin increased peak Ca_{1.2} current by a mean of 1.9±0.1-fold increase in cardiomyocytes isolated from the ΔNNAN-S1700A-T1704A mice, nearly identical to the 1.8±0.1-fold increase in pWT α_{1C} cardiomyocytes (Online Figure III).

Similar to the pWT α_{1C} transgenic mice, the magnitude of isoproterenol-induced increase in nisoldipine-insensitive Ca^{2+} current was inversely correlated with the basal total CaV_{1.2} current (Figure 5C and 5D). The slopes and intercepts of the 2 linear regression lines describing the relationship of total basal current density and response to isoproterenol of pWT and ΔNNAN-S1700A were not statistically different (Figure 5D). In cardiomyocytes with a total basal CaV_{1.2} current density <10 pA/pF, isoproterenol caused a 1.8±0.1-fold increase in Ca_{1.2} current (P=not significant compared with pWT transgenic α_{1C}). In cardiomyocytes with basal Ca_{1.2} current density >15 pA/pF, isoproterenol caused a 1.3±0.1-fold increase in Ca_{1.2} current (P=not significant compared with pWT α_{1C}). Stratifying the magnitude of the isoproterenol effect by the fraction of nisoldipine-resistant current also demonstrated that the increase in Ca^{2+} current was equivalent for WT α_{1C} and ΔNNAN-S1700A-T1704A α_{1C} (Figure 5E and 5F). Thus, phosphorylation of Ser^{1700} or Thr^{1704} is not required for isoproterenol- or forskolin-induced modulation of Ca_{1.2} current.

Isoproterenol-Induced Modulation of Fractional Shortening Is Preserved in Cardiomyocytes Isolated From ΔNNAN-S1700A-T1704A Mutant Mice

We incubated cardiomyocytes for at least 2 minutes in the superfusion solution containing 300-nmol/L nisoldipine, to ensure that all cardiomyocytes were exposed to nisoldipine. In nontransgenic cardiomyocytes, >95% of the cardiomyocytes failed to contract to electric field stimulation at 1-Hz, and in the remaining cardiomyocytes, contraction was reduced by 80% (Online Figure IVA and IVB). Isoproterenol increased the fractional shortening of the myocytes by 1.5-fold, both in the absence and presence of 300 nmol/L nisoldipine (Online Figure IVA and IVB). The cardiomyocytes isolated from both FLAG-tagged pWT and FLAG-tagged ΔNNAN-S1700A-T1704A, DHPr-resistant transgenic mice were relatively resistant to the effects of nisoldipine (Online Figure IVA and IVC). More than 90% of cardiomyocytes demonstrated sustained contraction to electric field stimulation at 1-Hz. Isoproterenol increased the fractional shortening of myocytes, in the presence of nisoldipine, in both pWT and ΔNNAN-S1700A-T1704A transgenic lines by 1.6- and 1.7-fold, respectively (Online Figure IVA and IVC). Thus, phosphorylation of either Ser^{1700} or Thr^{1704} is not required for β-adrenergic modulation of excitation–contraction coupling in murine cardiomyocytes.
Discussion

In this study, we have developed an approach to probe molecular aspects efficiently and reliably of CaV1.2 regulation within the context of freshly isolated cardiomyocytes, approximating the ease and power of a heterologous expression system. In prior studies, overexpression of $\alpha_{1C}$ or $\beta$ subunits markedly reduced the $\beta$-adrenergic regulation of the channel and induced cardiac dysfunction or apoptosis. To circumvent these problems, we created inducible, tissue-specific, transgenic mice expressing, DHP-resistant, FLAG-epitope–tagged $\alpha_{1C}$. This approach preserves hormonal regulation of CaV1.2 by limiting its overexpression. The channels containing the transgenic $\alpha_{1C}$ are transported appropriately to the dyad and can initiate excitation–contraction coupling.

Using this newly developed approach, we now show that $\beta$-adrenergic regulation of cardiac CaV1.2 is robust in doxycycline-regulated transgenic mice. The isoproterenol-induced increase in current in the cardiomyocytes from transgenic mice is similar to previously reported studies. Muth et al. reported that isoproterenol (100 nmol/L) induced a 1.7±0.2-fold increase in peak Ca$^{2+}$ current, but only a 1.2±0.1-fold increase in cardiomyocytes isolated from $\alpha_{1C}$ overexpressing transgenic mice. Brandmayr et al. reported that isoproterenol (100 nmol/L) increased peak current by 1.9±0.25-fold in WT mice and 1.8±0.25-fold in mice with a deletion of the $\beta$-subunit C-terminus at Pro 501. Nichols et al. reported an approximate 2-fold increase in CaV1.2 currents by isoproterenol (100 nmol/L) in WT and AKAP5 knock-out mice. 

**Figure 5. Analysis of isoproterenol’s effects on transgenic CaV2 currents.**

A and C, Bar graphs of isoproterenol-induced increase in nisoldipine-resistant current binned by total basal current density before nisoldipine for pWT $\alpha_{1C}$ (A) and NNAN-S1700A-T1704A transgenic mice (C). Mean±SEM. *P<0.05 by ANOVA and Tukey post hoc test. B and D, Graphs of isoproterenol-induced increase in nisoldipine-resistant current stratified by total basal current density before nisoldipine for pWT $\alpha_{1C}$ (B), and pWT $\alpha_{1C}$ and NNAN-S1700A-T1704A transgenic mice (D). Lines fitted by linear regression. E, Bar graph of isoproterenol-induced increase in nisoldipine-resistant current binned by fraction of nisoldipine-resistant current for pWT $\alpha_{1C}$ and NNAN-S1700A-T1704A mice. Mean±SEM. P=not significant. F, Graph of isoproterenol-induced increase in nisoldipine-resistant current stratified by fraction of nisoldipine-resistant current for pWT $\alpha_{1C}$ and NNAN-S1700A-T1704A mice. The differences between the slopes and intercepts of pWT $\alpha_{1C}$ and NNAN-S1700A-T1704A are not significant. pWT indicates pseudowild-type; and TG, transgenic.
CaV1.2. Stratifying the magnitude of the effects of the mutations on dynamic range of modulation could theoretically minimize reduced with increased basal current density. Reducing the sites within expressing the β2a subunit. Thus, by limiting overexpression of CaV1.2, the magnitude of β-adrenergic–mediated upregulation of CaV1.2 current by total basal current density is 1.6-fold, but isoproterenol did not increase the current amplitude in transgenic mice over WT mice by 1.6-fold, but isoproterenol did not increase the current amplitude in transgenic mice overexpressing the β2a subunit. Thus, by limiting overexpression of α1c and only inducing expression of α1c for 1 to 5 days, we have developed a highly reliable system that can accurately and efficiently report the functional effects of mutations.

A New Approach to Study the Regulation of CaV1.2 in Cardiomyocytes

Although useful for investigating biophysical properties, heterologous expression systems have not been successful for exploring physiological modulation, especially as related to cardiomyocytes. Compared with creating a knock-in mouse, expressing transgenic DHP-resistant α1c mutants in the heart is rapid and cost-effective, and multiple sites within α1c can be mutated at 1 time, regardless of intron/exon boundaries. There are, however, drawbacks using the approach. Transgenic expression naturally increases the basal current density, potentially disrupting normal stoichiometry and regulation. In the case of β-adrenergic modulation of CaV1.2, the magnitude of β-adrenergic stimulation is reduced with increased basal current density. Reducing the dynamic range of modulation could theoretically minimize the effects of the mutations on β-adrenergic regulation of CaV1.2. Stratifying the magnitude of β-adrenergic–mediated upregulation of CaV1.2 current by total basal current density attenuates this confounding variable.

With or without stratification by basal current density, we found that acute β-adrenergic stimulation of CaV1.2 is not significantly altered by Ala-substitution of Ser1700, implying that phosphorylation of Ser1700 is not the primary mechanism for β-adrenergic regulation of CaV1.2. Could phosphorylation of Ser1700 play a small, secondary role in mediating β-adrenergic regulation of CaV1.2, especially under conditions of relatively low basal current density at which the effect of β-adrenergic stimulation is greatest? At low basal current density, the mean increase in current for cardiomyocytes isolated from pWT α1c transgenic mice was 2.11±0.25-fold, whereas for cardiomyocytes from the ΔNNAN-S1700A-T1704A transgenic mice, the mean increase was 1.84±0.25-fold, a nonsignificant relative difference of 13%. In this low basal current density group, the current density of the cardiomyocytes from the ΔNNAN-S1700A-T1704A transgenic mice was slightly higher than pWT α1c transgenic mice (6.5 pA/pF versus 5.5 pA/pF), which may have contributed to the slightly lower increase β-adrenergic stimulation in the ΔNNAN-S1700A-T1704A transgenic mice.

Assuming 7% of endogenous current is not blocked by nisoldipine (Figure 2) and 65% of DHP-insensitive transgenic channels are not blocked by nisoldipine (Online Figure I), the maximal contamination of nisoldipine-resistant currents by endogenous channels would be 8% at 40% nisoldipine-resistant current to total current and ≈14% at 30% nisoldipine-resistant current to total current (See Online Methods). At 40% fractional nisoldipine resistance in the cardiomyocytes isolated from ΔNNAN-S1700A-T1704A mice, the effects of β-adrenergic stimulation are identical to cardiomyocytes isolated from pWT α1c mice (Figure 5E).

Taken together, these findings imply that phosphorylation of Ser1700 and Thr1704 cannot be the primary mechanism by which β-adrenergic agonists activate CaV1.2 in the adult cardiomyocytes.

Proteolytic Cleavage Does Not Require the Conserved Motif NNAN1800

Because proteolytic cleavage cannot be reconstituted in heterologous expression, there is no effective way to study the process, other than in native tissues. Indirect evidence, consisting of mass spectrometric analysis of the skeletal muscle α1S proteolytic peptides and sequence alignments of α1s and α1c, was used to identify Ala1800 as the putative proteolytic site in α1c. Deletion of Ala1800 and the immediately adjacent conserved residues did not alter the proteolytic cleavage of α1c suggesting that either Ala1800 is not the site in cardiomyocytes or that there is redundancy. Within the region, there are other similar motifs including NNAN1797, which would combine with Asn1802 after 1798NNAN1801 is deleted to form a 1794NNANIN motif. Whether cleavage could occur at Ala1795 in ANN transgenic mouse is a question for future study.

In summary, we have developed an approach to test informative mutants of CaV1.2 in cardiomyocytes using a transgenic mouse approach. By limiting overexpression of the CaV1.2 α1c subunit, we can reliably assess sympathetic regulation of CaV1.2. These data demonstrate that phosphorylation of Ser1700 and Thr1704 are not the primary mechanisms mediating β-adrenergic modulation of both Ca2+ current and excitation–contraction coupling in adult cardiomyocytes.

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Disclosures

None.

References


What Is Known?

- The L-type Ca²⁺ channel (Cav1.2) plays a key role in cardiac excitation–contraction coupling and it is an important target of the sympathetic nervous system.
- It has been suggested that proteolytic cleavage of α₁C, at residue Ala¹⁸⁰⁰, and protein kinase A phosphorylation of Ser¹⁷⁰⁰ mediate β-adrenergic–induced enhancement of cardiac CaV1.2 current, but these concepts have not been tested in cardiomyocytes.
- Although heterologous expression of CaV₁.2 channels has proven useful for investigating biophysical properties, it has not been as successful for exploring physiological modulation, especially as related to cardiomyocytes.

What New Information Does This Article Contribute?

- Selective and inducible expression in mice of FLAG-epitope–tagged, dihydropyridine-resistant CaV1.2 channels harboring mutations at key regulatory sites can be used to assess the properties of α₁C mutants in freshly isolated adult cardiomyocytes.
- Adrenergic regulation of CaV₁.2 current and fractional shortening of cardiomyocytes do not require phosphorylation of either Ser¹⁷⁰⁰ or Thr¹⁷⁰⁴ of the α₁C subunit.

Novelty and Significance

- Deletion of β⁹⁸⁸NNAN¹⁸⁰¹, the previously proposed cleavage site, does not prevent distal α₁C C-terminal proteolysis.

Excitation–contraction coupling is controlled, in part, through the precise regulation of Ca²⁺ influx by several neurohormonal and second-messenger systems, including the β-adrenergic/protein kinase A signaling pathway; however, the molecular mechanisms of β-adrenergic regulation of CaV₁.2 in cardiomyocytes are incompletely understood. A key obstacle has been the failure to reconstitute adrenergic regulation reproducibly in heterologously expressed CaV₁.2. To circumvent this problem, we used doxycycline-inducible, cardiac-specific, transgenic mice—expressing FLAG-epitope–tagged, dihydropyridine-resistant α₁C. In this system, we examined the proposed roles of proteolytic cleavage of α₁C, at residue Ala¹⁸⁰⁰, and protein kinase A phosphorylation of Ser¹⁷⁰⁰ in mediating β-adrenergic–induced enhancement of cardiac CaV₁.2 current.

We tested these predictions in native cardiomyocytes by creating a transgenic mouse expressing 3 mutations within α₁C (S₁⁷₀⁰A, T₁⁷₀⁴A, and Δ¹⁷⁹⁸⁸NNAN¹⁸⁰¹). We found that in cardiomyocytes, the NNAN motif is not required for cleavage of α₁C, and that Ser¹⁷⁰⁰ and Thr¹⁷⁰⁴ are not required for the β-adrenergic modulation of both Ca²⁺ current and excitation–contraction coupling.
β-Adrenergic Regulation of the L-type Ca\(^{2+}\) Channel Does Not Require Phosphorylation of α\(_{1C}\) Ser\(^{1700}\)

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Online Figure I. DHP-resistant currents in tsA-201 cells. (A) Exemplar whole cell current traces recorded from -70 mV to +10 mV before and after 300 nM nisoldipine. (B) Semi-log plot of dose-response relationship of nisoldipine and fraction of current blocked. IC$_{50}$ = 12 nM for WT; 650 nM for DHP-resistant, pWT $\alpha_{1C}$. N = 11 for WT and N = 12 for DHP-resistant, pWT $\alpha_{1C}$. Mean ± SEM.
Online Figure II. Inducible, cardiac-specific FLAG-tagged α₁C-expressing transgenic mice. Anti-FLAG antibody (upper) and anti-α₁C antibody (lower) immunoblots showing FLAG-epitope tagged α₁C expression in tsA-201 cells transfected with FLAG-tagged α₁C and expression in isolated cardiomyocytes from either pWT α₁C or ΔNNAN-S1700A-T1704A transgenic mice before doxycycline and after doxycycline-impregnated food for 1, 3 and 5 days.
Online Figure III. Forskolin-induced stimulation of transgenic Ca\textsubscript{v}1.2 currents. (A-B) Current-voltage relationships of pWT \(\alpha_{1C}\) (A) and \(\Delta\text{NNAN-S1700A-T1704A}\) Ca\textsubscript{v}1.2 (B) acquired in 300 nM nisoldipine, before (red traces) and 3 minutes after superfusion of 10 \(\mu\)M forskolin (green traces). Insets: Series of whole-cell Ca\textsubscript{v}1.2 currents recorded from a series of pulses between -40 mV and +50 mV from a holding potential of -70 mV before (red traces) and 3 minutes after forskolin (green traces). (C) Bar graph depicting the forskolin-induced fold-increase in peak current. Mean \(\pm\) SEM. P= not significant by Student’s t-test.
Online Figure IV. Effect of isoproterenol on fractional shortening in non-transgenic mice and doxycycline-fed transgenic mice.  (A) Changes in sarcomere length in response to field stimulation at 1-Hz. Cardiomyocytes were incubated for at least 2 minutes with 300 nM nisoldipine-containing solution (red traces). Isoproterenol (200 nM) was then superfused with 300 nM nisoldipine (blue traces). Sarcomere length was determined after 3 minutes.  (B-C) Bar graphs of % shortening in absence and presence of nisoldipine and isoproterenol. Mean ± SEM. N= 14 cardiomyocytes for all conditions. One-way Anova with Sidak post hoc test. * P<0.05, ** P<0.01, **** P<0.0001
DETAILED METHODS:

Extent of non-transgenic current in fraction of nisoldipine-resistant current

The fraction of nisoldipine-resistant current is

\[ R = \frac{I_{\text{Nis}}}{I_{\text{Tot}}} \]  

(1)

where \( I_{\text{Tot}} \) is the total peak current at +10 mV before nisoldipine, and \( I_{\text{Nis}} \) is the peak current at +10 mV in the presence of 300 nM nisoldipine.

The nisoldipine-resistant current at +10 mV is

\[ I_{\text{Nis}} = I_{\text{Tot}} * X * m + I_{\text{Tot}} * (1-X) * n \]  

(2)

where \( X \) is the fraction of endogenous current of total current, \( m \) is the fraction of current remaining in non-transgenic cardiomyocytes in the presence of 300 nM nisoldipine, and \( n \) is the fraction of remaining current of DHP-resistant transgenic channels in the presence of 300 nM nisoldipine (assessed in tsA-201).

Dividing Eq. 2 by \( I_{\text{Tot}} \) and substituting Eq. 1 \( R \) in Eq. 2, we obtain

\[ R = X * m + (1-X) * n \]  

(3)

Solving for \( X \):

\[ X = \frac{(R-n)}{(m-n)} \]  

(4)

In our experiments, \( m = 0.07 \) (see Fig. 3), \( n = 0.66 \). Therefore, when \( R=0.4 \), \( X = 0.44 \) and when \( R=0.3 \), \( X = 0.61 \).

In the presence of nisoldipine, the fraction of non-transgenic (NTG) current of the total current is:

\[ \text{Fraction}_{\text{NTG}} = \frac{(X * 0.07)}{(1-X) * 0.66 + (X * 0.07)} \]  

(5)

For \( R=0.4 \): \( \text{Fraction}_{\text{NTG}} = 0.08 \)
For \( R=0.3 \): \( \text{Fraction}_{\text{NTG}} = 0.14 \)