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

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ABSTRACT

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

Objectives: To assess the role of Ala\(^{1800}\) in the cleavage of α\(_{1c}\) and the role of Ser\(^{1700}\) and Thr\(^{1704}\) in mediating the adrenergic regulation of Ca\(_{\text{v}1.2}\) in the heart.

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

Conclusions: These results show that PKA phosphorylation of α\(_{1c}\) Ser\(^{1700}\) does not have a major role in the sympathetic stimulation of Ca\(^{2+}\) current and contraction in the adult murine heart. Moreover, this new transgenic approach enables functional and reproducible screening of α\(_{1c}\) mutants in freshly isolated adult cardiomyocytes in a reliable, timely and cost-effective manner.

Keywords: Ion channels, molecular electrophysiology, calcium channels, sympathetic nervous system, phosphorylation, adrenergic, transgenic mice, excitation-contraction coupling

Nonstandard Abbreviations and Acronyms:
- Ala: alanine
- DHP: dihydropyridine
- DAD: delayed after-depolarization
- EAD: early after-depolarization
- MHC: myosin heavy chain
- PKA: protein kinase A
- pWT: pseudo-wild-type
- rtTA: reverse transcriptional transactivator
- Ser: serine
- TG: transgenic
- WT: wild-type
INTRODUCTION

Ca,v1.2 has a key role in cardiac muscle excitation-contraction coupling 1, and in determining the plateau phase of the action potential 2. In pathological conditions, Ca,v1.2 currents can trigger electrical instability, early after-depolarizations (EADs), arrhythmias, and sudden death frequently in the setting of adrenergic stimulation or decreased repolarizing currents 3, 4. Increased Ca,v1.2 activity can also lead to Ca2+ overload, which in turn can result in arrhythmogenic delayed after-depolarizations (DADs).

Ca,v1.2 channels are composed minimally of a pore-forming α1C and regulatory β and α2δ subunits. In the heart, Ca,v1.2 also associates with large supramolecular complexes that regulate channel trafficking, localization, turnover, and function 5-7. Proteolytic cleavage of the α1C C-terminus, occurring in greater than 80% of cardiac Ca,v1.2 channels, has been posited to play an essential role in setting the basal activity and enabling the adrenergic stimulation of Ca,v1.2 8-15.

The molecular mechanisms of β-adrenergic regulation of Ca,v1.2 in cardiomyocytes are incompletely known. A key obstacle for decades has been the failure to reproducibly reconstitute adrenergic regulation of heterologously expressed Ca,v1.2. Ser1928, in the α1C subunit, was originally identified as the sole α1C PKA phosphorylation site 11, 16-23. Phosphorylation of this residue, however, is not required for β-adrenergic agonist stimulation of Ca,v1.2, as shown in guinea pig cardiomyocytes infected with adenovirus expressing a relatively dihydropyridine (DHP)-resistant S1928A-α1C 24, and in α1C S1928A knock-in mice 25. Similarly, although β2a Ser459, Ser478 and Ser479 are PKA phosphorylated 26, these sites are not required for β-adrenergic stimulation of Ca,v1.2 in cardiomyocytes 24, 27, 28. Based upon heterologous expression studies, Ser1700 was recently reported to be the functionally relevant PKA phosphorylation site 15, 29.

Although heterologous expression of Ca,v1.2 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 perinatal period may preclude studies at later stages of development 14, 30. Although adenoviruses have been used to express Ca,v1.2 subunits in cardiomyocytes, creation of adenoviruses encoding α1C is difficult because of the α1C insert size and the cardiomyocytes need to be cultured for extended period, potentially inducing dedifferentiation. Since overexpression of α1C or β 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 α1C. The approach preserves hormonal regulation of Ca,v1.2 by limiting Ca,v1.2 over-expression.

Prominent roles for proteolytic cleavage of α1C, at residue Ala1800, and PKA phosphorylation of Ser1700, in the C-terminus of α1C (Fig. 1A), in mediating β-adrenergic-induced enhancement of cardiac Ca,v1.2 current have been proposed, based upon heterologous expression of Ca,v1.2 subunits 8, 14, 15. In the absence of proteolytic cleavage at Ala1800, PKA is unable to phosphorylate Ser1700 and upregulate the activity of heterologously expressed Ca,v1.2 15. Ala-substitution of the neighboring Thr1704, a residue that may be phosphorylated by casein kinase II, reduced heterologously expressed basal Ca,v1.2 channel activity in unstimulated tsA-201 cells, and when combined with Ala-substitution of Ser1700 more effectively reduced forskolin-induced stimulation of Ca,v1.2, compared to Ala-substitution of Ser1700 alone. These concepts have not been tested in cardiomyocytes. We tested these predictions in native cardiomyocytes by creating a transgenic mouse expressing three mutations within α1C (S1700A, T1704A, and Δ1798NNAN1801).
METHODS

Reagents.
Nisoldipine (Santa Cruz) was dissolved daily in 30 mM ethanol and was protected from light. All other chemicals were acquired from Sigma.

Animals.
The pWT α1C and ΔNNAN-S1700A-T1704A constructs were generated by fusing the rabbit CACNA1C cDNA (accession X15539) to the modified murine α-myosin heavy chain (MHC), tetracycline-inducible promoter (“responder” line) vector (gift of Drs. Jeffrey Robbins and Jeffrey Molkentin) 38, 39. A 3X FLAG-epitope was ligated in-frame to the N-terminus of α1C. The α1C subunit was engineered to be DHP-insensitive with the substitutions T1066Y and Q1070M 40, 41. Transgenic founder mice were identified with genomic DNA utilizing polymerase chain reactions. These mice were bred with cardiac specific (αMHC) doxycycline-regulated codon-optimized reverse transcriptional transactivator (rtTA) mice (obtained via MMRRC) 42 to generate double transgenic mice. We selected founder lines that did not express the transgenic α1C in the absence of doxycycline. To induce expression, animals received 0.2 g/kg doxycycline-impregnated food (Bio Serv Cat # S3888) for 1-5 days. The results presented were consistent across all founder lines and gender, 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 43 from 8-12 week-old non-transgenic and doxycycline-fed transgenic mice. Cardiomyocytes were homogenized in a 1% Triton X-100 buffer containing (in mM): 50 Tris-HCl (pH7.4) 150 NaCl, 10 EDTA, 10 EGTA and protease inhibitors. The lysates were incubated on ice for 30 min, centrifuged at 14K rpm at 4°C for 10 min and supernatants collected. Proteins were size-fractionated on SDS-PAGE, transferred to nitrocellulose membranes and probed with anti-FLAG (Sigma) or anti-α1C 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 FITC-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 24-48 hours after transfection. The isolated cardiomyocytes and tsA-201 cells were superfused with (in mM) 140 TEA-Cl, 1.8 CaCl2, 1 MgCl2, 10 glucose, and 10 HEPES, 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 mM) 135 CsCl, 10 EGTA, 1 MgCl2, 2 Mg-ATP, 2.0 CaCl2, and 10 HEPES, 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 5-10 seconds. To evaluate the current-voltage relationship for Ca2+ currents, the same protocol was repeated with steps between -50 mV to +50 mV in 10 mV increments.

Fractional shortening.
Freshly isolated myocytes were perfused with a Tyrode’s solution containing 1.8 mM CaCl2. Myocytes were field stimulated at 1-Hz. In one series of experiments, nisoldipine (300 nM) was superfused in the absence and presence of isoproterenol (200 nM). In the second series of experiments, cardiomyocytes were placed in a Tyrode’s solution containing 300 nM nisoldipine. A Tyrode’s solution containing 300 nM nisoldipine and 200
nM isoproterenol was superfused. Fractional shortening of sarcomere length was measured using the SarcLen module of Ionoptix.

**Statistical analysis.**
Results are presented as mean ± SEM. For multiple group comparisons, a one-way ANOVA followed by Tukey’s, Sidak’s or Dunnett’s post hoc tests were performed. For comparisons between two groups, an unpaired Student’s t-test was used. Statistical analyses were performed using Prism 6 (Graphpad Software). Differences were considered statistically significant at values of $P < 0.05$.

**RESULTS**

*Generation of inducible, cardiac-specific $\alpha_{1C}$ transgenic mice.*

We deleted the highly conserved $^{1708}$NNAN$^{1801}$ motif in $\alpha_{1C}$ (Fig. 1B) and co-expressed the cDNA with the $\beta_2$ 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 $\text{Ca}_{\text{V}1.2}$ (Fig. 1C-D). Since proteolytic cleavage of $\alpha_{1C}$ does not occur when wild-type (WT) $\alpha_{1C}$ is expressed heterologously, the effect of deletion of the putative cleavage site on proteolysis of $\alpha_{1C}$ could not be assessed using this approach (Fig. 1C).

We generated transgenic mice with inducible cardiomyocyte-specific expression of a N-terminal 3X FLAG-epitope-tagged dihydropyridine (DHP)-resistant $\alpha_{1C}$, designated pseudo-WT, [pWT $\alpha_{1C}$]) using a bitransgenic tetracycline-regulated system that permits robust expression only when both transgenes, and doxycycline are present (Fig. 2A). The $\alpha_{1C}$ subunit was engineered to be relatively DHP-insensitive with the substitutions $\text{T1066Y and Q1070M}$ $^{40,41}$. The IC$_{50}$ for nisoldipine block of heterologously expressed WT $\alpha_{1C}$ was 12 nM, whereas the IC$_{50}$ for pWT $\alpha_{1C}$ was 650 nM (Online Fig. I). We selected a concentration of 300 nM nisoldipine as optimal for further experiments since nisoldipine (300 nM) blocked >98% of heterologously expressed WT $\text{Ca}_{\text{V}1.2}$ current in tsA-201 cells, but only blocked $34.6 \pm 2.5\%$ of DHP-insensitive $\alpha_{1C}$ (Online Fig. I).

Seven pWT $\alpha_{1C}$ founder transgenic lines were originally generated. Two founder lines were lost due to mortality, possibly because of high levels of doxycycline-independent $\alpha_{1C}$ expression. Four founder lines, when crossed with $\alpha$MHC-rtTA mice, demonstrated doxycycline-induced expression of $\alpha_{1C}$, assessed by anti-FLAG antibody immunoblots (Fig. 2B, upper; Online Fig. II). One transgenic founder line, after crossing with $\alpha$MHC-rtTA mice, did not demonstrate doxycycline-induced $\alpha_{1C}$ expression. Of 59 pWT $\alpha_{1C}$ bitransgenic mice treated with doxycycline, 18 mice (31%) died within 5 days of doxycycline administration possibly due high levels of doxycycline-dependent $\alpha_{1C}$ expression.

We also generated a transgenic mouse line expressing three mutations within $\alpha_{1C}$, Ala-substitutions of Ser$^{1700}$ (S1700A) and Thr$^{1704}$ (T1704A), and deletion of the $^{1708}$NNAN$^{1801}$ motif ($\Delta$NNAN), in the background of a N-terminal 3X FLAG-epitope tag and DHP-resistance ($\Delta$NNAN-S1700A-T1704A). Six $\Delta$NNAN-S1700A-T1704A mutant transgenic founder lines were originally generated. Three founders, when crossed with $\alpha$MHC-rtTA, demonstrated doxycycline-induced $\alpha_{1C}$ expression (Fig. 2B, upper; Online Fig. II). The other 3 founders, after crossing with $\alpha-$MHC-rtTA mice, had either no or low levels of doxycycline-induced expression. Of the 35 $\Delta$NNAN-S1700A-T1704A mutant mice treated with doxycycline, 9 mice (26%) died within 5 days, possibly due to high levels of $\alpha_{1C}$ expression. The 41 pWT $\alpha_{1C}$ transgenic mice and the 26 $\Delta$NNAN-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 α_{1C} subunits consistent with t-tubular localization (Fig. 2D). No staining was detected in cardiomyocytes when the anti-FLAG antibody was omitted.

Cardiomyocyte contraction requires Ca^{2+} influx via Ca_{v}1.2, which triggers sarcoplasmic reticulum (SR) Ca^{2+} release. Superfusion of nisoldipine inhibited the contraction of non-transgenic cardiomyocytes to electric field stimulation at 1-Hz (Fig. 2E). In cardiomyocytes isolated from pWT α_{1C} transgenic mice, the effect of nisoldipine was greatly diminished (Fig. 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_{1C} in tsA-201 cells migrated as full-length α_{1C} without evidence of proteolytic processing, detected by immunoblots using anti-FLAG and anti-α_{1C} antibodies (Figs. 1C, 2B). In cardiomyocytes isolated from non-transgenic mice (C57Bl/6), native α_{1C} was detected as a full-length ~240 kDa band and a cleaved ~210 kDa band, using an anti-α_{1C} antibody created against an internal epitope within the intracellular loop of domains II and III. Native α_{1C} in non-transgenic mice cannot be detected using an anti-FLAG antibody (Fig. 2B). Both the pWT α_{1C} transgenic channels and the transgenic channels with a deletion of \textsuperscript{1798}NNAN\textsuperscript{1801} were proteolytically cleaved, detected using the anti-FLAG antibody (Fig. 2B, Online Fig. II). The ratios of cleaved to full-length pWT and ΔNNAN transgenic α_{1C} were 62% ± 4% and 72% ± 5% respectively, not significantly different than the 79% ± 5% cleavage of the native α_{1C} (Fig. 2C). Since deletion of the putative proteolytic cleavage site had no effect on the ratio of truncated to full-length α_{1C} in cardiomyocytes, we can conclude that the NNAN motif and Ala\textsuperscript{1800} are not required for post-translational cleavage of α_{1C}.

Functional, inducible expression of pWT and mutant DHP-insensitive transgenic α_{1C} in cardiomyocytes.

We measured Ca_{v}1.2 currents in adult cardiomyocytes from non-transgenic and transgenic mice (Fig. 3A-E). The mean current density was significantly larger in the doxycycline-fed transgenic mice than in the non-transgenic mice (5.9 ± 0.8 pA/pF [n=12] in non-transgenic cardiomyocytes, 12.9 ± 0.9 pA/pF in pWT α_{1C} cardiomyocytes [n=43, P<0.0001 compared to non-transgenic], and 9.9 ± 0.5 pA/pF in ΔNNAN-S1700A-T1704A mutant cardiomyocytes [n=82, P<0.05 compared to non-transgenic]) (Fig. 3F). Nisoldipine (300 nM) inhibited 92.4% ± 1.6% of endogenous peak Ca^{2+} current in cardiomyocytes isolated from non-transgenic mice (n=12), but 70% + 3.6% of peak current in cardiomyocytes isolated from doxycycline-fed pWT α_{1C} transgenic mice (n= 43, P ≤ 0.001 compared to non-transgenic) and 70% + 1.8% of peak current in the cardiomyocytes isolated from doxycycline-fed ΔNNAN-S1700A-T1704A mutant transgenic mice (n= 82, P ≤ 0.001 compared to non-transgenic). In other words, approximately 30% of the peak current in the cardiomyocytes isolated from doxycycline-treated transgenic mice was insensitive to nisoldipine (Fig. 3G). The voltage dependence of Ca_{v}1.2 activation for endogenous, transgenic pWT and ΔNNAN-S1700A-T1704A α_{1C} were equivalent (Fig. 3D-E), implying that at least under basal conditions, the modulation of transgenic Ca_{v}1.2 channels by accessory proteins was similar to endogenous Ca_{v}1.2 channels.

Adrenergic-modulation of Ca_{v}1.2 in WT α_{1C} transgenic mice.

In cardiomyocytes isolated from non-transgenic mice, we measured the effects of the β-adrenergic agonist, isoproterenol, in the presence of nisoldipine. Isoproterenol (200 nM) increased the small amount of residual Ca_{v}1.2 current by a mean of 2.5 ± 0.2- fold (Fig. 4A, F). 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 \(\alpha_{1C}\) transgenic mice, isoproterenol increased the nisoldipine-insensitive peak current by a mean of 1.7 \(\pm\) 0.1-fold (Fig. 4B, D, F) (P \(\leq 0.01\) compared to non-transgenic). In cardiomyocytes with a basal current density before nisoldipine of less than 10 pA/pF, which is similar to the basal current density of cardiomyocytes from non-transgenic mice, isoproterenol increased Ca\(_{\text{V}1.2}\) currents by 2.1 \(\pm\) 0.3-fold (Fig. 5A) (P=not significant compared to non-transgenic). In cardiomyocytes with peak Ca\(_{\text{V}1.2}\) currents greater than 15 pA/pF, in contrast, isoproterenol increased Ca\(_{\text{V}1.2}\) currents by only 1.4 \(\pm\) 0.1-fold (Fig. 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 Ca\(_{\text{V}1.2}\) current (Fig. 5B). The diminished adrenergic-modulation of the transgenic pWT Ca\(_{\text{V}1.2}\) current compared to endogenous Ca\(_{\text{V}1.2}\) is likely due to the increased basal Ca\(_{\text{V}1.2}\) current density in the transgenic cardiomyocytes. Cardiomyocytes may have a limited number of permissive sites on the membrane where PKA-mediated upregulation of Ca\(_{\text{V}1.2}\) current can occur and channels in excess of this limited number may be less responsive to \(\beta\)-adrenergic stimulation, thereby diluting the overall fold-increase in Ca\(_{\text{V}1.2}\) currents \(^{27}\).

**Phosphorylation of Ser\(^{1700}\) and Thr\(^{1704}\) are not required for isoproterenol- and forskolin-induced stimulation of Ca\(_{\text{V}1.2}\) currents.**

Freshly isolated cardiomyocytes were isolated from doxycycline-treated \(\Delta\text{NNAN-S1700A-T1704A}\) transgenic mice. In the presence of nisoldipine, isoproterenol increased peak Ca\(_{\text{V}1.2}\) current by a mean of 1.7 \(\pm\) 0.1-fold, identical to the isoproterenol-induced augmentation of current in pWT \(\alpha_{1C}\) transgenic cardiomyocytes (P= not significant, pWT \(\alpha_{1C}\) vs. \(\Delta\text{NNAN-S1700A-T1704A}\)) (Fig. 4C, E-F). In the presence of nisoldipine, forskolin increased peak Ca\(_{\text{V}1.2}\) current by a mean of 1.9 \(\pm\) 0.1-fold increase in cardiomyocytes isolated from the \(\Delta\text{NNAN-S1700A-T1704A}\) mice, nearly identical to the 1.8 \(\pm\) 0.1-fold increase in pWT \(\alpha_{1C}\) cardiomyocytes (Online Fig. III).

Similar to the pWT \(\alpha_{1C}\) transgenic mice, the magnitude of isoproterenol-induced increase in nisoldipine-insensitive Ca\(_{2+}\) current was inversely correlated with the basal total Ca\(_{\text{V}1.2}\) current (Fig. 5C, D). The slopes and intercepts of the two linear regression lines describing the relationship of total basal current density and response to isoproterenol of pWT \(\alpha_{1C}\) transgenic cardiomyocytes (P= not significant, pWT\(\alpha_{1C}\) vs. \(\Delta\text{NNAN-S1700A-T1704A}\)) (Fig. 4C, E-F). In the presence of nisoldipine, forskolin increased peak Ca\(_{\text{V}1.2}\) current by a mean of 1.9 \(\pm\) 0.1-fold increase in cardiomyocytes isolated from the \(\Delta\text{NNAN-S1700A-T1704A}\) mice, nearly identical to the 1.8 \(\pm\) 0.1-fold increase in pWT \(\alpha_{1C}\) cardiomyocytes (Online Fig. III).

Isoproterenol-induced modulation of fractional shortening is preserved in cardiomyocytes isolated from \(\Delta\text{NNAN-S1700A-T1704A}\) mutant mice.

We incubated cardiomyocytes for at least 2 minutes in the superfusion solution containing 300 nM nisoldipine, in order to ensure that all cardiomyocytes were exposed to nisoldipine. In non-transgenic 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% (IVFig. 4A-B). Isoproterenol increased the fractional shortening of the myocytes by 1.5-fold, both in the absence and presence of 300 nM nisoldipine (Online Fig. IVA-B). The cardiomyocytes isolated from both FLAG-tagged pWT and FLAG-tagged \(\Delta\text{NNAN-S1700A-T1704A}\) DHP-resistant transgenic mice were relatively resistant to the effects of nisoldipine (Online
Fig. IVA, C). Greater 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 Fig. IVA, C). Thus, phosphorylation of either Ser\textsuperscript{1700} or Thr\textsuperscript{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 efficiently and reliably probe molecular aspects of Ca\textsubscript{v}1.2 regulation within the context of freshly isolated cardiomyocytes, approximating the ease and power of a heterologous expression system. In prior studies, overexpression of α\textsubscript{1C} or β subunits markedly reduced the β-adrenergic regulation of the channel, and induced cardiac dysfunction or apoptosis\textsuperscript{31, 32, 34-36}. To circumvent these problems, we created inducible, tissue-specific, transgenic-mice expressing, DHP-resistant, FLAG-epitope-tagged α\textsubscript{1C}. This approach preserves hormonal regulation of Ca\textsubscript{v}1.2 by limiting its over-expression. The channels containing the transgenic α\textsubscript{1C} are transported appropriately to the dyad and can initiate excitation-contraction coupling.

Using this newly developed approach, we now show that β-adrenergic regulation of cardiac Ca\textsubscript{v}1.2 channels is unaltered by Ala-substitution of Ser\textsuperscript{1700} or Thr\textsuperscript{1704}, indicating that these sites are dispensable for this purpose in adult cardiomyocytes. Ser\textsuperscript{1700} was recently reported to be the functionally relevant PKA site in heterologously expressed Ca\textsubscript{v}1.2\textsuperscript{15, 29}. Phosphorylation of Thr\textsuperscript{1704}, a consensus site for casein kinase II, increases the basal activity of heterologously expressed Ca\textsubscript{v}1.2\textsuperscript{15}. It may also play a role in adrenergic modulation of Ca\textsubscript{v}1.2, since forskolin-induced stimulation of heterologously expressed Ca\textsubscript{v}1.2 was more attenuated with the double mutant S1700A-T1704A than for S1700A alone\textsuperscript{15}. Although Ser\textsuperscript{1928} is PKA phosphorylated\textsuperscript{11, 16-23}, it is not required for β-adrenergic stimulation of Ca\textsubscript{v}1.2\textsuperscript{24, 25}, and forskolin-induced stimulation of the heterologously expressed triple mutant S1700A-T1704A-S1928A was not different than the double mutant S1700A-T1704A\textsuperscript{15}. It is based upon these experiments\textsuperscript{15} that we chose the S1700A-T1704A mutations for testing in transgenic mice. We were unable to assess the role of Thr\textsuperscript{1704} on basal activity in cardiomyocytes, however, since the basal activity of heterologously expressed Ca\textsubscript{v}1.2 was determined by comparing the coupling efficiency of pore opening to gating charge movement\textsuperscript{15}.

**β-adrenergic stimulation of Ca\textsubscript{v}1.2 currents 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. Schwartz and colleagues reported that isoproterenol (100 nM) induced a 1.7 ± 0.2-fold increase in peak Ca\textsuperscript{2+} current, but only a 1.2 ± 0.1-fold increase in cardiomyocytes isolated from α\textsubscript{1C} over-expressing transgenic mice\textsuperscript{32}. Moosmang and Hofmann reported that isoproterenol (100 nM) 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 β-subunit C-terminus at Pro\textsuperscript{501}\textsuperscript{28}. McKnight, Santana and Catterall reported an approximate 2-fold increase in Ca\textsubscript{v}1.2 currents by isoproterenol (100 nM) in WT and AKAP5 knock-out mice\textsuperscript{44}. Chen and Houser reported that isoproterenol increased Ca\textsubscript{v}1.2 currents in WT mice by 1.6-fold, but isoproterenol did not increase the current amplitude in transgenic mice overexpressing the β\textsubscript{2a} subunit\textsuperscript{45}. Thus, by limiting over-expression of α\textsubscript{1C} and only inducing expression of α\textsubscript{1C} for 1-5 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 Ca\textsubscript{v}1.2 in cardiomyocytes.**

Although useful for investigating biophysical properties, heterologous expression systems have not been
successful for exploring physiological modulation, especially as related to cardiomyocytes\textsuperscript{14, 27, 30, 46}. Compared to creating a knock-in mouse, expressing transgenic DHP-resistant $\alpha_{1C}$ mutants in the heart is rapid and cost-effective, and multiple sites within $\alpha_{1C}$ can be mutated at one 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 $\beta$-adrenergic modulation of $\text{Ca}_{\text{v}}1.2$, the magnitude of $\beta$-adrenergic stimulation is reduced with increased basal current density. Reducing the dynamic range of modulation could theoretically minimize the effects of the mutations on $\beta$-adrenergic regulation of $\text{Ca}_{\text{v}}1.2$. Stratifying the magnitude of $\beta$-adrenergic-mediated upregulation of $\text{Ca}_{\text{v}}1.2$ current by total basal current density attenuates this confounding variable.

With or without stratification by basal current density, we found that acute $\beta$-adrenergic stimulation of $\text{Ca}_{\text{v}}1.2$ is not significantly altered by Ala-substitution of Ser$^{1700}$, implying that phosphorylation of Ser$^{1700}$ is not the primary mechanism for $\beta$-adrenergic regulation of $\text{Ca}_{\text{v}}1.2$. Could phosphorylation of Ser$^{1700}$ play a small, secondary role in mediating $\beta$-adrenergic regulation of $\text{Ca}_{\text{v}}1.2$, especially under conditions of relatively low basal current density at which the effect of $\beta$-adrenergic stimulation is greatest? At low basal current density, the mean increase in current for cardiomyocytes isolated from pWT $\alpha_{1C}$ transgenic mice was $2.11 \pm 0.25$-fold, whereas for cardiomyocytes from the $\Delta$NNAN-S1700A-T1704A transgenic mice, the mean increase was $1.84 \pm 0.25$-fold, a non-significant relative difference of 13%. In this low basal current density group, the current density of the cardiomyocytes from the $\Delta$NNAN-S1700A-T1704A transgenic mice was slightly higher than pWT $\alpha_{1C}$ transgenic mice (6.5 pA/pF vs. 5.5 pA/pF), which may have contributed to the slightly lower increase $\beta$-adrenergic stimulation in the $\Delta$NNAN-S1700A-T1704A transgenic mice.

Assuming 7% of endogenous current is not blocked by nisoldipine (Fig. 2) and 65% of DHP-insensitive transgenic channels are not blocked by nisoldipine (Online Fig. I), the maximal contamination of nisoldipine-resistant currents by endogenous channels would be $\sim$8% at 40% nisoldipine-resistant current to total current and $\sim$14% at 30% nisoldipine-resistant current to total current (See Online Methods). At 40% fractional nisoldipine resistance in the cardiomyocytes isolated from $\Delta$NNAN-S1700A-T1704A mice, the effects of $\beta$-adrenergic stimulation are identical to cardiomyocytes isolated from pWT $\alpha_{1C}$ mice (Fig. 5E). Taken together, these findings imply that phosphorylation of Ser$^{1700}$ and Thr$^{1704}$ cannot be the primary mechanism by which $\beta$-adrenergic agonists activate $\text{Ca}_{\text{v}}1.2$ in the adult cardiomyocytes.

**Proteolytic cleavage does not require the conserved motif 1798NNAN1801.**

Since 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 $\alpha_{1S}$ proteolytic peptides and sequence alignments of $\alpha_{1S}$ and $\alpha_{1C}$, was used to identify Ala$^{1800}$ as the putative proteolytic site in $\alpha_{1C}$\textsuperscript{8}. Deletion of Ala$^{1800}$ and the immediately adjacent conserved residues did not alter the proteolytic cleavage of $\alpha_{1C}$, suggesting that either Ala$^{1800}$ is not the site in cardiomyocytes or that there is redundancy. Within the region, there are other similar motifs including 1794NANI1797, which would combine with Asn$^{1802}$ after 1798NNAN1801 is deleted to form a 1794NANIN motif. Whether cleavage could occur at Ala$^{1795}$ in $\Delta$NNAN transgenic mouse is a question for future study.

In summary, we have developed an approach to reproducibly and efficiently test informative mutants of $\text{Ca}_{\text{v}}1.2$ in cardiomyocytes using a transgenic mouse approach. By limiting over-expression of the $\text{Ca}_{\text{v}}1.2$ $\alpha_{1C}$ subunit, we can reliably assess sympathetic regulation of $\text{Ca}_{\text{v}}1.2$. These data demonstrate that phosphorylation of Ser$^{1700}$ and Thr$^{1704}$ are not the primary mechanisms mediating $\beta$-adrenergic modulation of both Ca$^{2+}$ current and excitation-contraction coupling in adult cardiomyocytes.
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DISCLOSURES
None.

REFERENCES
1. Fabiato A, Fabiato F. Calcium and cardiac excitation-contraction coupling. Annu Rev Physiol. 1979;41:473-484
7. Dai S, Hall DD, Hell JW. Supramolecular assemblies and localized regulation of voltage-gated ion channels. Physiol Rev. 2009;89:411-452
24. Ganesan AN, Maack C, Johns DC, Sidor A, O'Rourke B. Beta-adrenergic stimulation of l-type ca2+ channels in cardiac myocytes requires the distal carboxyl terminus of alpha1c but not serine 1928. *Circ Res.* 2006;98:e11-18

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**Figure Legends**

**Figure 1.** Deletion of proteolytic cleavage site does not affect heterologously expressed \( \alpha_{1C} \) channel expression or function. (A) Schematic of cardiac \( \alpha_{1C} \) subunit topology. The putative proteolytic cleavage site, \( ^{1798} \text{NNAN}^{1801} \) 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. (C) Anti-\( \alpha_{1C} \) antibody immunoblot of extracts from WT \( \alpha_{1C} \) and \( \Delta \text{NNAN} \alpha_{1C} \) expressing tsA-201 cells. (D) WT (black) and \( \Delta \text{NNAN} \alpha_{1C} \) (red) current-voltage relationships and current traces (inset). Currents elicited by 400-ms test pulses between -60 mV to +60 mV from a holding potential of -70 mV.

**Figure 2.** Inducible, cardiac-specific FLAG-tagged \( \alpha_{1C} \)-expressing transgenic mice. (A) Schematic representation of the binary transgene system. The \( \alpha \text{MHC-rtTA} \) is the standard cardiac-specific reverse tetracycline-controlled transactivator system. The \( \alpha \text{MHC}_{\text{MOD}} \) construct is a modified \( \alpha \text{MHC} \) promoter containing the tet-operator for regulated expression of FLAG-tagged DHP-resistant (DHP*) \( \alpha_{1C} \). (B) Anti-FLAG antibody (upper) and anti-\( \alpha_{1C} \) antibody (lower) immunoblots showing FLAG-epitope tagged \( \alpha_{1C} \) expression in tsA-201 cells transfected with FLAG-tagged \( \alpha_{1C} \) and expression in isolated cardiomyocytes from either pWT \( \alpha_{1C} \) or \( \Delta \text{NNAN-S1700A-T1704A} \) transgenic mice. (C) Bar graph of densitometries of cleaved \( \alpha_{1C} \) band divided by truncated + full-length \( \alpha_{1C} \) bands. \( N=4 \) non-transgenic (NTG) mice; \( N=10 \) pWT \( \alpha_{1C} \) mice; \( N=6 \) \( \Delta \text{NNAN-S1700A-T1704A} \) mice. \( P \) = not significant by Anova. (D) Immunostaining of pWT \( \alpha_{1C} \) and \( \Delta \text{NNAN-S1700A-T1704A} \) cardiomyocytes with or without (negative control) anti-FLAG antibody and FITC-conjugated secondary antibody, and nuclear labeling with Hoechst stain. Images obtained with confocal microscope at 40X magnification. (E-F) Time course of changes in sarcomere length after superfusion of nisoldipine (300 nM) containing solution. Cardiomyocytes were field-stimulated at 1-Hz.

**Figure 3.** Dihydropyridine-resistant currents in transgenic mice. (A-C) Exemplar whole-cell Ca\(_{1.2}\) currents recorded from pulses from -70 mV to +10 mV before (black traces) and 3 minutes after (red traces) of 300 nM nisoldipine. (D-E) Current-voltage relationships of pWT \( \alpha_{1C} \) (D) and \( \Delta \text{NNAN-S1700A-T1704A} \) Ca\(_{1.2}\) (E) acquired before (black traces) and 3 minutes after superfusion of 300 nM nisoldipine. Insets: Series of whole-cell Ca\(_{1.2}\) currents recorded from a series of pulses between -40 mV and + 50 mV from a holding potential of -70 mV in the absence of nisoldipine (black traces) and 3 minutes after 300 nM nisoldipine (red traces). (E-F) Combined bar graph and column scatter plot for total peak current density (pA/pF) and peak DHP-resistant current density (pA/pF). Bar graphs are mean + SEM. ***\( P<0.0001 \), *** \( P<0.001 \), * \( P<0.05 \) by one-way Anova and Sidak’s post-hoc test.

**Figure 4.** \( \beta \)-adrenergic stimulation of Ca\(_{1.2}\) current does not require phosphorylation of Ser\(^{1700}\). (A-C) Exemplar whole-cell Ca\(_{1.2}\) currents recorded from pulses from -70 mV to +10 mV before (red traces) and 3 minutes after (blue traces) superfusion of 200 nM isoproterenol, in the presence of nisoldipine. (D-E) Ca\(_{2+}\) current-voltage relationships before (red trace) and after (blue trace) 200 nM isoproterenol, in the presence of 300 nM nisoldipine in cardiomyocytes isolated from pWT \( \alpha_{1C} \) (\( N=4 \)) and \( \Delta \text{NNAN-S1700A-T1704A} \) mice (\( N=8 \)). Mean ± SEM. Insets: Series of whole-cell Ca\(_{1.2}\) currents recorded from a series of pulses between -50 mV to +50 mV from a holding potential of -70 mV in the presence of nisoldipine, before (red trace) and 3 minutes after (blue trace) 200 nM isoproterenol. (F) Combined bar and column scatter plot depicting the fold increase in peak current caused by isoproterenol. Bar graphs are mean ± SEM. **\( P<0.01 \) by Anova and Tukey’s post-hoc test. \( N=6 \) non-transgenic cardiomyocytes, \( N=24 \) pWT \( \alpha_{1C} \), \( N=56 \) \( \Delta \text{NNAN-S1700A-T1704A} \) cardiomyocytes.

**Figure 5.** Analysis of isoproterenol’s effects on transgenic Ca\(_{2+}\) currents. (A, 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 \( \Delta \text{NNAN-S1700A-T1704A} \) transgenic mice (C). Mean ± SEM. * \( P<0.05 \) by Anova and Tukey’s post hoc test. For pWT \( \alpha_{1C} \), \( N=9 \) cardiomyocytes for 1-10 pA/pF, \( N=9 \) cardiomyocytes for 10-15 pA/pF, \( N=8 \)
cardiomyocytes for >15 pA/pF. For ΔNNAN-S1700A-T1704A, N= 33 cardiomyocytes for 1-10 pA/pF, N=17 for 10-15 pA/pF, N=6 for >15 pA/pF. (B, D) Graphs of isoproterenol-induced increase in nisoldipine-resistant current stratified by total basal current density before nisoldipine for pWT α1C (B), and pWT α1C and ΔNNAN-S1700A-T1704A transgenic mice (D). Lines fitted by linear regression. The differences between the slopes and intercepts of pWT α1C and ΔNNAN-S1700A-T1704A are not significant. (E) Bar graph of isoproterenol-induced increase in nisoldipine-resistant current binned by fraction of nisoldipine-resistant current for pWT α1C and ΔNNAN-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 α1C and ΔNNAN-S1700A-T1704A mice. The differences between the slopes and intercepts of pWT α1C and ΔNNAN-S1700A-T1704A are not significant.
Novelty and Significance

What Is Known?

- The L-type Ca\(^{2+}\) 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 \(\alpha_{1C}\), at residue Ala\(^{1800}\) and protein kinase A (PKA) phosphorylation of Ser\(^{1700}\) mediate \(\beta\)-adrenergic-induced enhancement of cardiac Ca\(_{\text{V1.2}}\) current, but these concepts have not been tested in cardiomyocytes.
- Although heterologous expression of Ca\(_{\text{V1.2}}\) 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 (DHP)-resistant Ca\(_{\text{V1.2}}\) channels harboring mutations at key regulatory sites can be used to assess the properties of \(\alpha_{1C}\) mutants in freshly isolated adult cardiomyocytes.
- \(\beta\)-adrenergic regulation of Ca\(_{\text{V1.2}}\) current and fractional shortening of cardiomyocytes do not require phosphorylation of either Ser\(^{1700}\) or Thr\(^{1704}\) of the \(\alpha_{1C}\) subunit.
- Deletion of 1798NNAN1801, the previously proposed cleavage site, does not prevent distal \(\alpha_{1C}\) C-terminus proteolysis.

Excitation-contraction coupling is controlled in part through the precise regulation of Ca\(^{2+}\) influx by several neurohormonal and second-messenger systems, including the \(\beta\)-adrenergic/PKA signaling pathway; however, the molecular mechanisms of \(\beta\)-adrenergic regulation of Ca\(_{\text{V1.2}}\) in cardiomyocytes are incompletely understood. A key obstacle has been the failure to reproducibly reconstitute \(\beta\)-adrenergic regulation in heterologously expressed Ca\(_{\text{V1.2}}\). To circumvent this problem, we used doxycycline-inducible, cardiac-specific, transgenic-mice-expressing FLAG-epitope-tagged, DHP-resistant \(\alpha_{1C}\). In this system, we examined the proposed roles of proteolytic cleavage of \(\alpha_{1C}\), at residue Ala\(^{1800}\), and PKA phosphorylation of Ser\(^{1700}\) in mediating \(\beta\)-adrenergic-induced enhancement of cardiac Ca\(_{\text{V1.2}}\) current. In addition, we tested these predictions in native cardiomyocytes by creating a transgenic mouse expressing three mutations within \(\alpha_{1C}\) (S1700A, T1704A, and Δ1798NNAN1801). We found that in cardiomyocytes, the NNAN motif is not required for cleavage of \(\alpha_{1C}\), and that Ser\(^{1700}\) and Thr\(^{1704}\) are not required for the \(\beta\)-adrenergic modulation of both Ca\(^{2+}\) current and excitation-contraction coupling.
Figure 2

A

\[ \alpha\text{MHC "driver" (rtTA)} \]

\[ \alpha\text{MHC}_{\text{MOD} \text{ "responder"}} \]

\[ \text{tetO} \]

\[ \text{FLAG-DHP*-}\alpha\text{1C} \]

B

anti-FLAG Ab

Full-length \( \alpha\text{1C} \)

Cleaved \( \alpha\text{1C} \)

kDa

200

130

95

72

anti-\( \alpha\text{1C} \) Ab

Full-length \( \alpha\text{1C} \)

Cleaved \( \alpha\text{1C} \)

C

Fraction of cleaved \( \alpha\text{1C} \)

NTG

TG pWT \( \Delta\text{NNAN-S1700A-T1704A} \)

D

pWT \( \alpha\text{1C} \)

Anti-FLAG

No primary Ab

\( \Delta\text{NNAN-S1700A-T1704A} \)

Anti-FLAG

No primary Ab

E

sarcotome length (\( \mu\text{M} \))

Non-transgenic

Nisoldipine

20 s

F

pWT \( \alpha\text{1C} \) transgenic

Nisoldipine

20 s
Figure 3

A) Non-transgenic

B) pWT α1C

C) ΔNNAN-S1700A-T1704A

D) pWT α1C

E) ΔNNAN-S1700A-T1704A

F) Total I_{Ca} (pA/pF)

G) DHP-resistant : total I_{Ca} (fraction)

NTG, TG pWT, ΔNNAN-S1700A-T1704A
β-Adrenergic Regulation of the L-type Ca^{2+} Channel Does Not Require Phosphorylation of α1C 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 $\pm$ 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 µM 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 + 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

\[ R = \frac{I_{\text{Nis}}}{I_{\text{Tot}}} \]  \hspace{1cm} (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

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

\[ R = X \times m + (1-X) \times n \]  \hspace{1cm} (3)

Solving for \( X \):

\[ X = \frac{(R-n)}{(m-n)} \]  \hspace{1cm} (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:

\[ \text{Fraction}_{\text{NTG}} = \frac{(X \times 0.07)}{((1-X) \times 0.66) + (X \times 0.07)} \]  \hspace{1cm} (5)

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