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β-Adrenergic Regulation of the L-type Ca^{2+} Channel does not Require Phosphorylation of α_{1C} Ser^{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, $Ca_V1.2$, which plays a key role in cardiac excitation-contraction coupling. The molecular mechanisms of β-adrenergic regulation of $Ca_V1.2$ 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 $Ca_V1.2$.

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 $Ca_V1.2$ in the heart.

Method and Results—Using a transgenic approach that enables selective and inducible expression in mice of FLAG-epitope tagged, dihydropyridine-resistant $Ca_V1.2$ channels harboring mutations at key regulatory sites, we show that adrenergic regulation of $Ca_V1.2$ 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 ^{1798}NNAN^{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 $Ca_V1.2$ 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.

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Keywords

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

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 Ca^{2+} overload, which in turn can result in arrhythmogenic delayed after-depolarizations (DADs).

$Ca_V1.2$ channels are composed minimally of a pore-forming α_{1C} and regulatory β and $\alpha_2\delta$ 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 α_{1C} 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 $Ca_V1.2$. Ser^{1928}, in the α_{1C} subunit, was originally identified as the sole α_{1C} 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-α_{1C} [24], and in α_{1C} S1928A knock-in mice[25]. Similarly, although β_{2a} Ser^{459}, Ser^{478} and Ser^{479} 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, Ser^{1700} 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 α_{1C} is difficult because of the α_{1C} insert size and the cardiomyocytes need to be cultured for extended period, potentially inducing dedifferentiation. Since overexpression of α_{1C} 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 α_{1C}. The approach preserves hormonal regulation of $Ca_v1.2$ by limiting $Ca_V1.2$ over-expression.

Prominent roles for proteolytic cleavage of α_{1C}, at residue Ala^{1800}, and PKA phosphorylation of Ser^{1700}, in the C-terminus of α_{1C} (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 Ala^{1800}, PKA is unable to phosphorylate Ser^{1700} and upregulate the activity of heterologously expressed $Ca_V1.2$ [15]. Ala-substitution of the neighboring Thr^{1704}, 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 Ser^{1700} more effectively reduced forskolin-induced stimulation of $Ca_V1.2$, compared to 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 three mutations within α_{1C} (S1700A, T1704A, and Δ^{1798}NNAN^{1801}).

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 α_{1C} 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 α_{1C}. The α_{1C} 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 α_{1C} 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-α_{1C} 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 [43] and tsA-201 cells were superfused with (in

mM) 140 TEA-Cl, 1.8 $CaCl_2$, 1 $MgCl_2$, 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 CsCI, 10 EGTA, 1 $MgCl_2$, 2 Mg-ATP, 2.0 $CaCl_2$, 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 Ca^{2+} 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 Ca^{2+} 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 $CaCl_2$. 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, Sidek'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 α_{1C} transgenic mice

We deleted the highly conserved ^{1798}NNAN^{1801} motif in α_{1C} (Fig. 1B) and co-expressed the cDNA with the β_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 $Ca_V1.2$ (Fig. 1C-D). Since proteolytic cleavage of α_{1C} does not occur when wild-type (WT) α_{1C} is expressed heterologously, the effect of deletion of the putative cleavage site on proteolysis of α_{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 α_{1C}, designated pseudo-WT, [pWT α_{1C}]) using a bitransgenic tetracycline-regulated system that permits robust expression only when both transgenes, and doxycycline are present (Fig. 2A). The α_{1C} subunit was engineered to be relatively DHP-insensitive with the substitutions T1066Y and Q1070M [40, 41]. The IC_{50} for nisoldipine block of heterologously expressed WT α_{1C} was 12 nM, whereas the IC_{50} for pWT α_{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 $Ca_V1.2$ current in tsA-201 cells, but only blocked 34.6 ± 2.5% of DHP-insensitive α_{1C} (Online Fig. I).

Seven pWT α_{1C} founder transgenic lines were originally generated. Two founder lines were lost due to mortality, possibly because of high levels of doxycycline-independent α_{1C} expression. Four founder lines, when crossed with αMHC-rtTA mice, demonstrated doxycycline-induced expression of α_{1C}, assessed by anti-FLAG antibody immunoblots (Fig.

2B, upper; Online Fig II). One transgenic founder line, after crossing with αMHC-rtTA mice, did not demonstrate doxycycline-induced α_{1C} expression. Of 59 pWT α_{1C} bitransgenic mice treated with doxycycline, 18 mice (31%) died within 5 days of doxycycline administration possibly due high levels of doxycycline-dependent α_{1C} expression.

We also generated a transgenic mouse line expressing three mutations within α_{1C}, Ala-substitutions of Ser^{1700} (S1700A) and Thr^{1704} (T1704A), and deletion of the ^{1798}NNAN^{1801} motif (ΔNNAN), in the background of a N-terminal 3X FLAG-epitope tag and DHP-resistance (ΔNNAN-S1700A-T1704A). Six ΔNNAN-S1700-T1704A mutant transgenic founder lines were originally generated. Three founders, when crossed with αMHC-rtTA, demonstrated doxycycline-induced α_{1C} expression (Fig. 2B, upper; Online Fig. II). The other 3 founders, after crossing with α–MHC-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 due to high levels of α_{1C} expression. The 41 pWT α_{1C} 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 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 $Ca_V1.2$, 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 ^{1798}NNAN^{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^{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 $Ca_V1.2$ 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 $Ca_V1.2$ 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 $Ca_V1.2$ channels by accessory proteins was similar to endogenous $Ca_V1.2$ channels.

Adrenergic-modulation of $Ca_V1.2$ 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 $Ca_V1.2$ 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 α_{1C} transgenic mice, isoproterenol increased the nisoldipine-insensitive peak current by a mean of 1.7 ± 0.1-fold (Fig. 4B, D, F) (P ≤ 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 $Ca_V1.2$ currents by 2.1 ± 0.3-fold (Fig. 5A) (P=not significant compared to non-transgenic). In cardiomyocytes with peak $Ca_V1.2$ currents greater than 15 pA/pF, in contrast, isoproterenol increased $Ca_V1.2$ currents by only 1.4 ± 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 $Ca_V1.2$ current (Fig. 5B). The diminished adrenergic-modulation of the transgenic pWT $Ca_V1.2$ current compared to endogenous $Ca_V1.2$ is likely due to the increased basal $Ca_V1.2$ current density in the transgenic cardiomyocytes. Cardiomyocytes may have a limited number of permissive sites on the membrane where PKA-mediated upregulation of $Ca_V1.2$ 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 $Ca_V1.2$ currents [27].

Phosphorylation of Ser^{1700} and Thr^{1704} are not required for isoproterenol- and forskolin-induced stimulation of $Ca_V1.2$ currents

Freshly isolated cardiomyocytes were isolated from doxycycline-treated ΔNNAN-S1700A-T1704A transgenic mice. In the presence of nisoldipine, isoproterenol increased peak $Ca_V1.2$ current by a mean of 1.7 ± 0.1-fold, identical to the isoproterenol-induced augmentation of current in pWT α_{1C} transgenic cardiomyocytes (P= not significant, pWTα_{1C} vs. ΔNNAN-S1700A-T1704A) (Fig. 4C, E-F). In the presence of nisoldipine, forskolin increased peak $Ca_V1.2$ 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 Fig. 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 $Ca_V1.2$

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 and ΔNNAN-S1700A-T1704A α_{1C} were not statistically different (Fig. 5D). In cardiomyocytes with a total basal $Ca_V1.2$ current density less than 10 pA/pF, isoproterenol caused a 1.8 ± 0.1-fold increase in $Ca_V1.2$ current (P= not significant compared to pWT TG α_{1C}). In cardiomyocytes with basal $Ca_V1.2$ current density greater than 15 pA/pF, isoproterenol increased $Ca_V1.2$ currents by 1.3 ± 0.1 fold (P= not significant compared to 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} (Fig. 5E-F). Thus, phosphorylation of Ser^{1700} or Thr^{1704} is not required for isoproterenol or forskolin-induced modulation of $Ca_V1.2$ 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 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 ΔNNAN-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^{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 efficiently and reliably probe molecular aspects of $Ca_V1.2$ regulation within the context of freshly isolated cardiomyocytes, approximating the ease and power of a heterologous expression system. In prior studies, overexpression of α_{1C} or β subunits markedly reduced the β-adrenergic regulation of the channel, and induced cardiac dysfunction or apoptosis [31, 32, 34-36]. To circumvent these problems, we created inducible, tissue-specific, transgenic-mice expressing, DHP-resistant, FLAG-epitope-tagged α_{1C}. This approach preserves hormonal regulation of $Ca_V1.2$ by limiting its over-expression. The channels containing the transgenic α_{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 $Ca_V1.2$ channels is unaltered by Ala-substitution of Ser^{1700} or Thr^{1704}, indicating that these sites are dispensable for this purpose in adult cardiomyocytes. Ser^{1700} was recently reported to be the functionally relevant PKA site in heterologously expressed $Ca_V1.2$ [15, 29]. Phosphorylation of Thr^{1704}, a consensus site for casein kinase II, increases the basal activity of heterologously expressed $Ca_V1.2$ [15]. It may also play a role in adrenergic-modulation of $Ca_V1.2$, since forskolin-induced stimulation of heterologously expressed $Ca_V1.2$ was more attenuated with the double mutant S1700A-T1704A than for S1700A alone [15]. Although Ser^{1928} is PKA phosphorylated [11, 16-23], it is not required for β-adrenergic stimulation of $Ca_V1.2$ [2425], and forskolin-induced stimulation of the heterologously expressed triple mutant S1700A-T1704A-S1928A was not different than the double mutant S1700A-T1704A [15]. It is based upon these experiments [15] that we chose the S1700A-T1704A mutations for testing

in transgenic mice. We were unable to assess the role of Thr^{1704} on basal activity in cardiomyocytes, however, since the basal activity of heterologously expressed $Ca_V1.2$ was determined by comparing the coupling efficiency of pore opening to gating charge movement [15].

β-adrenergic stimulation of $Ca_V1.2$ 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^{2+} current, but only a 1.2 ± 0.1-fold increase in cardiomyocytes isolated from α_{1C} over-expressing transgenic mice [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^{501} [28]. McKnight, Santana and Catterall reported an approximate 2-fold increase in $Ca_V1.2$ currents by isoproterenol (100 nM) in WT and AKAP5 knock-out mice [44]. Chen and Houser reported that isoproterenol increased $Ca_V1.2$ currents in WT mice by 1.6-fold, but isoproterenol did not increase the current amplitude in transgenic mice overexpressing the β_{2a} subunit [45]. Thus, by limiting over-expression of α_{1C} and only inducing expression of α_{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 $Ca_V1.2$ in cardiomyocytes

Although useful for investigating biophysical properties, heterologous expression systems have not been successful for exploring physiological modulation, especially as related to cardiomyocytes [14, 27, 30, 46]. Compared to creating a knock-in mouse, expressing transgenic DHP-resistant α_{1C} mutants in the heart is rapid and cost-effective, and multiple sites within α_{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 β-adrenergic modulation of $Ca_V1.2$, 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 $Ca_V1.2$. Stratifying the magnitude of β-adrenergic-mediated upregulation of $Ca_V1.2$ 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 $Ca_V1.2$ is not significantly altered by Ala-substitution of Ser^{1700}, implying that phosphorylation of Ser^{1700} is not the primary mechanism for β-adrenergic regulation of $Ca_V1.2$. Could phosphorylation of Ser^{1700} play a small, secondary role in mediating β-adrenergic regulation of $Ca_V1.2$, 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 α_{1C} 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 non-significant 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 α_{1C} transgenic mice (6.5 pA/pF vs. 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 (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 ~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 α_{1C} mice (Fig. 5E). Taken together, these findings imply that phosphorylation of Ser^{1700} and Thr^{1704} cannot be the primary mechanism by which β-adrenergic agonists activate $Ca_V1.2$ in the adult cardiomyocytes.

Proteolytic cleavage does not require the conserved motif ^{1798}NNAN^{1801}

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 α_{1S} proteolytic peptides and sequence alignments of α_{1S} and α_{1C}, was used to identify Ala^{1800} as the putative proteolytic site in α_{1C} [8]. Deletion of Ala^{1800} and the immediately adjacent conserved residues did not alter the proteolytic cleavage of α_{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 ^{1794}NANI^{1797}, which would combine with Asn^{1802} after ^{1798}NNAN^{1801} is deleted to form a ^{1794}NANIN motif. Whether cleavage could occur at Ala^{1795} in ΔNNAN transgenic mouse is a question for future study.

In summary, we have developed an approach to reproducibly and efficiently test informative mutants of $Ca_V1.2$ in cardiomyocytes using a transgenic mouse approach. By limiting over-expression of the $Ca_V1.2$ α_{1C} subunit, we can reliably assess sympathetic regulation of $Ca_V1.2$. These data demonstrate that phosphorylation of Ser^{1700} and Thr^{1704} are not the primary mechanisms mediating β-adrenergic modulation of both Ca^{2+} current and excitation-contraction coupling in adult cardiomyocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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

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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 α_{1C}, at residue Ala^{1800} and protein kinase A (PKA) phosphorylation of Ser^{1700} mediate β–adrenergic-induced enhancement of cardiac $Ca_V1.2$ current, but these concepts have not been tested in cardiomyocytes
- 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.

What New Information Does This Article Contribute?

- Selective and inducible expression in mice of FLAG-epitope tagged, dihydropyridine (DHP)-resistant $Ca_V1.2$ channels harboring mutations at key regulatory sites can be used to assess the properties of α_{1C} mutants in freshly isolated adult cardiomyocytes
- Adrenergic regulation of $Ca_V1.2$ current and fractional shortening of cardiomyocytes do not require phosphorylation of either Ser^{1700} or Thr^{1704} of the α_{1C} subunit
- Deletion of ^{1798}NNAN^{1801}, the previously proposed cleavage site, does not prevent distal α_{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 β-adrenergic/PKA signaling pathway; however, the molecular mechanisms of β-adrenergic regulation of $Ca_V1.2$ in cardiomyocytes are incompletely understood. .A key obstacle has been the failure to reproducibly reconstitute adrenergic regulation in heterologously expressed $Ca_V1.2$. To circumvent this problem, we used doxycycline-inducible, cardiac-specific, transgenic-mice-expressing FLAG-epitope-tagged, DHP-resistant α_{1C}. In this system, we examined the proposed roles of proteolytic cleavage of α_{1C}, at residue Ala^{1800}, and PKA phosphorylation of Ser^{1700} in mediating β-adrenergic-induced enhancement of cardiac $Ca_V1.2$ current. In addition, we tested these predictions in native cardiomyocytes by creating a transgenic mouse expressing three mutations within α_{1C} (S1700A, T1704A, and Δ^{1798}NNAN^{1801}). We found that in cardiomyocytes, the NNAN motif is not required for cleavage of α_{1C}, and that Ser^{1700} and Thr^{1704} are not required for the β-adrenergic modulation of both Ca^{2+} current and excitation-contraction coupling.

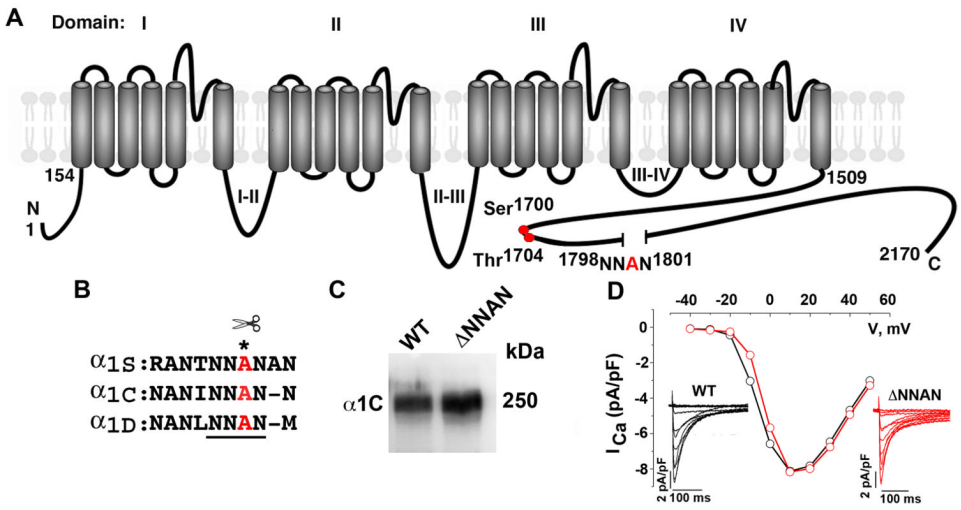

Figure 1. Deletion of proteolytic cleavage site does not affect heterologously expressed $Ca_V1.2$ channel expression or function

(A) Schematic of cardiac α_{1C} subunit topology. The putative proteolytic cleavage site, ^{1798}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-α_{1C} antibody immunoblot of extracts from WT α_{1C} and ΔNNAN α_{1C} expressing tsA-201 cells. (D) WT (black) and ΔNNAN α_{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.

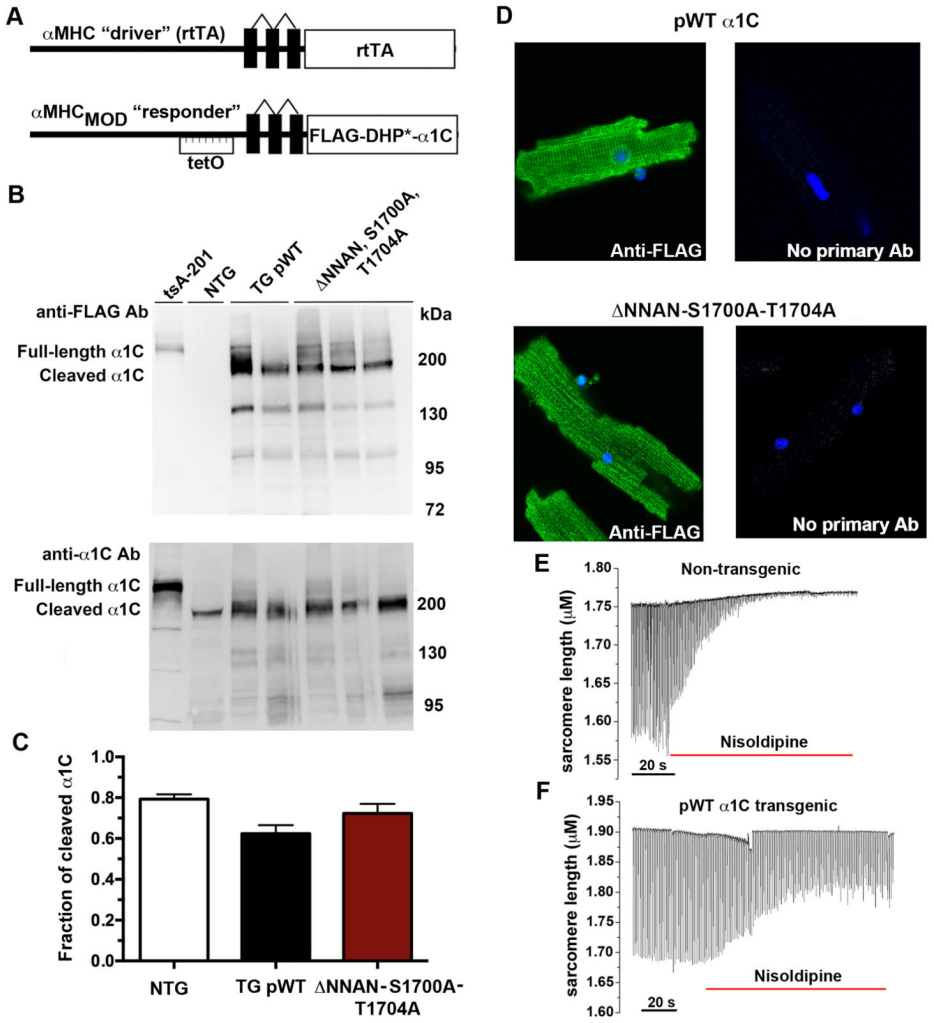

Figure 2. Inducible, cardiac-specific FLAG-tagged α_{1C}-expressing transgenic mice

(A) Schematic representation of the binary transgene system. The αMHC-rtTA is the standard cardiac-specific reverse tetracycline-controlled transactivator system. The αMHC_{MOD} construct is a modified αMHC promoter containing the tet-operon for regulated expression of FLAG-tagged DHP-resistant (DHP*) α_{1C}. (B) Anti-FLAG antibody (upper) and anti-α_{1C} antibody (lower) immunoblots showing FLAG-epitope tagged α_{1C} expression in tsA-201 cells transfected with FLAG-tagged α_{1C} and expression in isolated cardiomyocytes from either pWT α_{1C} or ΔNNAN-S1700A-T1704A transgenic mice. (C) Bar graph of densitometries of cleaved α_{1C} band divided by truncated + full-length α_{1C} bands. N=4 non-transgenic (NTG) mice; N= 10 pWT α_{1C} mice; N=6 ΔNNAN-S1700A-T1704A mice. P = not significant by Anova. (D) Immunostaining of pWT α_{1C} and ΔNNAN-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.

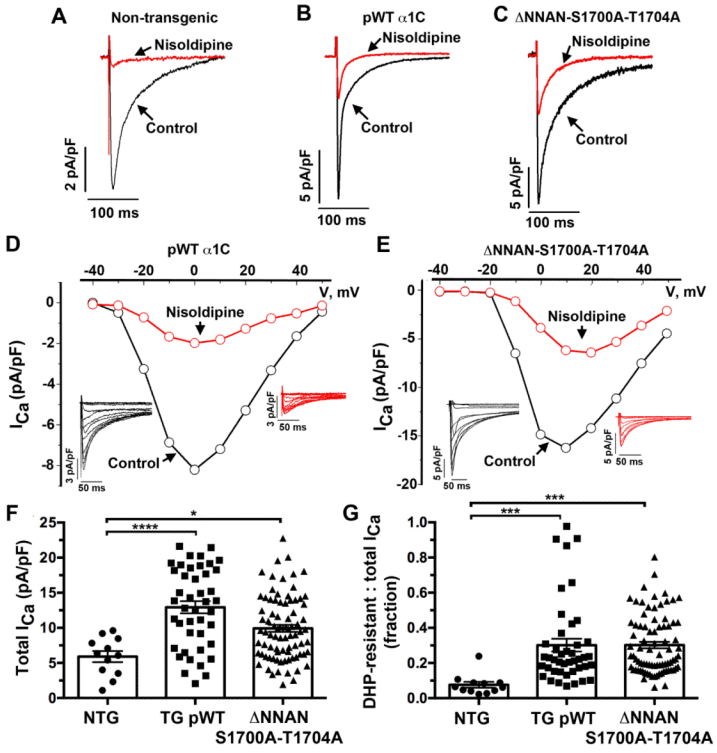

Figure 3. Dihydropyridine-resistant currents in transgenic mice

(A-C) Exemplar whole-cell $Ca_V1.2$ 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 α_{1C} (D) and ΔNNAN-S1700A-T1704A $Ca_V1.2$ (E) acquired before (black traces) and 3 minutes after superfusion of 300 nM nisoldipine. Insets: Series of whole-cell $Ca_V1.2$ 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.

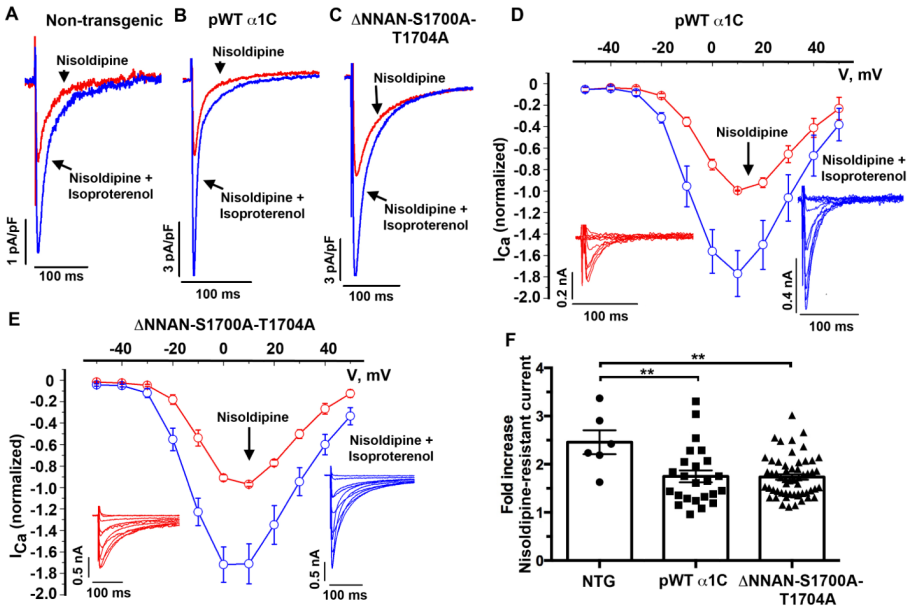

Figure 4. β-adrenergic stimulation of $Ca_V1.2$ current does not require phosphorylation of Ser^{1700}

(A-C) Exemplar whole-cell $Ca_V1.2$ 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 α_{1C} (N=4) and ΔNNAN-S1700A-T1704A mice (N=8). Mean ± SEM. Insets: Series of whole-cell $Ca_V1.2$ 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 α_{1C}, N= 56 ΔNNAN-S1700A-T1704A cardiomyocytes.

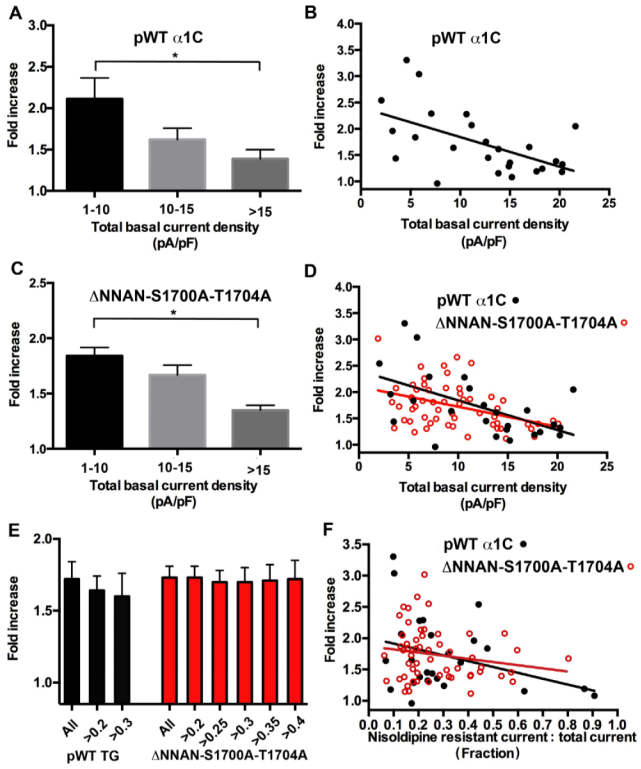

Figure 5. Analysis of isoproterenol's effects on transgenic Ca^{2+} currents

(A, C) Bar graphs of isoproterenol-induced increase in nisoldipine-resistant current binned by total basal current density before nisoldipine for pWT α_{1C} (A) and ΔNNAN-S1700A-T1704A transgenic mice (C). Mean ± SEM. * P<0.05 by Anova and Tukey's post hoc test. For pWT α_{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 α_{1C} (B), and pWT α_{1C} and ΔNNAN-S1700A-T1704A transgenic mice (D). Lines fitted by linear regression. The differences between the slopes and intercepts of pWT α_{1C} 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 α_{1C} and ΔNNAN-S1700A-T1704A mice. Mean ± SEM. P= not significant. (F) Graph of isoproterenol-induced increase in nisoldipine-resistant current stratified by fraction of nisoldiipine-resistant current for pWT α_{1C} and ΔNNAN-S1700A-T1704A mice. The differences between the slopes and intercepts of pWT α_{1C} and ΔNNAN-S1700A-T1704A are not significant.