L-Type Calcium Channel C Terminus Autoregulates Transcription

Elizabeth Schroder, Miranda Byse, Jonathan Satin

Abstract—Calcium homeostasis is critical for cardiac myocyte function and must be tightly regulated. The guiding hypothesis of this study is that a carboxyl-terminal cleavage product of the cardiac L-type calcium channel (CaV1.2) autoregulates expression. First, we confirmed that the CaV1.2 C terminus (CCt) is cleaved in murine cardiac myocytes from mature and developing ventricle. Overexpression of full-length CCt caused a 34±8% decrease of CaV1.2 promoter activity, and truncated CCt caused an 80±3% decrease of CaV1.2 promoter (n=12). The full-length CCt distributes into cytosol and nucleus. A deletion mutant of CCt has a greater relative affinity for the nucleus than full-length CCt, and this is consistent with increased repression of CaV1.2 promoter activity by truncated CCt. Chromatin immunoprecipitation analysis revealed that CCt interacts with the CaV1.2 promoter in adult ventricular cardiac myocytes at promoter modules containing Nkx2.5/Mef2, C/EBP, and a cis regulatory module. The next hypothesis tested was that CCt contributes to transcriptional signaling associated with cellular hypertrophy. We explored whether fetal cardiac myocyte CaV1.2 was regulated by serum in vitro. We tested atrial natriuretic factor promoter activity as a positive control and measured the serum response of CaV1.2 promoter, protein, and L-type current (I_Ca,L) from fetal mouse ventricular myocytes. Serum increased atrial natriuretic factor promoter activity and cell size as expected. Serum withdrawal increased CaV1.2 promoter activity, mRNA, and I_Ca,L. Moreover, serum withdrawal decreased the relative nuclear localization of CCt. A combination of promoter deletion mutant analyses, and the response of promoter mutants to serum withdrawal support the conclusion that CCt, a proteolytic fragment of CaV1.2, autoregulates CaV1.2 expression in cardiac myocytes. These data support the novel mechanism that a mobile segment of CaV1.2 links Ca handling to nuclear signaling.

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Key Words: L-type Ca^{2+} channels ■ L-type Ca^{2+} current ■ Ca^{2+} channels ■ Ca^{2+} regulation ■ transcriptional regulation

The beat-to-beat function of the L-type calcium channel (LTCC), CaV1.2, is to provide trigger Ca for excitation–contraction coupling in cardiac myocytes. Over a longer time frame, LTCC Ca current (I_{Ca,L}) provides Ca that ultimately contributes to cellular Ca homeostasis.1 On first principles, CaV1.2 expression and function must be tightly regulated to maintain Ca homeostasis in cardiac myocytes. We recently showed that LTCC block by sustained in vivo pharmacological treatment results in an upregulation of I_{Ca,L}, CaV1.2 protein, and mRNA2 in agreement with earlier studies.3–5 There are also precedents for physiological perturbations that alter I_{Ca,L} and CaV1.2. LTCC channel levels change in response to Ca,V6 angiotensin II,7 cardiac denervation in heart failure,8 or prolonged exposure to catecholamines.9 In atrial fibrillation, I_{Ca,L}10,11 and CaV1.2 decrease.12–14 Late stage hypertrophic failing hearts show significant reduction of LTCC density,15–20 and this decline can be rescued by left ventricular assist devices.21 Thus, cellular hypertrophic signals contribute to CaV1.2 expression levels, and such signals may be reversible. However, we have limited information regarding mechanisms of regulation of CaV1.2 expression. The goal of this study was to test a new mechanism for control of LTCC expression.

CaV1.2 encodes the pore-forming subunit of the LTCC complex at the surface membrane. CaV1.2 is posttranslationally processed22 with a functionally important cleavage of its carboxyl terminus.23–25 The CaV1.2 C terminus (CCt) is a 300-aa protein that reassociates with the main body of CaV1.2 at the surface membrane.26 In neurons and in heterologous expression systems, fragments of CCt also localize to the nucleus, and display transcriptional activity.27 This study tested whether native CCt and CCt fragments show similar nuclear localization in cardiac myocytes. To test for involvement of CCt in CaV1.2 expression, we probed for CCt–CaV1.2 promoter interactions. Our data suggest that CCt is a repressor of CaV1.2 promoter activity. Cellular hypertrophy mediated by serum coordinately regulates CCt nuclear localization, CCt repression of CaV1.2 promoter activity and, concomitantly, downregulation of CaV1.2 protein and current. Taken together, these data show that CCt, a segment of
the LTCC, autoregulates LTCC expression in cardiac myocytes.

Materials and Methods

Cell Culture

All animal procedures used in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee. Embryonic day (E16) mouse (ICR outbred strain, Harlan) hearts were dissected free of connective tissues, and ventricles were separated from conotruncus and sinus venosus. Cells were enzymatically digested and cultured as previously described. Briefly, 10 to 40 embryos were minced and quickly transferred to nominally Ca-free digestion buffer containing 0.5 mg/mL collagenase (type II, Worthington) and 1 mg/mL pancreatin for two 15-minute cycles. Digested tissue yielded a large fraction of single spontaneously beating cells. Cells were cultured in DMEM with or without 10% FBS, 100 μg/mL penicillin, 100 μg/mL streptomycin, and 2 μM/L L-glutamine.

Adult Ventricular Myocyte Isolation Procedures

Single ventricular myocytes were enzymatically isolated following a modified Alliance for Cellular Signaling protocol P00000125. Briefly, 4- to 6-month-old female ICR mice were anesthetized and hearts were rapidly excised and retrogradely perfused at 3 mL/min at 37°C for 4 to 8 minutes with a Ca2+-free bicarbonate-based perfusion buffer containing (in mmol/L) NaCl 113; KCl 4.7; KH2PO4 0.6; MgSO4 1.2; NaH2PO4 0.6; glucose 5.5; NaHCO3 12; KHCO3 10; HEPES 10; phenol red 0.032; 2.3-butanediol monoxide 10; and taurine 30. The perfusion buffer was gassed with 95% O2/5% CO2 for at least 30 minutes before use. Enzymatic digestion began using 0.25 mg/mL liberase blendzyme (Roche), and 12.5 μmol/L CaCl2 was added to the perfusion buffer for approximately 13 minutes until the heart was swollen and pale in color. The heart was then cut from the canula. Ventricular tissue was placed in a dish with enzyme buffer and gently dissociated for several minutes. After the addition of stop buffer (perfusion buffer containing 10% FBS and 12.5 μmol/L CaCl2), dissociation continued until large pieces of heart tissue were gently dispersed into the cell suspension. Cells were allowed to sediment by gravity for 10 minutes followed by centrifugation at 180 g for at least 30 minutes before use. Enzymatic digestion was repeated several times. After the addition of stop buffer, the cells were gently resuspended in the perfusion buffer. Cells were resuspended in perfusion buffer containing 5% FBS and 12.5 μmol/L CaCl2. External Ca2+ was added incrementally back to the solution to 2.0 mmol/L. Only rod-shaped, quiescent myocytes with clear margins were selected for current recording. For the nuclear extraction protocol cells were paced at 1.0 Hz (37°C) for 6 hours in media with or without 10% FBS.

Western Blots

Whole cell lysates were prepared from cells isolated from adult hearts. SDS-PAGE (4% to 15% separating gel, Bio-Rad), and immunoblotting were carried out following routine protocols. Affinity purified LTCC α-subunit polyclonal antibody (custom designed for L-III, III epitope), Pol II (Santa Cruz Biotechnology), tissue transglutaminase (Developmental Hybridoma Bank), and custom antibodies (ECM Biosciences, Versailles, Ky) for the Ser1928 phosphorylation site on the CCT and for the terminal piece that were generated against epitopes as described in Hulme et al. Antibody localization was visualized with the appropriate (rabbit or mouse) horseradish peroxidase–conjugated secondary antibody (Chemicon) and Super Signal West Pico Chemiluminescence (Pierce). Each lane contained 60 μg of total protein. All Western blot experiments for a given animal were repeated at least 3 times to ensure that experimental observations were reproducible. Loading was confirmed by stripping (Restore Western Blot Stripping Buffer, Pierce) and reprobing blots with GAPDH monoclonal antibody (Ambion). Immunoblots were scanned on an Epson Perfection 1650 and quantified using densitometry (Scion Image, Scion Corporation).

RNA Extraction

Isolated cells from embryonic or adult cultures were pelleted and washed with PBS. Cells were then either snap frozen at −80°C or used immediately for RNA isolation. Total RNA was isolated using the RNAqueous-4PCR Kit (Ambion) and quantitated spectrophotometrically at 260 nm. Contaminating genomic DNA was eliminated by DNase treatment (Ambion). A portion of the resulting RNA (1 μg) was immediately used as a template for cDNA synthesis. Reverse transcription was performed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Removal of genomic DNA was confirmed by preparing a no reverse transcription control for each sample. cDNA was then stored at −20°C.

Chromatin Immunoprecipitation Assay

Isolation of nuclei from transfected E16 and adult heart isolated cells was adapted from a method recently described. Samples were incubated overnight at 4°C with the custom CCT antibody (adult) (ECM Biosciences, Versailles, Ky) or green fluorescent protein (GFP) antibody (E16 transfected cells) (Abcam, Cambridge, Mass) or no antibody as a negative control. After clearing, samples were treated with Proteinase K (10 mg/mL), and DNA was recovered for PCR. Primers (Tm of ~60°C) were designed to amplify ~100-bp regions that were identified with in silico analysis (Figure 3A) in the Ca1.2 promoter region using the high fidelity AccuPrime P-Taq polymerase (Invitrogen, Carlsbad, Calif) for 32 to 35 cycles. Primer sequences for the amplification regions are as follows (forward and reverse, respectively): CRE: GGTGTAACATTAGCAACGAGACGACATGTGGTGATTTGGATTCATCTATT; cta regulatory module 1 (CRM1): TCTACGTCAAGTTCGTATGTTGGATTCATCTATT; cis regulatory module 2 (CRM2): GATAAACTTTGTTTTTGATTTGAATTCATCTATT; cta regulatory module 3 (CRM3): TCTACGTCAAGTTCGTATGTTGGATTCATCTATT. Loading was confirmed by PCR using forward and reverse primers designed according to the published sequence (accession no., AF221551). The PCR product was subcloned into the PGL3 basic vector at the HindIII and Xhol sites. Two deletion constructs were made using a similar strategy. All constructs were confirmed by DNA sequencing (Davis Sequencing). The atrial natriuretic factor (ANF) promoter reporter construct was a kind gift from Dr Ginell Post.

CCT Enhanced GFP Fusion Constructs

eGFPf1906–2171 was a kind gift from Douglas Andres. eGFPf1821–2171 was created by amplifying amino acids 1821 to 2171 from a pE GFP-C1 plasmid containing the rabbit Cav1.2 sequence from amino acid 1507 to 2171 (D. Andres). Amino acids 1821 to 2171 were ligated into the multiple cloning site of pEGFPC1 and verified by sequencing (Davis Sequencing).

Transfection and Luciferase Assay

E16 cardiac myocytes were transfected with 0.4 μg of PGL3-Ca1.2 promoter DNA and 0.2 μg of PRL (Renilla luciferase expression vector) using Lipofectamine 2000 (Invitrogen). Transfections were also performed with the PGL3 control vector and PGL3 basic vector as both positive and negative controls, respectively. Seventy-two hours posttransfection, cells were washed with PBS and lysed with 100 mL of passive Lysis buffer (Promega). Luciferase activity was measured with the dual luciferase assay kit (Promega) on a Lumat LB 9507 luminometer.

Translocation Assay

E16 ventricular cardiomyocytes were isolated and transfected within 24 hours with eGFPf1906–2171 or eGFPf1821–2171. Twenty-four hours posttransfection, cells were treated with serum or serum-free media for 24 hours. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton, and blocked with 1% BSA in PBS.
Cardiomyocytes were stained with mouse sarcomeric anti-α-actinin (Sigma) and Alexa Fluor-594 goat anti-mouse IgG1 (Invitrogen) to identify cardiomyocytes and mounted with Vecta Shield with DAPI (Vector Laboratories) to identify nuclei. A LSM 5 Live (Zeiss) was used to obtain confocal images of 1-μm thickness focused on the center of the nuclei. Nuclear and cytosolic fluorescence was quantified using ImageJ 1.38w with the LSM Toolbox version 4.0b. The area within the nucleus and cytosol containing the greatest fluorescent intensity was used to measure the nuclear to cytosolic ratio. Representative images were obtained using a Leica TSP SP5 confocal microscope.

**Ca²⁺ Current Measurements**

Calcium current was recorded from Ca²⁺-tolerant adult female mouse ventricular cells at 37°C 1 to 6 hours after isolation. After establishment of the whole cell configuration the cells were perfused with sodium and potassium-free solution containing (in mmol/L): 140 N'-methyl-D-glucamine; MgCl₂ 1.0; CaCl₂ 2.5; HEPES 10; glucose 10; 4-aminopyridine 5 at pH 7.4. Pipettes had tip resistances of 1 to 2 MΩ after filling with pipette solution composed of (in mmol/L): CsCl 125; TEA-Cl 10; MgCl₂ 1.0; EGTA 10; Mg-ATP 5; HEPES 5 (pH 7.2). Current was recorded with an Axopatch-200B amplifier (Axon Instruments). The series resistance was typically 4 to 8 MΩ before compensation (50% to 75%). Data were digitized at 20 kHz using an A/D converter (Digidata-1200, Axon Instruments) under software control (pClamp 8.2, Axon Instruments).

**Nuclear Extracts**

Adult cells were paced at 1.0 Hz (IonOptix Cell Pacer) in the presence or absence of serum (37°C) for 6 hours before fractionation. E16 cells were treated with serum or serum-free media for 72 hours before fractionation. Isolated, treated cells were pelleted, washed with PBS, and snap-frozen. Nuclear and cytoplasmic fractions were isolated using the Ne-Per Nuclear and Cytoplasmic Extraction Reagent Kit (Pierce) following standard protocols. A minimum of 3 separate isolations were performed for each experiment. Western blot analysis confirmed the fractionation method successfully isolated cytoplasmic and nuclear components by using Pol II (nuclear) or TGM2 (tissue trans glutaminase II, cytoplasmic) antibody (Figure I, A, in the online data supplement, available at http://circres.ahajournals.org).

**Graphing and Statistics**

Origin 7 was used to create a box plot with the box denoting the 25th and 75th percentiles, the whiskers denoting the 5th and 95th percentiles, and the stars denoting the 1st and 99th percentile. Cell
size was determined by drawing a circle around the borders of the cell and measuring total area. Significant differences were determined using Student’s t test.

Results

CCt in Murine Heart Is Cleaved

CaV1.2 protein undergoes C-terminal cleavage at residue 1821,30 which is downstream of the CB/IQ motif but upstream of Ser1928, a putative phosphorylation substrate for PKA/C modulation. tsA-201 cells transfected with CaV1.2, Caβ2a, and GFP do not process the full length Cav1.2 to generate CCt.30 This was confirmed by a custom designed CCt-antibody recognizing only the full length channel (Figure 1). However, Western blotting of tsA-201 cells transfected with HA-CCt and probed with the same CCt antibody detects a ~37kDa CCt band. Figure 1 shows that in murine cardiac myocytes we can only detect the proteolytically cleaved CaV1.2 in the E16 ventricle, and in the adult ventricle.

Autoregulation of Transcription

Given the recent identification of CCt as a calcium sensitive transcription cofactor in neurons,27 we next tested whether CCt also functioned as a transcriptional regulator in cardiac myocytes. More specifically, we tested the hypothesis that CCt autoregulates CaV1.2 expression. We used eGFP-CCt fusion constructs to explore CCt regulation of the CaV1.2 promoter, and to report subcellular localization of CCt in cardiac myocytes. E16 myocytes or tsA-201 cells were cotransfected with the CaV1.2 promoter-reporter construct, and the full-length CCt (1821 to 2171) or a truncated CCt (1906 to 2171). Consistent with previous studies,31–33 CaV1.2 promoter activity was tissue specific with minimal activity observed in tsA-201 cells. Overexpression of full-length CCt caused a 34±8% decrease of CaV1.2 promoter activity, whereas truncated CCt caused 80±3% decrease of CaV1.2 promoter (Figure 2A).

The enhanced (e)GFP tag on the CCt piece enabled us to examine the subcellular localization in CCt overexpressing E16 cardiac myocytes. Truncated and full-length CCt pieces showed nuclear and cytosolic localization (Figure 2B). A truncated form of CCt had a greater relative affinity for the nucleus than the full-length CCt (Figure 2C). These data are consistent with increased repression of CaV1.2 promoter activity by truncated CCt (Figure 2A); that is, increased nuclear localization correlates with increased repression of CaV1.2 expression.

The chromatin immunoprecipitation (ChIP) assay is useful for identifying DNA sequences that interact with transcriptional modulators. We designed PCR primers to amplify in silico–predicted promoter elements of CaV1.2 including CRE, Nkx2.5, Mef2, CRM2, C/EBp, and CRM1 (Figure 3A). The CRE element served as a positive control; CREB is known to bind to the CRE element. We, therefore, used a commercially available CREB antibody to immunoprecipitate CREB (and bound DNA) and assay for CRE interaction via PCR. The upstream CRE site on the CaV1.2 promoter tested positive for CRE/CREB interaction. To determine whether CCt interacts with CaV1.2 promoter we used a CCt antibody (Figure 1) for immunoprecipitation. The CRE element does not interact with CCt. CCt does interact with modules harboring Nx and Mef2, C/EBP, and CRM1 (a region of the promoter with an enriched number of in silico predicted trans factor binding sites; Figure 3B). We repeated the ChIP assay in transfected E16 cardiac myocytes overexpressing the CaV1.2 promoter-reporter construct and CCt (1906 to 2171). We reasoned that as the truncated CCt, which shows the greatest degree of repression should show the strongest binding to the promoter. CCt (1906 to 2171) interacted with the Nxx, C/EBP, and CRM1 sites (Online Figure I, B), supporting our data from the native adult rat.

Figure 2. A, Relative CaV1.2 promoter luciferase activity in E16 ventricular myocytes overexpressing the promoter construct and eGFP or the truncated CCt (1906) or the full-length CCt (1821). tsA-201 cells transfected with the CaV1.2 promoter construct show minimal promoter activity. There is no significant difference between eGFP-only–transfected and untransfected myocytes (lower 2 bars). *P<0.01. B, Representative images of E16 ventricular myocytes overexpressing eGFP or the full-length CCt (1821) (scale bars=40 μm). C, Summary data showing nuclear/cytosolic localization with overexpression of eGFP (1.82±0.04), the full-length CCt (1821) (2.09±0.05), or the truncated CCt (1906) (2.29±0.15).
Serum Regulation

We next explored whether embryonic cardiac myocyte CaV1.2 was regulated by serum as an in vitro model of cardiac myocyte cellular hypertrophy. We used ANF promoter activity as a positive control and measured the serum response of CaV1.2 promoter, mRNA, protein, and L-type current ($I_{\text{Ca,L}}$) from embryonic mouse ventricular myocytes. As expected, serum increased ANF promoter activity and cell size. Serum repressed CaV1.2 promoter activity, CaV1.2 message, CaV1.2 protein, and $I_{\text{Ca,L}}$ (Figure 4A through 4D).

Further evidence was garnered from Western blot data showing greater expression of Cct in the nuclear extracts of adult myocytes treated with 10% serum (FBS) for 6 hours after isolation (Figure 5A). In cells exposed to serum, 31% of the total Cct was found in the nuclear fraction, whereas in myocytes incubated without serum, only 14% of the total Cct was located in the nuclear fraction. Serum caused a significant increase of Cct nuclear localization in E16 myocytes exposed as well (Figure 5B).

We made promoter deletion constructs to evaluate the putative elements responsible for controlling serum-sensitive CaV1.2 promoter activity (Figure 6A). These deletion constructs were transiently transfected into E16 ventricular myocytes. The deletion construct Δ1 removes the CRE, Nkx2.5,
and Mef2 elements. In the presence of serum, Δ1 promoter activity was significantly reduced compared to the full-length promoter (Figure 6B). In the absence of serum, there was no significant difference between Δ1 and full-length promoter constructs (Figure 6C). This is consistent with earlier studies demonstrating positive regulation by humoral factors occurs via the CRE module of the CaV1.2 promoter.32 A further deletion (Δ2) reduced promoter activity independently of serum.

Next, the effect of CCt overexpression was tested on the deletion mutants. CCt repression of Δ1 promoter activity was stronger in the presence than in the absence of serum (83.81 ± 7.14% versus 65.74 ± 6.24%; Figure 6B and 6C). The CCt deletion mutant 1906 to 2171 caused significantly more repression than full-length CCt from the full-length CaV1.2 promoter (Figure 6B and 6C).

CCt is known to inhibit I_{Ca,L}.23,26 This raises the possibility that the promoter response effect of CCt overexpression is secondary to a decrease of Ca signaling. Therefore, we tested the effect of bath Ca-removal. We measured mRNA and protein after 48 hours of Ca-free bath incubation. In contrast, to CCt overexpression, bath-Ca removal resulted in an increase of mRNA and protein (Figure 6D and 6E). Thus, CCt suppression of transcription, mRNA, and protein cannot be secondary to a CCt overexpression–induced decrease of Ca entry, because Ca entry decrease causes a compensatory upregulation of CaV1.2 (for comparison, see Schroder et al²). Taken together, these data suggest that CCt responds to hypertrophic stimuli and mediates phenotypic changes in response to serum.

Serum triggers hypertrophic signaling pathways in cardiac myocytes. The most noticeable change in phenotype is cellular hypertrophy. To test for a contribution of CCt in causing cellular hypertrophy, we overexpressed CCt in E16 myocytes and then measured cell size and ANF expression. A 25% increase in cell size was observed in the presence of serum; however, CCt attenuated the FBS-induced cellular hypertrophy (Figure 7A through 7D). CCt overexpression also attenuated serum-induced increase of ANF expression (Figure 7E).

**Discussion**

We show that CCt, a proteolytic fragment of CaV1.2, interacts with the CaV1.2 promoter in vivo and autoregulates CaV1.2 transcription in cardiac myocytes. CCt repression of CaV1.2 promoter activity suggests a negative-feedback loop whereby upregulated CaV1.2 (hence upregulated CCt) results in additional repression. The serum response suggests more complex CaV1.2 promoter regulation: CCt antagonizes serum response elements on the distal segment of the CaV1.2 promoter. Finally, serum was shown to promote cellular hypertrophy (manifested as an increase of cell size), which was antagonized by CCt. Taken together, these data suggest that CCt responds to hypertrophic stimuli and mediates phenotypic changes in response to serum.

In neurons, CCt localizes to the nucleus²⁷,³⁴ and transcriptionally regulates connexin 31.1.²⁷ Microarray studies suggest that CCt may also downregulate the Na/Ca exchanger.²⁷ This is consistent with the hypothesis that Na/Ca exchanger and I_{Ca,L} are coordinately regulated to maintain cellular Ca homeostasis.³⁵,³⁶ CCt truncations increase current,²³ and in heterologous expression systems, CCt overexpression inhibits I_{Ca,L}.²⁶ Given that Na/Ca exchanger is the main plasma membrane Ca efflux pathway in cardiac cells, Ca balance is maintained by a reduced capacity for extrusion with reduced
Ca entry. A rigorous test of this hypothesis will be an important extension of this work.

A major point of the present study was the demonstration that CCt is a transcriptional repressor of CaV1.2. In effect, proteolytically cleaved CCt becomes a de facto auxiliary subunit of the CaV1.2 surface membrane channel complex. DREAM/Calsenilin/KChIP3 analogously interacts with Kv4 channels at the cell surface and is a transcriptional repressor, albeit not necessarily autoregulatory. However, with respect to Ca homeostasis, DREAM is indirectly regulatory by virtue of its ability to repress Na/Ca exchange expression in neurons. We show evidence for CCt interaction with endogenous chromatin of ventricular cardiac myocytes and for CCt repression of CaV1.2 transcription. CCt repression of CaV1.2 was then superimposed on the effect of serum factors. This interpretation is consistent with the finding that CCt forms transcriptional complexes with other nucleoproteins, such as p54(nrb)/NonO, and raises the likelihood that multiple factors converge on the CaV1.2 promoter to produce an integrated response. CaV1.2 promoter mutation analysis supports a positive promoter interaction at the distal CRE element. Previous CaV1.2 promoter deletion analyses are not in complete agreement. Thus, whereas deletion of distal promoter elements results in a serum-independent decrease of activity in our study, as well as for human promoter constructs, rat promoter constructs showed a 1.2- to 1.5-fold increase of activity following deletion of the distal segment. Differences between these studies may reflect species differences and the different cell models used for promoter–reporter expression. We chose to use native primary cultured embryonic mouse ventricular myocytes rather than neonatal cultured rat ventricular myocytes (NRVMs) or genetically modified HL-1 cell line. We could not reproduce the NRVM experiments because of poor transfection efficiencies. Despite this, our studies qualitatively agree with myocyte specificity for promoter activity and for deletions >1000 base pairs across species and transfection systems.

We were surprised that N-terminal truncated CCt (1906 to 2171) showed the strongest degree of nuclear localization, and raises the likelihood that multiple factors converge on the CaV1.2 promoter to produce an integrated response. CaV1.2 promoter mutation analysis supports a positive promoter interaction at the distal CRE element. Previous CaV1.2 promoter deletion analyses are not in complete agreement. Thus, whereas deletion of distal promoter elements results in a serum-independent decrease of activity in our study, as well as for human promoter constructs, rat promoter constructs showed a 1.2- to 1.5-fold increase of activity following deletion of the distal segment. Differences between these studies may reflect species differences and the different cell models used for promoter–reporter expression. We chose to use native primary cultured embryonic mouse ventricular myocytes rather than neonatal cultured rat ventricular myocytes (NRVMs) or genetically modified HL-1 cell line. We could not reproduce the NRVM experiments because of poor transfection efficiencies. Despite this, our studies qualitatively agree with myocyte specificity for promoter activity and for deletions >1000 base pairs across species and transfection systems. It will be important in follow-up studies to attempt to elucidate CCt nucleoprotein binding partners to more fully understand CaV1.2 promoter regulation.

We were surprised that N-terminal truncated CCt (1906 to 2171) showed the strongest degree of nuclear localization,
because the data disagree with a recent study showing that CCt is a neuronal transcriptional regulator. Our studies were mainly performed in cardiac myocytes, whereas previous studies mainly used HEK cells and neurons. To further examine localization, we overexpressed the eGFP-tagged CCt pieces in both E16 ventricular myocytes and tsA-201 cells (data not shown). Truncated CCt showed a greater degree of nuclear localization when compared with the native piece. In contrast, Gomez-Ospina et al., using a yellow fluorescent protein–tagged CCt (rat sequence), found no nuclear localization with a similarly truncated CCt in neurons and HEK cells. Our CCt constructs were based on the rabbit sequence, which might suggest species differences, although this seems unlikely based on the high degree of homology (>80%) across species for the C-terminal portion of the LTCC. Nonetheless, in the present study, the degree of nuclear localization and the degree of CaV1.2 transcriptional repression were positively correlated.

Sites on the promoter used for PCR amplification in the ChIP assay were selected based on in silico analysis focusing on regions containing sites for transcription factor binding that are important for determining cardiac development and hypertrophic signaling such as members of the GATA, NK, MEF2, and T-box families. Building on the fact that the truncated CCt showed stronger repression of luciferase activity, and a greater degree of nuclear localization, we coexpressed the eGFP tagged CCt (1906 to 2171) with the promoter construct in E16 cultured cells and performed a ChIP assay (online data supplement). Similar results to that of the native system were shown with the exception of an absence of binding the C/EBP region and the presence of binding the CRM2 (Cis regulatory module 2). This altered binding pattern may provide an explanation for the increased repression of promoter activity and nuclear localization observed with the truncated form of CCt.

In summary, we report that the C terminus of the LTCC in ventricular myocytes is cleaved, and, in turn, functions as a transcription factor regulating CaV1.2 expression. Future studies will evaluate CCt contributions to cellular hypertrophy and the regulation of CCt nuclear–cytosolic shuttling.

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Disclosures

None.

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Supplemental Figures

**Online Figure I.** (A) Representative Western blots validating nuclear and cytosolic fractions with Pol II (nuclear) and TGM2 (tissue transglutaminase 2, cytosolic).  (B) Representative ChIP data showing potential cis binding regions in E16 ventricular myocytes over-expressing the CaV1.2 promoter construct and the 1906-2171 eGFP tagged CCt construct.  (C) Relative ANF promoter activity in E16 ventricular myocytes in the presence or absence of 10% FBS.

**Online Figure II.** (Text Figure 2B with separate DAPI panels) Representative images of E16 ventricular myocytes over-expressing eGFP or the full-length CCt (1821), or the truncated CCt (1906) (scale bars = 40 microns).

**Online Figure III.** (Text Figure 7A-C with separate DAPI panels) (A-C) Representative images of E16 ventricular myocytes over-expressing: (A) eGFP alone; (B) full-length CCt (1821-2171); or (C) truncated CCt (1906-2171).  scale bars = 40 microns.
Online Figure I. Schroder, Byse, and Satin
Online Figure II. Schroder, Byse, and Satin
Online Figure III. Schroder, Byse, and Satin