Physical and Functional Interaction Between Calcineurin and the Cardiac L-Type Ca\(^{2+}\) Channel

Samvit Tandan, Yanggan Wang, Thomas T. Wang, Nan Jiang, Duane D. Hall, Johannes W. Hell, Xiang Luo, Beverly A. Rothermel, Joseph A. Hill

Abstract—The L-type Ca\(^{2+}\) channel (LTCC) is the major mediator of Ca\(^{2+}\) influx in cardiomyocytes, leading to both mechanical contraction and activation of signaling cascades. Among these Ca\(^{2+}\)-activated cascades is calcineurin, a protein phosphatase that promotes hypertrophic growth of the heart. Coimmunoprecipitations from heart extracts and pulldowns using heterologously expressed proteins provided evidence for direct binding of calcineurin at both the N and C termini of \(\alpha_1\). At the C terminus, calcineurin bound specifically at amino acids 1943 to 1971, adjacent to a well-characterized protein kinase (PK)/PKC/PKG phospho-acceptor site Ser1928. In vitro assays demonstrated that calcineurin can dephosphorylate \(\alpha_1\). Channel function was increased in voltage-clamp recordings of \(I_{\text{Ca,L}}\) from cultured cardiomyocytes expressing constitutively active calcineurin, consistent with previous observations in cardiac hypertrophy in vivo. Conversely, acute suppression of calcineurin pharmacologically or with specific peptides decreased \(I_{\text{Ca,L}}\). These data reveal direct physical interaction between the LTCC and calcineurin in heart. Furthermore, they demonstrate that calcineurin induces robust increases in \(I_{\text{Ca,L}}\) and highlight calcineurin as a key modulator of pathological electrical remodeling in cardiac hypertrophy. (Circ Res. 2009;105:51-60.)

Key Words: action potential remodeling ■ Ca\(^{2+}\) channels ■ calcineurin ■ electrophysiology ■ hypertrophy

Perturbation of intracellular Ca\(^{2+}\) signaling and prolongation of the myocyte action potential accompany many forms of heart disease and contribute to the pathogenesis of cardiac hypertrophy and failure.\(^1\) The voltage-gated L-type Ca\(^{2+}\) channel (LTCC) (\(\alpha_1\)) is the major pathway of Ca\(^{2+}\) influx into cardiomyocytes and an important determinant of action potential morphology. Indeed, the LTCC transduces membrane potential changes into local intracellular calcium transients that initiate a host of physiological events. We and others have reported an increase in LTCC activity in various models of cardiac hypertrophy.\(^2,3\) However, molecular mechanisms responsible for the observed increase in channel function in hypertrophy are unknown, although evidence suggests that it involves an increase in channel availability and open probability.\(^4\)

L-type channel function is governed by an array of reversible phosphorylation and dephosphorylation events. Among the best characterized reactions is a robust, transient activation of L-type Ca\(^{2+}\) current (\(I_{\text{Ca,L}}\)) by protein kinase (PK)A.\(^5,6\) More complex regulation by PKC has been reported, with both channel activation and inhibition observed.\(^5,6\) Other work has demonstrated \(I_{\text{Ca,L}}\) inhibition by PKG.\(^7\) However, despite a growing understanding of the role of phosphor-
ylation in the regulation of \(I_{\text{Ca,L}}\), less is known regarding the role of protein phosphatases in these reactions. Some reports have described PP2A\(^8,9\) and PP1-mediated\(^10\) channel regulation.

Calcineurin (PP2B) is a cytoplasmic Ca\(^{2+}\)/calmodulin-dependent protein phosphatase that contributes to hypertrophic signaling in many models of cardiac hypertrophy including that induced by elevated afterload.\(^1\) Calcineurin links cytoplasmic Ca\(^{2+}\) to transcriptional regulation of multiple genes involved in the hypertrophic and failure programs, and expression of constitutively active calcineurin in transgenic mice is sufficient to drive robust hypertrophy and failure.\(^11\) Calcineurin is activated in human heart failure and participates in hypertrophic signal transduction in models of cardiac\(^1\) and skeletal muscle,\(^12\) biomechanical stress, and fiber type-specific gene expression in skeletal muscle.\(^12\)

We previously reported that the activity and abundance of the LTCC are altered in pressure-overload cardiac hypertrophy through a calcineurin-dependent pathway.\(^2\) Based on these findings, we hypothesized that calcineurin is a component of the LTCC macromolecular complex in heart and participates in stress-dependent regulation of channel function.

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From the Departments of Internal Medicine (Cardiology) (S.T., Y.W., T.T.W., N.J., X.L., B.A.R., J.A.H.) and Molecular Biology (J.A.H.), University of Texas Southwestern Medical Center, Dallas; and Department of Pharmacology (D.D.H., J.W.H.), University of Iowa, Iowa City. Present address for Y.W.: Department of Pediatrics, Emory University, Atlanta, Ga.

Correspondence to Joseph A. Hill, MD, PhD, Division of Cardiology, UT Southwestern Medical Center, NB11.200, 5323 Harry Hines Blvd, Dallas, TX 75390-8573. E-mail joseph.hill@UTSouthwestern.edu

From the Departments of Internal Medicine (Cardiology) (S.T., Y.W., T.T.W., N.J., X.L., B.A.R., J.A.H.) and Molecular Biology (J.A.H.), University of Texas Southwestern Medical Center, Dallas; and Department of Pharmacology (D.D.H., J.W.H.), University of Iowa, Iowa City. Present address for Y.W.: Department of Pediatrics, Emory University, Atlanta, Ga.

Correspondence to Joseph A. Hill, MD, PhD, Division of Cardiology, UT Southwestern Medical Center, NB11.200, 5323 Harry Hines Blvd, Dallas, TX 75390-8573. E-mail joseph.hill@UTSouthwestern.edu

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control.

lysate and immunoblots (IB) for both. IgG used as a negative

cisneurin is a component of the LTCC macromolecular com-

isoforms prevalent in heart. Conversely, immunoprecipitation

and 190 kDa, consistent with the full-length and processed

S

Glutathione

To map the CnA binding site, we engineered overlapping

Calcineurin Colocalizes With the LTCC

Averaged data are reported as means ±SEM. Statistical significance

was analyzed using a Student’s unpaired t test or 1-way ANOVA

followed by Bonferroni’s method for post hoc pair-wise multiple

comparisons.

All protocols were approved by the Institution’s Animal Care and

Use Committee. An expanded Materials and Methods section is avail-

able in the Online Data Supplement at http://circres.ahajournals.org.

Results

Calcineurin Colocalizes With the LTCC

Calcineurin is a heterodimer of catalytic A (CnA) and Ca\(^{2+}\)

binding regulatory B subunits.\(^{13}\) To determine whether calcin-

eurin is a component of the LTCC macromolecular complex,

we immunoprecipitated \(\alpha_{1.2}\) from rat ventricular lysates and probed for CnA. A strong band migrating at \(\approx 61\)

kDa was readily identified (Figure 1). As a positive control,

we immunoblotted for \(\alpha_{1.2}\), which revealed bands at 240

and 190 kDa, consistent with the full-length and processed

isoforms prevalent in heart. Conversely, immunoprecipitation

with anti-CnA antibody pulled down \(\alpha_{1.2}\). Immunocyto-

chemical studies revealed colocalization of CnA and \(\alpha_{1.2}\) at murine myocyte Z bands (data not shown).

Direct Interaction Between \(\alpha_{1.2}\) and Calcineurin

To map the CnA binding site, we engineered overlapping

glutathione S-transferase (GST) fusion proteins spanning the

intracellular domains of \(\alpha_{1.2}\). The C terminus (CT) was

further subdivided into numbered CT fragments (Figure 2A; Online Table I). Equal quantities of \(\alpha_{1.2}\) GST fusion

proteins were used to pull down CnA from ventricular (Figure 2B) or brain tissue (data not shown) lysates. A consistent subset of GST fusion proteins (CT-4, CT-8, CT-D) corresponding to overlapping regions of \(\alpha_{1.2}\) pulled down CnA from both cytosolic and membrane fractions (Figure 2B). Each of these peptides overlaps considerably, suggesting

the presence of a specific calcineurin-binding site within the

\(\alpha_{1.2}\) sequence. The N terminus of \(\alpha_{1.2}\) also pulled down calcineurin, suggesting that calcineurin and \(\alpha_{1.2}\) may have multiple sites of interaction.

To test whether the interaction between calcineurin and

\(\alpha_{1.2}\) is direct or mediated by intervening proteins, GST-

\(\alpha_{1.2}\) fusion proteins were incubated with purified recombi-

nant calcineurin (CnA/B, expressed as a complex of catalytic A and regulatory B subunits). Immunoblots probed for CnA revealed an identical pattern of GST fusion protein interac-

tions as was seen with native lysates from heart or brain

(CT-4, CT-8, CT-D, and N terminus) (Figure 2C). These data,

then, demonstrate a specific and direct interaction between

CnA and \(\alpha_{1.2}\) without intermediary scaffolding elements.

The minimal interaction domain (amino acids 1909 to

2029) contains several potential PxIxIT motifs, a calcineurin-

binding domain found in many calcineurin-interacting part-

ners (Online Figure I, A). We tested whether one such potential motif was responsible for calcineurin binding to

\(\alpha_{1.2}\) by mutating residues PSI to AAA (amino acids 2018 to

2020) on CT-4. In GST-pulldown experiments, CnA bound

wild-type CT-4 and CT-4 PSI-AAA with similar efficiency, suggesting this region is not the binding site. As a second test, we performed competition-binding assays using overlapping peptides spanning amino acids 1976 to 2029 of CT-8, the region containing the potential PxIxIT motifs (Online Figure I, C). Despite the presence of several-fold molar excess of peptide relative to both CnA and CT-8, CnA bound avidly to CT-8 (Online Figure I, B).

Robson–Garnier algorithms\(^{14}\) applied to the primary se-

quence of CT-8 suggested the presence of 2 distinct domains:

an N-terminal domain (amino acids 1909 to 1968) containing an \(\alpha\)-helix, and CT domain (amino acids 1969 to 2029),

containing the potential PxIxIT motifs and manifesting no

definitive structure (Online Figure I, D). Because our findings

(above) suggested that the unstructured region is not respon-

sible for CnA binding, we analyzed the N-terminal \(\alpha\)-helix.

To do this, we engineered serial truncations of CT-8 (Online

Figure I, D). GST pulldowns revealed binding of CnA to

amino acids 1909 to 1971 and 1943 to 2029 within CT-8,

implicating the \(\alpha\)-helix as necessary to bind CnA (Online

Figure I, E). Furthermore, disruption of helical structure by

introduction of a proline rendered CT-8 incapable of binding

CnA (Figure 2D). Together, these data establish amino acids

1943 to 1971 as the minimal region required for interaction

between CnA and the CT of \(\alpha_{1.2}\).

Finally, as heterologous expression of mammalian proteins in

bacteria often leads to early termination events during

synthesis (Figure 2B), we expressed \(\alpha_{1.2}\) CT constructs

fused to myc-epitope tags in mammalian cells. Immunopre-

cipitation using anti-myc antibodies in HEK-293 cells that

coeXpressed calcineurin fused to GFP and probed for GFP-

CnA revealed a specific band at \(\approx 75\) kDa (the expected mass of

CnA plus GFP) only for peptides containing amino acids

1909 to 1969 (Online Figure I, F). These data, then, are

consistent with our findings using proteins expressed in

bacteria and point to this CT region of \(\alpha_{1.2}\), as required for calcineurin binding.
Calcineurin Binds α_1.2 CT With High Affinity

To obtain a semiquantitative estimate of the affinity of interaction between α_1.2 and CnA, we performed GST-pulldown experiments using varying amounts of CT-8 while maintaining a constant, low concentration of recombinant calcineurin (15 nmol/L) (Online Figure II). Purified immobilized CT-8 was added in increasing concentrations, and supernatants were collected after each pulldown to assess bound (pellet) versus unbound (supernatant) calcineurin. Approximately equivalent amounts of CnA cosedimented when CT-8 was present at half the concentration of CnA (CT-8:CnA, 0.5), whereas almost all the CnA was bound when more than 3 times as much CT-8 was present (CT-8:CnA, ≈3). These data lend credence to the specificity of the pulldown assays, and they suggest that the interaction between α_1.2 and CnA occurs at relatively high (approximately nanomolar) affinity.

CnA Regulatory Region Is Required for α_1.2 Binding

Experiments reported so far were performed in the presence of EGTA, which inactivates calcineurin by chelating Ca^{2+}. We observed similar levels of binding in the absence of EGTA (Figure 3A), a condition where calcineurin is expected...
to be active. We also observed that CnA was able to bind CT-8 in the presence of cyclosporine A (CsA 10 μmol/L, Figure 3B), an inhibitor that obstructs the calcineurin catalytic domain. To test further the role of the catalytic domain, we expressed full-length CnA (F.L.) or a constitutively active mutant (CnA*) lacking the CT autoinhibitory domain that obstructs access to the catalytic site of the enzyme (Figure 3C). In 6 independent experiments, only F.L. (61 kDa) bound CT-8, whereas no signal was detected with lysates from cells expressing CnA* (48 kDa) (Figure 3D) or from cells coexpressing both F.L. and CnA* (Figure 3E). Taken together, these findings suggest that calcineurin A binds the channel via its CT regulatory region.

Phospho-α1.2 Is a Calcineurin Substrate

Next, we set out to determine whether calcineurin recognizes α1.2 as a substrate. GST fusion proteins were phosphorylated in vitro by recombinant PKA or PKC and then tested for dephosphorylation by calcineurin. As expected, PKA readily phosphorylated both CT-4 and CT-8 (Figure 4A), proteins that harbor Ser1928. CT-B, which is adjacent to CT-8, was phosphorylated to only a modest extent. As a further control,
the N terminus of α1,2, which binds CnA and can be phosphorylated by PKC but does not contain consensus sequences for PKA phosphorylation (Figure 4). PKC phosphorylated CT-8 and the N terminus but did not phosphorylate CT-B (Figure 4C).

We then incubated the 32P-phosphorylated GST fusion proteins with purified recombinant calcineurin (CnA/B). Here, we observed marked decreases in the phosphorylation of CT-4 and CT-8 (Figure 4A and 4C), consistent with their serving as substrates for calcineurin phosphatase. Phosphate incorporation in either CT-B by PKA or N terminus by PKC was unchanged in the presence of recombinant calcineurin. To test the specificity of CT-8 phosphorylation, an alanine was substituted for serine at position 1928 (CT-8*). PKA failed to induce phosphorylation of CT-8* (Figure 4B), indicating that PKA-mediated phosphorylation is specific to Ser1928. However, PKC induced phosphorylation of CT-8*, suggesting additional unidentified phosphorylation sites within α1,2, which calcineurin recognizes as substrate (Figure 4C). Together, these data demonstrate that calcineurin is capable of recognizing α1,2 as a substrate in vitro.

To corroborate these findings in a more physiological setting, we tested whether calcineurin targets endogenous α1,2 by studying cultured neonatal rat cardiomyocytes (NRCMs) exposed to isoproterenol (Iso) in the presence of CsA (Figure 4D). As expected, Western blot using anti-Ser1928 phospho-specific antibody demonstrated a time-dependent increase in Ser1928 phosphorylation after Iso treatment (Online Figure III). Importantly, inhibition of calcineurin with CsA elicited no difference in Iso-induced Ser1928 phosphorylation (Figure 4D). Furthermore, overexpression of a constitutively active mutant of calcineurin similarly did not antagonize Iso-induced phosphorylation of Ser1928 (data not shown). Together, these data suggest that endogenous calcineurin does not specifically antagonize PKA-mediated phosphorylation of α1,2 Ser1928 and point to the presence of additional calcineurin target sites on α1,2 that remain to be resolved.

Calcineurin Activation Increases $I_{Ca,L}$

To test whether calcineurin regulates L-type channel function, we infected NRCMs with adenovirus expressing constitutively active calcineurin (AdCnA*). Overexpression of activated calcineurin provoked hypertrophic cell growth, as evidenced by an 84% (±9%, $P<0.05$; $n=100$ AdCnA*, $n=100$ AdGFP) increase in cell cross-sectional area (Figure 5A), increased sarcomeric organization, and upregulated $I_{Ca,L}$ (Figure 5B). Control and AdCnA* cardiomyocytes were infected with adenovirus expressing constitutively active calcineurin (AdCnA*) or GFP (AdGFP) and visualized with fluorescent tracer (CellTracker). AdCnA* led to the expected increase in cell size and associated myocyte hypertrophy. To test whether calcineurin regulates $I_{Ca,L}$, we infected NRCMs with adenovirus expressing constitutively active calcineurin (AdCnA*) or GFP (AdGFP) and visualized with fluorescent tracer (CellTracker). AdCnA* led to the expected increase in cell size and associated myocyte hypertrophy. $I_{Ca,L}$ was expressed as the increase in peak $I_{Ca,L}$ density (AdCnA* [12 pA/pF at +10 mV, $n=10$] vs control [10 pA/pF at +10 mV, $n=6$]; $P<0.05$), with no shift in I-V relationship.
ANF expression (data not shown). Recordings of $I_{\text{Ca,L}}$ in AdCnA*-infected myocytes revealed a 26% (±4%, $P<0.05$; $n=10$ AdCnA*, $n=6$ AdGFP) increase in $I_{\text{Ca,L}}$ density with no significant shift in the steady-state current–voltage (I–V) relationship (Figure 5B). Hypertrophy-associated increases in $I_{\text{Ca,L}}$ exceeded that expected from simple increases in cell size, because current density was significantly increased.

**Inhibition of Calcineurin Diminishes $I_{\text{Ca,L}}$.**
Calcineurin-dependent increases in $I_{\text{Ca,L}}$ do not exclude the possibility that changes in channel activity are secondary to the hypertrophic phenotype. To address this, we inhibited calcineurin acutely with CsA and evaluated changes in $I_{\text{Ca,L}}$ in voltage-clamp recordings from dissociated ventricular myocytes. To minimize current rundown or dialysis of intracellular contents, we used an amphotericin B perforated patch technique. Cells were held at $-80$ mV, stepped transiently to $-50$ mV to inactivate sodium currents, and then $I_{\text{Ca,L}}$ was measured (0.1 Hz) at test potentials between $-30$ and $+50$ mV. Under these conditions, addition of 10 μmol/L CsA significantly inhibited $I_{\text{Ca,L}}$ at all potentials without altering the potential at which peak current was observed (Figure 6A). Similar results were observed using tacrolimus (FK-506), a structurally distinct calcineurin inhibitor (Figure 6B). Suppression of peak (+10 mV) $I_{\text{Ca,L}}$ occurred over a time course of several minutes and was partially reversible following drug washout (Figure 6C). Together, these findings suggest that the effects of CsA or FK-506 on $I_{\text{Ca,L}}$ are mediated by calcineurin suppression rather than by nonspecific actions of either compound.

CsA inhibited $I_{\text{Ca,L}}$ without inducing a shift in the steady-state I–V relationship (Online Figure IV, A). Moreover, the kinetics of $I_{\text{Ca,L}}$ inactivation were not altered (Online Figure IV, B), suggesting that the effects of calcineurin do not involve antagonism of PKA-dependent phosphorylation of the channel. To test this further, we investigated the effects of Iso in the presence of calcineurin activation (Figure 6D). Iso (1 μmol/L) induced similar ($P=NS$) increases in $I_{\text{Ca,L}}$ density in cells infected with AdGFP (51.9 ± 3%, $n=3$), AdCnA* (54.1 ± 7.4%, $n=5$), and AdRCAN1 (regulator of calcineurin protein 1) (a protein inhibitor of calcineurin) (46.1 ± 7%, $n=3$) (Figure 6D). These data, then, provide strong evidence that PKA and calcineurin activate the channel independently.
Inhibition of $I_{\text{Ca,L}}$ by CsA Is Calcineurin-Dependent

To determine whether the inhibitory effects of CsA on $I_{\text{Ca,L}}$ are calcineurin-dependent, we studied $I_{\text{Ca,L}}$ in the context of negligible calcineurin activity. Calcineurin is inactive in the absence of Ca$^{2+}$, so we hypothesized that pharmacological suppression of calcineurin should have no effect on $I_{\text{Ca,L}}$ in the presence of BAPTA (because calcineurin is inactivated by elimination of intracellular Ca$^{2+}$). $I_{\text{Ca,L}}$ was recorded in dissociated ventricular myocytes using whole-cell methods, where the pipette filling solution included 10 mmol/L BAPTA, a chelator that binds Ca$^{2+}$ nearly 2 orders of magnitude faster than EGTA.$^{16}$ Under these conditions, where intracellular Ca$^{2+}$ is irreversibly chelated, addition of 10 $\mu$mol/L CsA did not diminish $I_{\text{Ca,L}}$ (Figure 7A); $I_{\text{Ca,L}}$ declined by only 2.5% ($0.8\%$, $n=7$, $P=\text{NS}$) in the presence of BAPTA. In the presence of Ca$^{2+}$ chelation, and consequent calcineurin inactivation, 10 $\mu$mol/L CsA had no effect on $I_{\text{Ca,L}}$ (10 mmol/L BAPTA [+]; control [-]). B, Representative recordings of $I_{\text{Ca,L}}$ density from dissociated ventricular myocytes treated with 5 mmol/L EGTA. With incomplete Ca$^{2+}$ buffering, 10 $\mu$mol/L CsA induced a partial reduction in $I_{\text{Ca,L}}$ (5 mmol/L EGTA [+]; control [-]). C, Representative time course of $I_{\text{Ca,L}}$ recorded from a ventricular myocyte dissociated from cardiomyocyte-overexpressing RCAN1 transgenic mice. D, Mean data of percentage inhibition of $I_{\text{Ca,L}}$ induced by 10 $\mu$mol/L CsA vs control from dissociated adult ventricular myocytes (perforated patch, 44±5%, $n=5$, $P<0.05$), when 5 mmol/L EGTA or 10 mmol/L BAPTA is dialyzed through the patch pipette (21±5%, $n=9$, $P<0.05$ and 2.5±0.8%, $n=7$, $P<0.05$, respectively), or recordings from ventricular myocytes isolated from RCAN1 transgenic hearts (3±0.8%, $n=6$, $P<0.05$).
of intracellular BAPTA as compared to 44% (±5%, n=5, P<0.05) without BAPTA (Figure 7D). To determine whether the loss of CsA-induced inhibition in the presence of BAPTA was simply an artifact of the disrupted-membrane recording method, we studied I_{Ca,L} under conditions where intracellular Ca^{2+} was only partially buffered (pipette filled with 5 mmol/L EGTA). Under these conditions, calcineurin could be partially activated owing to incomplete buffering of intracellular Ca^{2+}, and CsA suppressed I_{Ca,L} to an intermediate extent (21±5%, n=9, P<0.05) (Figure 7B and 7D).

As another test for nonspecific actions of CsA, we measured Ba^{2+} current in the setting of buffered intracellular Ca^{2+} (10 mmol/L BAPTA). Under these conditions, CsA had no effect on I_{Ca,L} (data not shown). Finally, we evaluated the effects of CsA on I_{Ca,L} in myocytes dissociated from transgenic (Tg) mouse hearts overexpressing RCAN1, a calcineurin-inhibitory protein.15 CsA had no effect on I_{Ca,L} (3.0±0.8% decline, n=6, P=NS) in myocytes in which calcineurin activity was suppressed by overexpression of RCAN1 (Figure 7C and 7D).

**Targeted Inhibition of Calcineurin Diminishes I_{Ca,L}**

We tested the effects of nonpharmacologic suppression of calcineurin by intracellular dialysis of the calcineurin-inhibitory peptide autoinhibitory domain (AID), derived from the autoinhibitory domain of calcineurin. Calcineurin inhibition by pipette dialysis of 10 μmol/L AID induced a 36% (±3%, n=5, P<0.05) decrease in I_{Ca,L}. By contrast, no declines in I_{Ca,L} were seen with intracellular dialysis of a control peptide (peptide 2010; 1.7±4%, n=4, P=NS) or no peptide at all (6.1±2.5%, n=6, P<0.05) (Figure 8A, 8B, and 8D). Peptide 2010 was chosen as a control because it did not interfere with calcineurin-binding (Online Figure I, B) and was of a similar molecular mass and pI to AID. Consistent with our findings when calcineurin was suppressed pharmacologically, AID-induced inhibition of I_{Ca,L} to a similar extent (36% versus 44%, P=NS) and was not associated with shifts in the steady-state I–V relationship (Figure 8C). Furthermore, AID did not elicit reduction in I_{Ca,L} recorded from RCAN1 transgenic myocytes (2.6±1.6%, n=5, P<0.05) or in the presence of BAPTA (7.4±0.8%, n=4, P<0.05). All experiments were performed on myocytes isolated from at least 3 different mice of the same strain.

**Figure 8. Targeted inhibition of calcineurin decreases I_{Ca,L}**

A, Representative peak I_{Ca,L} densities (+10 mV) and time course (B) recorded from ventricular myocytes after peptide dialysis of 10 μmol/L control peptide (2010, black line) or AID (red line) peptide. C, I–V relationship demonstrating that calcineurin inhibition by AID induced a 36% decrease in peak inward I_{Ca,L} density (4.08±1 pA/pF at +10 mV, n=4; P<0.05) vs control peptide (6.42±0.32 pA/pF at +10 mV, n=4), with no shift in I–V relationship. D, Mean data demonstrating that AID induced a 36% (±3%, n=5, P<0.05) decrease in I_{Ca,L} density vs control peptide (1.7±4.3%, n=4, P<0.05) or no peptide (4.1±2.5%, n=6, P<0.05). AID had no effect on I_{Ca,L} density in RCAN1 transgenic myocytes (2.6±1.6%, n=5, P<0.05) or in the presence of BAPTA (7.4±0.8%, n=4, P<0.05). All experiments were performed on myocytes isolated from at least 3 different mice of the same strain.

**Discussion**

The LTCC is an element central to multiple processes in the heart, including action potential repolarization, excitation–contraction coupling, and activation of Ca^{2+}-responsive signaling pathways. The L-type channel is also a prominent target of disease-related remodeling, being up- or downregulated in a variety of pathological states. In this study, we demonstrate that (1) calcineurin copurifies with the LTCC in the ventricle; (2) calcineurin interacts directly with N-terminal and CT intracellular domains of the pore-forming subunit α1.2; (3) calcineurin recognizes α1.2 as an enzymatic substrate in vitro; and (4) calcineurin upregulates L-type channel function, because acute inhibition of calcineurin decreases I_{Ca,L} in a calcineurin phosphatase-dependent manner. Together, these data uncover a previously uncharacterized regulatory mechanism governing cardiac LTCC function.

**The LTCC Macromolecular Complex**

An emerging theme in cardiovascular biology emphasizes the importance of specific localization of signaling molecules in proximity to their targets.17 The LTCC comprises a macromolecular complex of various enzymes and anchoring proteins, most of which associate with the long intracellular CT of the α1.2 subunit.6 One study in neurons implicated...
calcineurin, anchored to the L-type channel by the scaffolding protein AKAP79/150, in antagonizing PKA-mediated activation of \( I_{Ca,L} \). Here, we demonstrate that calcineurin binds to both the N terminus and CT of \( \alpha_1,2 \) and that this interaction is direct and not mediated by an intermediary protein. We have identified residues 1943 to 1971 as the minimal region required for specific calcineurin binding. That this interaction occurs with approximately nanomolar affinity is consistent with its being physiologically relevant.

**Phosphorylation-Dependent Regulation of \( Ca^{2+} \) Channel Function**

The \( Ca^{2+} \) channel is subject to regulation by multiple hormones and neurotransmitters largely through activation of kinases and phosphatases. Prominent among these is a transient increase in channel activity triggered by PKA phosphorylation. Interestingly, recent evidence suggests that much of PKA-dependent activation of \( I_{Ca,L} \) in ventricular myocytes does not involve Ser1928. PKC-dependent mechanisms are similarly complex, acting on 2 threonine residues at the N terminus of \( \alpha_1,2 \), as well as at Ser1928. In the case of N-terminal phosphorylation, an initial transient activation of the channel is followed by sustained inhibition. Furthermore, a recent report mapped PKG-mediated inhibition of heterologously expressed L-type channel activity to Ser496 on the \( \beta_2a \) subunit. Thus, there is considerable variance in the literature regarding the effects and mechanisms of \( Ca^{2+} \) channel phosphorylation.

Whereas a great deal of work has focused on channel regulation by kinases, less is known about the countervailing actions of protein phosphatases. PP2A and PP1 have been reported to dephosphorylate the channel, but the functional significance of these actions remains poorly characterized. Indeed, we have localized calcineurin binding to a region of \( \alpha_1,2 \) close to a site where PP2A binds. Some studies have suggested that PP2A inhibits \( Ca^{2+} \) channel function, possibly by antagonizing the phosphorylation of Ser1928. Here, we show that calcineurin elicits robust upregulation of channel activity. Although still unresolved, one possible mechanism may be interference with PP2A binding and consequent antagonism of PP2A-dependent dephosphorylation.

**Calcineurin Inhibition Decreases \( I_{Ca,L} \)**

We have previously shown that targeted inhibition of calcineurin, either pharmacologically with CsA or genetically via RCAN1 overexpression, attenuated action potential prolongation and eliminated the increase in heart mass associated with pressure-overload hypertrophy. Here, we extend these observations to demonstrate direct effects of calcineurin on the L-type channel. These findings, which are consistent with our overall hypothesis, cannot be explained by simple channel blockade, because 2 entirely divergent means of calcineurin inhibition (pharmacological inhibition with small molecules [CsA, FK-506] of distinct molecular structure; intracellular peptide [AID] exposure) manifested the same response. Because the effect of calcineurin inhibitors was partially reversible, current rundown cannot account for these findings. We hypothesize that calcineurin suppression induces progressive increases in channel phosphorylation because the cognate kinase is no longer antagonized by calcineurin. Accordingly, we are not surprised to observe only partial washout of the inhibitory effects, because a return to steady-state levels of \( \alpha_1,2 \) phosphorylation would be expected to take time. Intriguingly, CsA and AID induced remarkably similar degrees of \( I_{Ca,L} \) inhibition (44% and 36%, respectively).

There are reports of potentially nonspecific actions of CsA on LTCC function. Here, we report evidence against this. Under conditions where calcineurin is inactive by complete chelation of intracellular \( Ca^{2+} \), CsA had no effect on \( I_{Ca,L} \). Also, electrophysiological recordings in ventricular myocytes isolated from RCAN1 transgenic mice demonstrated no effect of acute CsA exposure or peptide-inhibitor dialysis in the context of negligible calcineurin activity. These data, then, indicate that the effects of CsA we observe are mediated by direct inhibition of calcineurin enzymatic activity.

**Precedents in Other Systems**

In a wide variety of cell types, calcineurin, activated by intracellular \( Ca^{2+} \), feeds back to regulate \( Ca^{2+} \) homeostasis through its interaction with other \( Ca^{2+} \)-regulatory proteins. In various tissues including heart, calcineurin directly regulates the inositol triphosphate and ryanodine receptors and the \( Na^+/Ca^{2+} \) exchanger. Calcineurin also regulates voltage-gated \( Ca^{2+} \) channels in a variety of systems, although there is disagreement regarding the nature and extent of its regulatory function (Online Table II).

**\( Ca^{2+} \) Channel Remodeling and Significance in Cardiac Hypertrophy and Failure**

Cardiac hypertrophy is associated with significantly increased risk of both heart failure and arrhythmia and poses a major public health problem. There is evidence to suggest that alterations in transmembrane \( Ca^{2+} \) fluxes, and consequent perturbations of \( Ca^{2+} \) homeostasis, contribute to the pathogenesis of hypertrophy by abnormally activating \( Ca^{2+} \)-responsive signaling pathways. In several forms of heart disease, increases in LTCC activity are a proximal trigger that activates pathological signaling cascades, including mitogen-activated protein kinases, PKC, and calcineurin. Eventually, such abnormal profiles of signal transduction lead to disturbances of gene regulation, which may promote disease progression. Indeed, various transgenic models of L-type channel overexpression reveal the development of hypertrophy and severe cardiomyopathy associated with ventricular fibrosis, myocyte necrosis, and remodeling.

We also report that various other factors, such as 

We also report that various other factors, such as...
Perspective and Limitations

Specific mechanisms underlying the functional regulation of I_{Ca,L} by calcineurin remain to be elucidated. One possibility may involve direct protein–protein interaction and conformational changes in α1.2 subunit protein. However, our pull-down experiments revealed equivalent binding of calcineurin to α1.2 in the presence of CsA, suggesting that inhibition of I_{Ca,L} by CsA is not attributable to disruption of protein–protein interaction. Also, we report that calcineurin is capable of recognizing α1.2 as a substrate in vitro, although the specific enzymatic substrate remains unknown. Ser1928 is an unlikely target as PKA-dependent phosphorylation of this site is associated with increased channel activity and a hyperpolarizing shift in the steady-state I–V relationship. With that said, both PKC and PKG have been shown to target Ser1928, and PKG-dependent phosphorylation of Ser496 on the β2a subunit inhibits I_{Ca,L}. Finally, actions mediated indirectly by downstream targets of calcineurin may exist.33

Acknowledgments

We gratefully acknowledge Jun Cheng and Joel Feekes for technical expertise and advice.

Sources of Funding

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Disclosures

None.

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10. duBell WH, Rogers TB. Protein phosphatase 1 and an opposing protein kinase regulate steady-state L-type Ca^{2+} current in mouse cardiac myocytes. J Physiol. 2004;556(Pt 1):79–93.
Physical and Functional Interaction Between Calcineurin and the Cardiac L-Type Ca\(^{2+}\) Channel
Samvit Tandan, Yanggan Wang, Thomas T. Wang, Nan Jiang, Duane D. Hall, Johannes W. Hell, Xiang Luo, Beverly A. Rothermel and Joseph A. Hill

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Supplement Material

Materials and Methods

Fusion proteins and pulldowns — Rat brain and rabbit cardiac cDNAs were used to engineer GST-fusion proteins spanning the intracellular domains of α1.2. The C-terminus was further subdivided into shorter overlapping fragments and designated alphabetically (CT-B through CT-E) for clones expressing the rat brain α1.2 isoform (Genbank M67515) and numerically (CT-1 through CT-23) for clones expressing the rabbit cardiac isoform (Genbank CAA33546). GST-fusion constructs containing intracellular loops I, II, and III were expressed from rabbit cardiac α1.2 (Online Table I). The sequences were amplified by polymerase chain reaction (PCR) and cloned into pGEX-4T1 vector (Amersham Biosciences) in frame with GST, at the BamHI and NotI sites as described previously1. Clones were transformed in E. coli BL-21 strain, and fusion-protein expression was induced by isopropyl-β-D-thiogalactoside (IPTG). The proteins were solubilized in buffer containing (mM) 15 Tris-HCl, pH 7.4, 0.1mg/mL lysozyme, 1.5% N-lauroyl-sarcosine, 150 NaCl, 15 DTT, 10 EDTA, and protease inhibitors, and purified on Glutathione-Sepharose beads (Amersham Biosciences). The immobilized fusion proteins were then incubated with extracts from rat ventricle in the presence of 10 mM EGTA or 10 µM cyclosporine A, where indicated. Pulldowns were also performed from purified, recombinant calcineurin Aα and B subunits, prepared and purified as described2, or from HEK 293 cell lysates over-expressing constitutively active (CnA*) or full-length (F.L.) calcineurin tagged with HA (see Cell transfections below). For peptide competition assays, recombinant calcineurin was pre-incubated with peptide for up to 1 hr before pulldowns were performed with the GST fusion proteins. For affinity studies, 15 nM recombinant calcineurin was mixed with 7.5, 22.5 and 45 nM of immobilized and purified CT-8 in a final volume of 100 µL. Protein concentration was estimated using a standard curve of recombinant carbonic anhydrase and albumin (Sigma). The mixtures were incubated at 4°C for 2 hours and centrifuged at 5,000 rpm for 2 min. The supernatant fractions were aliquoted and pellets washed twice in the same buffer. Pellet and supernatant fractions were resolved by SDS-PAGE and analyzed by western blot.

Myc-tagged fusion proteins encoding the rabbit cardiac α1.2 C-terminus of various lengths were sub-cloned into a pCMV-Tag1 (Stratagene) mammalian expression vector. Clones were confirmed by sequencing and then expressed in HEK 293 cells (see Cell
transfections). Cell lysates were incubated with anti-myc agarose conjugate (Sigma) for 2 hrs. at 4°C, washed in lysis buffer, and resolved by SDS-PAGE followed by western blot.

**Adenoviral infection and primary culture of neonatal rat ventricular myocytes** — Cardiomyocytes were isolated from the ventricles of 1-2 day old rat pups (Sprague Dawley) and plated (1250 cells/mm²) in medium containing 10% fetal calf serum as described³. Myocyte cultures obtained from this differential plating method contained less than 5% non-cardiomyocytes as determined microscopically using a myocyte-specific α-actinin antibody (data not shown). Cells were transferred to serum-free medium containing the serum supplement Nutridoma (Roche) at 0.5x concentration 24 hours before treatment or infection with adenovirus. For expression of constitutively-active calcineurin and RCAN1, cells were infected with either AdCMVCnA*iresGFP or AdCMVMCIP, respectively, which contained the cDNAs for human calcineurin or RCAN1. Expression of each gene was driven by the CMV promoter, and AdCMVCnA*iresGFP co-expressed GFP from the bicistronic IRES transcription unit to ascertain infection efficiency and localize infected cells for voltage-clamp experiments. Experiments were performed at a multiplicity-of-infection (MOI) of 100 (≥95% infection efficiency), and voltage-clamp experiments (see **Electrophysiology**) were performed on plated cells 48 hours after infection.

For Western blot time course studies using a phospho-specific Ser-1928 antibody, cultured cardiomyocytes were treated with Isoproterenol (Iso; 1 µM) for time points as indicated — 1, 2, 4, 6 and 12 hrs. After each time point, cells were washed in PBS, harvested with membrane extraction buffer (see **Co-immunoprecipitation**) supplemented with phosphatase inhibitor cocktails (Sigma), and analyzed by SDS-PAGE. Where indicated, cells were pre-treated with Cyclosporine A (CsA; 10 µM) for 30 mins., and then supplemented with Iso for 4 hrs.

**Cell transfections** — HEK 293 cells were cultured (37°C) in standard DMEM supplemented with 10% FBS. Cells were transfected at 60-70% confluency using Lipofectamine Plus reagent (Invitrogen) with constructs encoding the murine calcineurin Aα subunit (full-length; tagged with HA or GFP) and constitutively active mutant (CnA*; tagged with HA) truncated at amino acid
398 (Figure 3C). When using the HA-tagged constructs, cells were co-transfected with pEGFP-C1 (BD Biosciences Clontech) to localize and quantify transfection efficiency. Twenty-four hours after transfection, cells were harvested and lysed in buffer containing (mM) 10 Tris, pH 7.4, 150 NaCl, 1 DTT, 20% glycerol, 0.1% Triton-X 100, protease inhibitors (Roche) and phosphatase inhibitor cocktails I and II (Sigma), and used for experiments.

**Site-directed mutagenesis** — PCR was performed on cloned plasmid encoding the GST fusion protein CT-8 (α1.2 aa. 1909-2029), using the forward primer 5'-CTGGGTGAAGGCTGCCTTCCACCTGGAG-3' and reverse primer 5'-CTCCAGGTGGAAGGCAGCCCTCGACCCAG-3' to replace serine-1928 with alanine. The template vector was digested with Dpn I, and the remaining mutant plasmid was directly transformed into *E. coli* XL1-Blue Supercompetent cells (Stratagene). Mutant plasmid was purified and sequenced to confirm mutation. A similar PCR approach was also used to mutate the putative PxIxIT motif in GST fusion protein CT-4 (Psi → AAA; α1.1.2 aa. 2018-2020), using the forward primer 5'-CAACAGCAGCTTCGCGGCCGCCCACTGCGGC-3' and reverse primer 5'-GCCGCAGTGGGCGGCCGACCACTGCTGTTG-3'. Two C-terminal truncations of CT-8 (aa. 1909-1946 and 1909-1971) were made via substitution of Gln1946 and Arg1971, respectively, into stop codons. The N-terminal truncations of CT-8 (1943-2029 and 1969-2029) were made via PCR using primers 5'-CGAGGATCCGACATCTCTCAGAAGACAGTCC-3' and 5'-TTGGATCCCTGCAGAGAAGCCATTCCC-3' along with the same reverse primer 5'-TCCGGGAGCTGCATGTGTCAGAGG-3', respectively. Finally, an alanine to proline mutation was introduced at residue 1959 within the α helix region (α1.2 aa. 1952-1966) using the mutagenic oligonucleotide forward primer 5'-GTCCACCACAGCATTGGGAGTGGG-3' and reverse primer 5'-CGCCACTGCAAATGGCTGTGGTGGAC-3' to generate “CT-8 helix-Pro”.

**In vitro kinase/phosphatase assay** — *In vitro* phosphorylation and dephosphorylation assays on select GST fusion proteins were performed using recombinant PKA and calcineurin. Briefly, immobilized GST fusion proteins were phosphorylated with the recombinant murine PKA catalytic subunit (α isoform; Calbiochem) in the presence of 32P-ATP (30 min, 30°C) in kinase buffer containing (mM) 50 Tris, pH 7.2, 10 MgCl2, 0.5 DTT, protease inhibitors. The kinase and unincorporated radiolabeled ATP were removed by thorough washing, and the proteins were divided into 2 aliquots, one of which was put aside for subsequent gel analysis. The other
aliquot was treated with a recombinant heterodimer of human calcineurin Aα + calcineurin B subunits (60 min, 30°C) in calcineurin buffer containing (mM) 100 Tris, pH 7.5, 200 NaCl, 12 MgCl₂, 1 DTT, 1 CaCl₂, 0.05% NP-40, and 2 μM calmodulin. Reactions were stopped in SDS buffer followed by PAGE and autoradiography. For PKC phosphorylation reactions, GST fusion proteins were incubated as described above with recombinant PKC (ζ isoform; Invitrogen) in kinase buffer containing (mM) 20 HEPES, pH 7.4, 10 MgCl₂, 0.1 EGTA, supplemented with 200 μg/mL DAG (Sigma), 1 mg/mL phosphatidylserine (Sigma), and 50 μM ATP.

Cardiomyocyte Isolation — Adult mouse ventricular cardiomyocytes were isolated after enzymatic dissociation as described with slight modification. Briefly, after retrograde perfusion with Krebs-Ringer solution at (2 mL/min, 5 min), the heart was perfused with fresh solution containing 0.8 mg/mL collagenase (Worthington type II) for another 12-15 min. The LV was removed and cut into small pieces in “KB” solution [(mM) taurine 10, glutamic acid 70, KCl 25, KH₂PO₄ 10, Glucose 22, EGTA 0.5, pH 7.2]. After trituration, cells were studied within 4 to 6 hours. All isolation steps were carried out at 36°C with continuous gassing with 95% O₂ + 5% CO₂. Only Ca²⁺-tolerant, quiescent and rod-shaped cells, showing clear cross striations were used.

Electrophysiology — Cultured neonatal rat cardiomyocytes or acutely isolated adult cardiomyocytes were studied in a continuously superfused (1.5 mL/min) recording chamber fixed to an inverted microscope. For recordings of L-type Ca²⁺ current (I_{Ca,L}), the whole-cell voltage clamp configuration was used on cells bathed in (mM) 135 tetraethylammonium (TEA) Cl, 0.53 MgCl₂, 1.8 CaCl₂, 20 CsCl, 5 HEPES. The pipette solution for I_{Ca,L} recording contained (mM): 110 CsOH, 90 aspartic acid, 20 CsCl, 10 TEACl, 10 HEPES, 10 EGTA, 5 Mg-ATP, 5 Na₂ creatine phosphate, 0.4 GTP (Tris), 0.1 leupeptin, pH 7.2 with CsOH. 5 mM EGTA or 10 mM BAPTA were substituted for 10 mM EGTA in this internal solution in some experiments. For certain studies, the amphotericin patch clamp technique was used. Here, patch clamp pipettes were prepared and the tips filled by dipping into internal solution containing (mM): 120 K-aspartate, 20 KCl, 5 MgCl₂, 10 HEPES, 1.8 CaCl₂, pH adjusted to 7.2 with KOH. The remainder of the pipette was back-filled with the same solution plus amphotericin B (200 μg/mL). Where indicated, bath solution was complemented with 10 μM CsA or FK-506. For peptide inhibitory
studies, peptides were dissolved in pipette solution and dialyzed into the cell during whole-cell configuration.

\[ I_{Ca,L} \] run-down, when present, typically occurs within the first 5 minutes of recording. In order to minimize the impact of run-down, we added 5 mmol/L Mg-ATP to the pipette solution (see above) and commenced data acquisition after 5-10 minutes of equilibration between pipette solution and intracellular contents\(^5\). Cells showing continuous current run-down (≈ 5%) were excluded from the analysis.

**Immunohistochemistry** – Mice were anesthetized with intraperitoneal injection of pentobarbital (Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA) and sacrificed by cervical dislocation in accordance with institutional and NIH animal use guidelines. A midline thoracotomy was performed and hearts excised while still beating. Hearts were grossly trimmed in the coronal plane and blotted free of excess blood prior to cryoembedding. Dorsal and ventral halves of heart specimens were placed in tissue freezing medium (TFM, Triangle BioScience, Raleigh, NC) and oriented for coronal sectioning in peelaway cryomolds (Polysciences, Warrington, PA). Cryoembedments were flash frozen by partial immersion in liquid-nitrogen-supercooled-isopentane and stored at -80°C until time of sectioning. Subsequently, samples were equilibrated to -22°C and eight-micron frozen sections were made on a Leica CM3000 cryostat (Wetzlar, Germany). Sections were air-dried and returned to frozen storage at -80°C until time of immunohistochemical staining.

For immunostaining, slides were thawed and sections fixed for 10 minutes in ice-cold methanol. Sections were rinsed in phosphate-buffered saline, pH 7.3 (PBS) and permeabilized in 0.1% Triton X-100/PBS. Residual Triton surfactant was removed with a PBS rinse and sections were blocked with Mouse IgG Blocking Reagent according to kit manufacturer’s instructions (Mouse on Mouse Kit, Vector Laboratories, Burlingame, CA). Sections were incubated with primary antibody (diluted in PBS) against \( \alpha_1.2 \), calcineurin and \( \alpha \)-actinin (overnight, 4°C). After rinsing with PBS, bound primary antibody was detected with Alexa 488 or 555-labeled secondary antibody (1 hr, RT). Sections were rinsed in PBS and mounted with Vectashield (Vector Laboratories, Burlingame, CA). A Nikon (Eclipse TE2000-U) confocal microscope was used to scan the tissue sections viewed at 60X objective-lens magnification, and the images were obtained with the EZ-C1 version 2.0 software (Nikon Instruments).
**Peptides** – All but one peptide used were synthesized (>70% purity) and HPLC analyzed by the Protein Chemistry Technology Center at UT Southwestern Medical Center. The peptides were designed based on the primary amino acid sequence of the C-terminus of the cardiac L-type Ca\(^{2+}\) channel \(\alpha_1.2\) subunit (and corresponding to CT-8 GST fusion protein). The peptides overlapped by three residues as follows:

peptide 1976: TSLPRPCATPPATPGSRGWP (aa. 1976-1995 of \(\alpha_1.2\))

peptide 1993: GWPPQPQPIPTLRLEGADSSEK (aa. 1993-2012 of \(\alpha_1.2\))

peptide 2010: SEKLNSSFPSIHCWSWGEN (aa. 2010-2029 of \(\alpha_1.2\))

Calcineurin-inhibitory peptide was purchased from Sigma (#C-3937) and Santa Cruz (#sc-3055).

peptide AID: ITSFEEAKGLDRINERMPPRR (aa. 457-482 derived from autoinhibitory domain of rat brain calcineurin \(A_\alpha\))

**Reagents** — Antibodies were purchased from Alomone Labs (\(\alpha_1.2\) #ACC003), BD Biosciences (CnA #610259), Santa Cruz (PP2B-A #9070, AKAP79 #10764), Roche (HA #12CA5), Sigma (GST #A7340, \(\alpha\)-actinin #A7811), Invitrogen Molecular Probes (GFP #A11122), and Cell Signaling Technology (myc #2276). The phospho-specific Ser-1928 antibody was prepared as previously described\(^6\).
Figure Legends

Online Figure I: CnA- α1.2 interaction mapping. A. Potential PxIxIT motifs on α1.2 CT-4. Alignment of primary amino acid sequences of murine NFAT1 (mNFAT1), human NFAT3 (hNFAT3), rat brain α1.2, rabbit cardiac α1.2, and the mutated CT-4 construct. GST-pulldowns of recombinant CnA using CT-4 or CT-4 PSI→AAA revealed comparable binding of CnA to each fusion protein. The membrane was re-blotted with anti-GST to confirm equivalence of fusion protein concentrations. B. Competition-binding assays were performed with peptides spanning CT-8. Peptides were incorporated in molar excess (10 µM) relative to CnA/B (20 nM) and CT-8 (100 nM). C. Prediction of CT-8 secondary structure based on Robson-Garnier algorithm analysis of primary amino acid sequence. CT-8 comprises of aa. 1909-2029, with two distinct halves – aa. 1909-1968 comprised of one alpha-helix (cylinder), and aa. 1969-2029 containing no definitive secondary structure but containing potential PxIxIT motifs (underlined). Overlapping peptides (1976, 1993 and 2010) of 20 aa. length comprised of the α1.2 primary amino acid sequence are shown, with their respective PxIxIT motifs underlined. D. Schematic of CT-8 topology predicted by Robson-Garnier algorithm. CT-8 contains one alpha-helix (within 1943-1971) and an unstructured globular region (1969-2029). CT-8 truncations were used in pulldowns (E) from ventricular extracts. F. Immunoprecipitation experiments of myc-tagged α1.2 C-terminus constructs co-expressed in HEK 293 cells with GFP-CnAα. Immunoblots for GFP show CnA binds α1.2 only in the presence of aa. 1909-1969, consistent with GST pulldown results.

Online Figure II: CnA binds α1.2 with high affinity. Representative GST-pulldown experiment with recombinant CnA/B (15 nM) and increasing concentrations (7.5, 22.5 and 45 nM) of CT-8. After each pulldown, supernatant (S) was saved to compare unbound to bound CnA co-sedimenting with bead-immobilized GST-CT-8 pellet (P). CT-8:CnA = molar ratio of CT-8 to CnA.

Online Figure III: Isoproterenol-induced phosphorylation of endogenousα1.2. To test the efficacy and specificity of the anti-Ser-1928 phospho-specific antibody, we treated NRCMs with Iso (1 µM), a potent β-adrenergic receptor agonist. As expected, endogenous Ser-1928 was
minimally phosphorylated under basal conditions (0 hr), but robustly phosphorylated at all time points on exposure to Iso.

**Online Figure IV: Acute suppression of I\textsubscript{Ca,L} by CsA.** A. I-V relation depicting mean I\textsubscript{Ca,L} in the presence-vs-absence of CsA. CsA-induced suppression of I\textsubscript{Ca,L} elicited no shift in the steady-state I-V relation (n=8 myocytes from 3 hearts in each group). B. Mean data demonstrating that CsA-induced suppression of I\textsubscript{Ca,L} did not elicit changes in fast (τ1) or slow (τ2) time-constants of I\textsubscript{Ca,L} inactivation.
Tables

Online Table I. Amino acid residues in $\alpha_1.2$ GST-fusion proteins.

<table>
<thead>
<tr>
<th>Fusion</th>
<th>Rabbit cardiac $\alpha_1.2$</th>
<th>Rat brain $\alpha_1.2$</th>
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<tr>
<td>N-Term</td>
<td>1-154</td>
<td>1-124</td>
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<tr>
<td>Loop I</td>
<td>437-554</td>
<td>409-526</td>
</tr>
<tr>
<td>Loop II</td>
<td>785-930</td>
<td>754-901</td>
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<tr>
<td>Loop III</td>
<td>1196-1248</td>
<td>1165-1219</td>
</tr>
<tr>
<td>C-Term</td>
<td>1507-2171</td>
<td>1477-2140</td>
</tr>
<tr>
<td>CT-1</td>
<td>1507-1733</td>
<td>1477-1703</td>
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<tr>
<td>CT-4</td>
<td>1909-2171</td>
<td>1879-2140</td>
</tr>
<tr>
<td>CT-7</td>
<td>2030-2171</td>
<td>2000-2140</td>
</tr>
<tr>
<td>CT-8</td>
<td>1909-2029</td>
<td>1879-1999</td>
</tr>
<tr>
<td>CT-23</td>
<td>1622-1905</td>
<td>1592-1875</td>
</tr>
<tr>
<td>CT-B</td>
<td>1724-1847</td>
<td>1694-1817</td>
</tr>
<tr>
<td>CT-C</td>
<td>1834-1957</td>
<td>1804-1927</td>
</tr>
<tr>
<td>CT-D</td>
<td>1944-2067</td>
<td>1914-2037</td>
</tr>
<tr>
<td>CT-E</td>
<td>2054-2171</td>
<td>2024-2140</td>
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</table>

**PKA/PKC/PKG site**  
Ser 1928  
Ser 1898

Sequences listed in bold font were synthesized and studied here. Sequences not listed in bold are provided to facilitate comparison between rabbit cardiac and rat brain $\alpha_1.2$. 
Online Table II. Summary of reports in the literature on the effects of calcineurin on $I_{\text{Ca,L}}$.

<table>
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<tr>
<th>Author</th>
<th>Journal</th>
<th>Compound/Method</th>
<th>Effect</th>
<th>Species</th>
<th>Cell/Tissue</th>
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</thead>
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<tr>
<td>Studies that report inhibitory effects of calcineurin on $I_{\text{Ca,L}}$</td>
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</tr>
<tr>
<td>Chad JE and Eckert R</td>
<td><em>J. Physiol.</em> (1986) 378:31-51.</td>
<td>40 $\mu$g/mL PP2B dialysis</td>
<td>“small” ↓ $I_{\text{Ca,L}}$</td>
<td><em>Helix</em></td>
<td>neuron</td>
</tr>
<tr>
<td>Schuhmann K et al.</td>
<td><em>J. Gen. Physiol.</em> (1997) 110:503-513.</td>
<td>1 $\mu$g/mL PP2B diffusion (inside-out patch)</td>
<td>$\sim$50% ↓ $I_{\text{Ca,L}}$</td>
<td><em>Human</em></td>
<td>smooth muscle</td>
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<tr>
<td>Santana LF et al.</td>
<td><em>J. Physiol.</em> (2002) 544:57-69.</td>
<td>100 nM CsA</td>
<td>$\sim$33% ↑ $I_{\text{Ca,L}}$</td>
<td><em>Mouse</em></td>
<td>Ventriculocyte</td>
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<tr>
<td>Studies that report no effect of calcineurin on $I_{\text{Ca,L}}$</td>
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<tr>
<td>Frace AM et al.</td>
<td><em>J. Physiol.</em> (1993) 472:305-326.</td>
<td>$\mu$M inhibitory peptide</td>
<td>No effect on $I_{\text{Ca,L}}$</td>
<td><em>Frog</em></td>
<td>Cardiomyocyte</td>
</tr>
<tr>
<td>McCall E et al.</td>
<td><em>Circ. Res.</em> (1996) 79:1110-1121</td>
<td>5 $\mu$M FK-506</td>
<td>No effect on $I_{\text{Ca,L}}$</td>
<td><em>Rat</em></td>
<td>Ventriculocyte</td>
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<tr>
<td>duBell WH et al.</td>
<td><em>J. Physiol.</em> (1997) 501:509-516.</td>
<td>25 $\mu$M FK-506</td>
<td>No effect on $I_{\text{Ca,L}}$</td>
<td><em>Rat</em></td>
<td>Ventriculocyte</td>
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<tr>
<td>Zeilhofer HU et al.</td>
<td><em>Neurosci.</em> (2000) 95:235-241.</td>
<td>10 $\mu$M FK-506</td>
<td>No effect on $I_{\text{Ca,L}}$</td>
<td><em>Rat</em></td>
<td>GH3 cells</td>
</tr>
<tr>
<td>Yatani A et al.</td>
<td><em>J. Mol. Cell Cardiol.</em> (2001) 33:249-259.</td>
<td>1 $\mu$M CsA</td>
<td>No effect on $I_{\text{Ca,L}}$</td>
<td><em>Mouse</em></td>
<td>Ventriculocyte</td>
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<td>Studies that report activating effects of calcineurin on $I_{\text{Ca,L}}$</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mijares A et al.</td>
<td><em>J. Mol. Cell Cardiol.</em> (1997) 29:2067-2076.</td>
<td>15 mg/kg/day CsA</td>
<td>$\sim$25% ↓ $I_{\text{Ca,L}}$</td>
<td>Guinea pig</td>
<td>Cardiomyocyte</td>
</tr>
<tr>
<td>Norris CM et al.</td>
<td><em>Neurosci.</em> (2002) 110:213-225.</td>
<td>0.5 – 50 $\mu$M FK-506</td>
<td>$\sim$10 – 98% ↓ $I_{\text{Ca,L}}$</td>
<td>Rat</td>
<td>hippocampal cells</td>
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References


Online Figure I

A

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<tr>
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<tr>
<td>mNFAT1</td>
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<td>hNFAT3</td>
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<td>Brain α₁,1.2</td>
<td>FPSIHCSSWSSE</td>
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<tr>
<td>Cardiac α₁,1.2 (CT-4)</td>
<td>FPSIHCGSWSG</td>
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<td>CT-4 PsI-AAA</td>
<td>FAAHCGSWSG</td>
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B

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<td>CnA/B</td>
<td>1976</td>
</tr>
<tr>
<td></td>
<td>1993</td>
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<tr>
<td></td>
<td>2010</td>
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</tbody>
</table>

[Image of a gel showing bands for CnA and GST]
Peptides

1976-1995: TSLPRPCATPPATPGSRGW
1993-2012: GWPPQPIPTLRLEGADSEK
2010-2029: SEKLNSSFPSIHCWSGEN

alpha-helix shown in blue
Potential PxIxIT motifs are underlined
Residue Calcineurin Binding

1909-2029  
1909-1946  
1909-1971  
1943-2029  
1969-2029  
CT-8 helix-Pro

Online Figure I
Online Figure I
### Myc-tagged construct

<table>
<thead>
<tr>
<th>Description</th>
<th>a.a</th>
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<tbody>
<tr>
<td><strong>CT-8</strong> Entire $\alpha_1.2$ C-terminus</td>
<td>1507-2160</td>
<td></td>
</tr>
<tr>
<td><strong>CT-8</strong> Encoding half of CT-8; lacking alpha-helix</td>
<td>1507-1908</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2045-2160</td>
<td></td>
</tr>
<tr>
<td><strong>CT-8</strong> 1969-2160</td>
<td>1969-2160</td>
<td></td>
</tr>
</tbody>
</table>

**anti-GFP**

- 1969-2160
- 1507-2160
- 2045-2160
- 1507-1908

**anti-myc**

- (heavy chain) →

---

**Online Figure I**
Online Figure II

CT-8:CnA

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CnA

- 75
- 50

GST
Online Figure III

ISO (hr): 0 1 2 4 6 12

Ser-1928P

α₁.2

- 250 kD - 250 kD