Physical and Functional Interaction Between Calcineurin and the Cardiac L-Type Ca²⁺ Channel

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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 $\alpha_1 1.2$. At the C terminus, calcineurin bound specifically at amino acids 1943 to 1971, adjacent to a well-characterized protein kinase (PK)A/PKC/PKG phospho-acceptor site Ser1928. In vitro assays demonstrated that calcineurin can dephosphorylate $\alpha_1 1.2$. Channel function was increased in voltage-clamp recordings of $I_{Ca,L}$ 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 $I_{Ca,L}$. These data reveal direct physical interaction between the LTCC and calcineurin in heart. Furthermore, they demonstrate that calcineurin induces robust increases in $I_{Ca,L}$ and highlight calcineurin as a key modulator of pathological electrical remodeling in cardiac hypertrophy.

Keywords

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) ($Ca_V1.2$) 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 ($I_{Ca,L}$) 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 $I_{Ca,L}$ inhibition by PKG.[7] However, despite a growing understanding of the role of phosphorylation in the regulation of $I_{Ca,L}$, 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 reported previously 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.

Materials and Methods

Coimmunoprecipitation

Rat left ventricle was homogenized on ice using a Dounce homogenizer in buffer containing (mmol/L) 10 Tris (pH 7.4), 10 EDTA, 10 EGTA, 1% sucrose, supplemented with Mini-Complete protease inhibitor (Roche). Following gentle centrifugation (5000 rpm, 3 minutes) the supernatant was subjected to high-speed ultracentrifugation (200 000g, 15 minutes) to collect the cytosolic fraction. The pellet was rehomogenized in membrane extraction buffer containing (mmol/L) 10 Tris-HCL (pH 7.4), 20 EDTA, 10 EGTA, 150 NaCl, 1% Triton X-100, and protease inhibitors and centrifuged as before to collect the membrane fraction (supernatant).

Statistical Methods

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 available 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 calcineurin is a component of the LTCC macromolecular complex, we immunoprecipitated $\alpha_1 1.2$ from rat ventricular lysates and probed for CnA. A strong

band migrating at ≈61 kDa was readily identified (Figure 1). As a positive control, we immunoblotted for $\alpha_1 1.2$, 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 $\alpha_1 1.2$. Immunocytochemical studies revealed colocalization of CnA and $\alpha_1 1.2$ at murine myocyte Z bands (data not shown).

Direct Interaction Between $\alpha_1 1.2$ and Calcineurin

To map the CnA binding site, we engineered overlapping glutathione *S*-transferase (GST) fusion proteins spanning the intracellular domains of $\alpha_1 1.2$. The C terminus (CT) was further subdivided into numbered CT fragments (Figure 2A; Online Table I). Equal quantities of $\alpha_1 1.2$ 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 $\alpha_1 1.2$ 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 $\alpha_1 1.2$ sequence. The N terminus of $\alpha_1 1.2$ also pulled down calcineurin, suggesting that calcineurin and $\alpha_1 1.2$ may have multiple sites of interaction.

To test whether the interaction between calcineurin and $\alpha_1 1.2$ is direct or mediated by intervening proteins, GST-$\alpha_1 1.2$ fusion proteins were incubated with purified recombinant 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 interactions 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 $\alpha_1 1.2$ 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 partners (Online Figure I, A). We tested whether one such potential motif was responsible for calcineurin binding to $\alpha_1 1.2$ 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 sequence of CT-8 suggested the presence of 2 distinct domains: an N-terminal domain (amino acids 1909 to 1968) containing an α-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 responsible for CnA binding, we analyzed the N-terminal α-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 α-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 $\alpha_1 1.2$.

Finally, as heterologous expression of mammalian proteins in bacteria often leads to early termination events during synthesis (Figure 2B), we expressed $\alpha_1 1.2$ CT constructs fused to myc-epitope tags in mammalian cells. Immunoprecipitation using anti-myc antibodies in

HEK-293 cells that coexpressed calcineurin fused to GFP and probed for GFP-CnA revealed a specific band at ≈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$1.2, as required for calcineurin binding.

Calcineurin Binds $\alpha_1$1.2 CT With High Affinity

To obtain a semiquantitative estimate of the affinity of interaction between $\alpha_1$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 $\alpha_1$1.2 and CnA occurs at relatively high (approximately nanomolar) affinity.

CnA Regulatory Region Is Required for $\alpha_1$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.[13] 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-$\alpha_1$1.2 Is a Calcineurin Substrate

Next, we set out to determine whether calcineurin recognizes $\alpha_1$1.2 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 $\alpha_1$1.2, which binds CnA and can be phosphorylated by PKC[5] but does not contain consensus sequences for PKA phosphorylation, was not phosphorylated (Figure 4). PKC phosphorylated CT-8 and the N terminus but did not phosphorylate CT-B (Figure 4C).

We then incubated the ^{32}P-phosphorylated GST fusion proteins with purified recombinant calcineurin (CnA/B). Here, we observed marked decreases in the phosphorylation of $\alpha_1$1.2 fragments 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 $\alpha_1$1.2, which calcineurin

recognizes as substrate (Figure 4C). Together, these data demonstrate that calcineurin is capable of recognizing $\alpha_1 1.2$ as a substrate in vitro.

To corroborate these findings in a more physiological setting, we tested whether calcineurin targets endogenous $\alpha_1 1.2$ 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 $\alpha_1 1.2$ Ser1928 and point to the presence of additional calcineurin target sites on $\alpha_1 1.2$ 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% ($\pm 9\%$, $P<0.05$; n=100 AdCnA*, n=100 AdGFP) increase in cell cross-sectional area (Figure 5A), increased sarcomeric organization, and upregulated ANF expression (data not shown). Recordings of $I_{Ca,L}$ in AdCnA*-infected myocytes revealed a 26% ($\pm 4\%$, $P<0.05$; n=10 AdCnA*, n=6 AdGFP) increase in $I_{Ca,L}$ density with no significant shift in the steady-state current–voltage (I–V) relationship (Figure 5B). Hypertrophy-associated increases in $I_{Ca,L}$ exceeded that expected from simple increases in cell size, because current density was significantly increased.

Inhibition of Calcineurin Diminishes $I_{Ca,L}$

Calcineurin-dependent increases in $I_{Ca,L}$ 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 $I_{Ca,L}$ 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 $I_{Ca,L}$ was measured (0.1 Hz) at test potentials between −30 and +50 mV. Under these conditions, addition of 10 µmol/L CsA significantly inhibited $I_{Ca,L}$ 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) $I_{Ca,L}$ 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 $I_{Ca,L}$ are mediated by calcineurin suppression rather than by nonspecific actions of either compound.

CsA inhibited $I_{Ca,L}$ without inducing a shift in the steady-state I–V relationship (Online Figure IV, A). Moreover, the kinetics of $I_{Ca,L}$ 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 $I_{Ca,L}$ 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[15]) (46.1±7%, n=3) (Figure 6D). These data, then, provide strong evidence that PKA and calcineurin activate the channel independently.

Inhibition of $I_{Ca,L}$ by CsA Is Calcineurin-Dependent

To determine whether the inhibitory effects of CsA on $I_{Ca,L}$ are calcineurin-dependent, we studied $I_{Ca,L}$ 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 $I_{Ca,L}$ in the presence of BAPTA (because calcineurin is inactivated by elimination of intracellular Ca^{2+}). $I_{Ca,L}$ 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 µmol/L CsA did not diminish $I_{Ca,L}$ (Figure 7A); $I_{Ca,L}$ declined by only 2.5% ($\pm0.8\%$, n=7, P=NS) in the presence of intracellular BAPTA as compared to 44% ($\pm5\%$, 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 ($21\pm5\%$, 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 $I_{Ca,L}$ ($3.0\pm0.8\%$ 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% ($\pm3\%$, 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; $1.7\pm4\%$, n=4, P=NS) or no peptide at all ($6.1\pm2.5\%$, 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 ($2.6\pm1.6\%$, n=5, P=NS) or with intracellular BAPTA ($7.4\pm0.8\%$, n=4, P=NS) (Figure 8D), consistent with a specific requirement for calcineurin inhibition.

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 $\alpha_1 1.2$; (3) calcineurin recognizes $\alpha_1 1.2$ 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 $\alpha_1 1.2$ 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 $I_{Ca,L}$.[18] Here, we demonstrate that calcineurin binds to both the N terminus and CT of $\alpha_1 1.2$ 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.[5,6] 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.[19,20] PKC-dependent mechanisms are similarly complex, acting on 2 threonine residues at the N terminus of $\alpha_1 1.2$, as well as at Ser1928.[5] In the case of N-terminal phosphorylation, an initial transient activation of the channel is followed by sustained inhibition.[21] Furthermore, a recent report mapped PKG-mediated inhibition of heterologously expressed L-type channel activity to Ser496 on the β_{2a} subunit.[7] 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[8,9] and PP1[10] 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 $\alpha_1 1.2$ close to a site where PP2A binds.[8] Some studies have suggested that PP2A inhibits Ca^{2+} channel function,[22] 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 CsA2 or genetically via RCAN1 overexpression,[23] 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 $\alpha_1 1.2$ 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.[24] 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.[25–27] Calcineurin also regulates voltagegated 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.[28] 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.[29] 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.[30,31]

We[2] (also this report) and others document relatively modest increases in $I_{Ca,L}$ during hypertrophy. In many species, membrane impedance is relatively high during phase 2 of the action potential, so modest changes in $I_{Ca,L}$ may have important effects on action potential morphology and duration. Consistent with this, computational analyses[32] of $I_{Ca,L}$ demonstrated that as little as 22% increase in $I_{Ca,L}$ density induced significant depolarization of the phase 2 plateau and delayed phase 3 repolarization (data not shown). Furthermore, entry of a small amount of Ca^{2+} via L-type channels triggers the release of much larger amounts of Ca^{2+} from intracellular stores. As a result, modest changes in $I_{Ca,L}$ (mediated Ca^{2+} flux such as those elicited by calcineurin) are amplified within the cell.

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 $\alpha_1 1.2$ subunit protein. However, our pulldown experiments revealed equivalent binding of calcineurin to $\alpha_1 1.2$ 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 $\alpha_1 1.2$ 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 β_{2a} subunit inhibits $I_{Ca,L}$. Finally, actions mediated indirectly by downstream targets of calcineurin may exist.[33]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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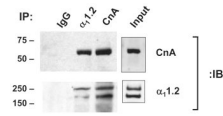
Figure 1.
Calcineurin associates with L-type Ca^{2+} channel. Immunoprecipitations (IP) of $\alpha_1 1.2$ and CnA from rat ventricular lysate and immunoblots (IB) for both. IgG used as a negative control.
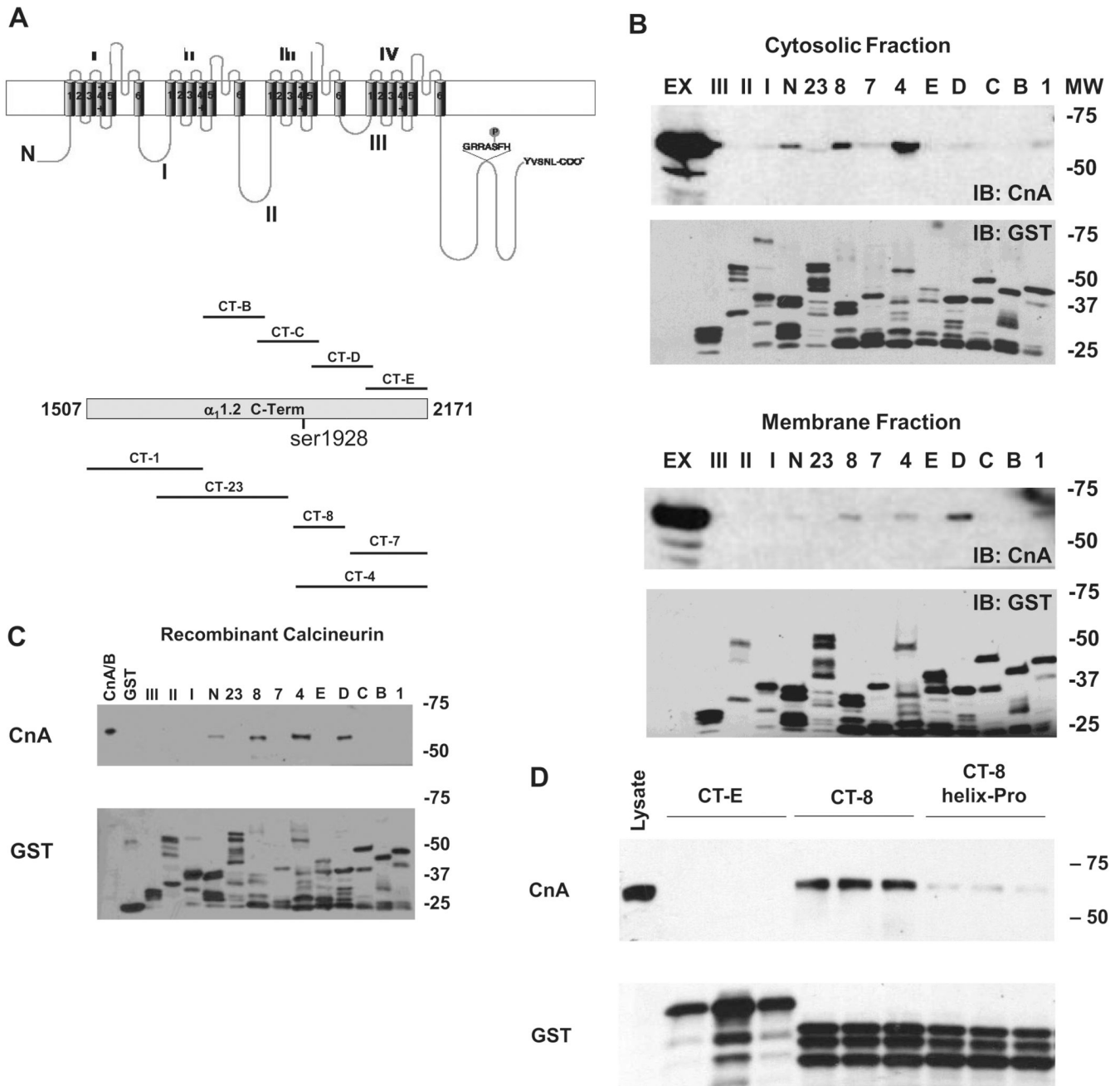
Figure 2.
Calcineurin binds directly to CT and N terminus of $\alpha_1 1.2$. A, Schematic diagram of $\alpha_1 1.2$. The CT was further subdivided into overlapping regions. GRRASFH contains Ser1928. B, Representative GST-pulldown experiments from rat ventricular lysate probed for CnA reveal binding to CT-4 and CT-8, and variable binding to CT-D and N terminus. Ex indicates tissue-extract input. C, GST pulldown with recombinant calcineurin A and B subunits (CnA/B). CnA/B indicates input. D, Pulldown experiments with mutant CT-8 containing a single proline insertion in the α-helix.
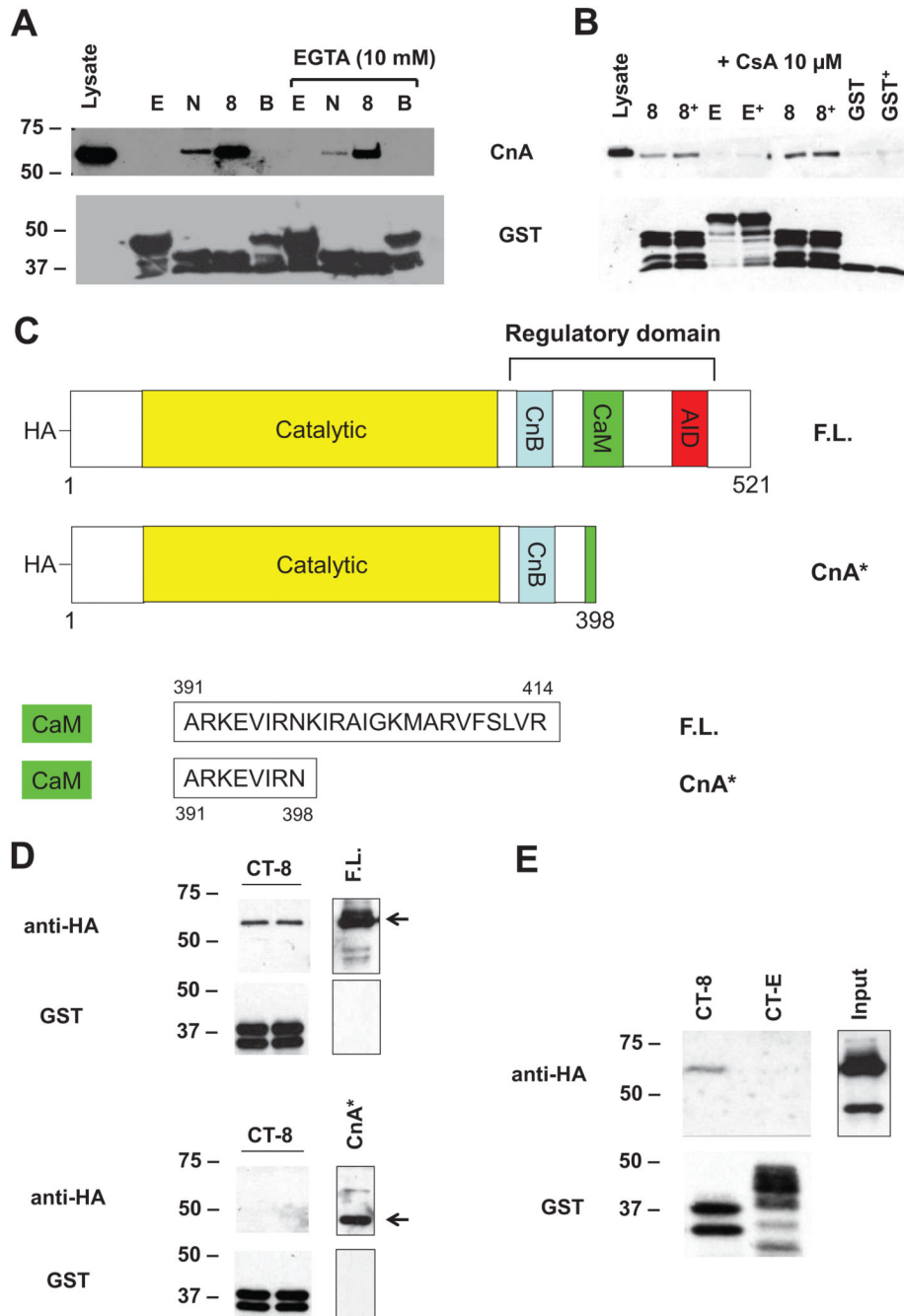
Figure 3.
$\alpha_1 1.2$ binds calcineurin regulatory region. Representative GST-pulldown experiment using ventricular lysates prepared in the presence vs absence of 10 mmol/L EGTA (A) or 10 µmol/L CsA (B) revealing equivalent interaction of endogenous CnA. C, Schematic representation of F.L. and constitutively active (CnA*) calcineurin constructs. CnA* is truncated at amino acid 398 and lacks the AID and most of the CaM domain. CnB indicates calcineurin B–binding domain; CaM, calmodulin-binding domain. GST pulldowns with lysates from HEK-293 cells expressing either F.L. or CnA* (D) or coexpressing both F.L. and CnA* (E).
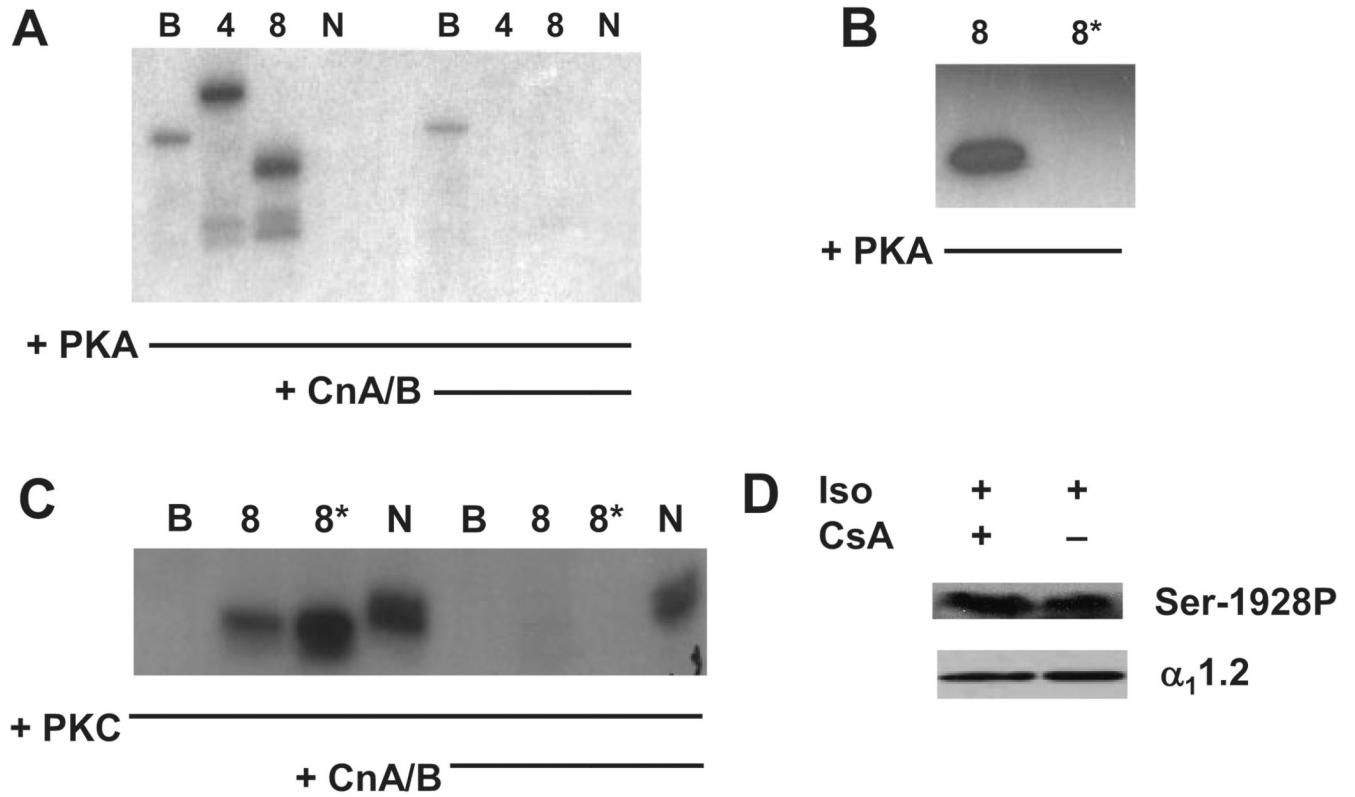
Figure 4.
Calcineurin recognizes phospho-$\alpha_1$1.2 as substrate. Representative autoradiogram of $\alpha_1$1.2 GST fusion proteins phosphorylated with recombinant catalytic PKA (A) or PKC (C) and subsequently treated with recombinant calcineurin (CnA/B). B, Representative autoradiogram demonstrating that PKA does not phosphorylate mutant CT-8 containing a S1928A substitution (CT-8*). D, Representative phosphospecific $\alpha_1$1.2 Ser1928 Western blot of NRCMs treated with Iso and/or CsA.
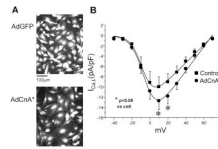
Figure 5.
Activated calcineurin increases $I_{Ca,L}$. A, Neonatal rat cardiomyocytes were infected with adenovirus expressing constitutively active calcineurin (AdCnA*) or GFP (AdGFP) and visualized with fluorescent tracer (CellTracker). AdCnA* led to the expected increase in cell size and associated myocyte hypertrophy. B, Voltage-clamp recordings of $I_{Ca,L}$ at increasing membrane potentials. AdCnA* was associated with an 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.
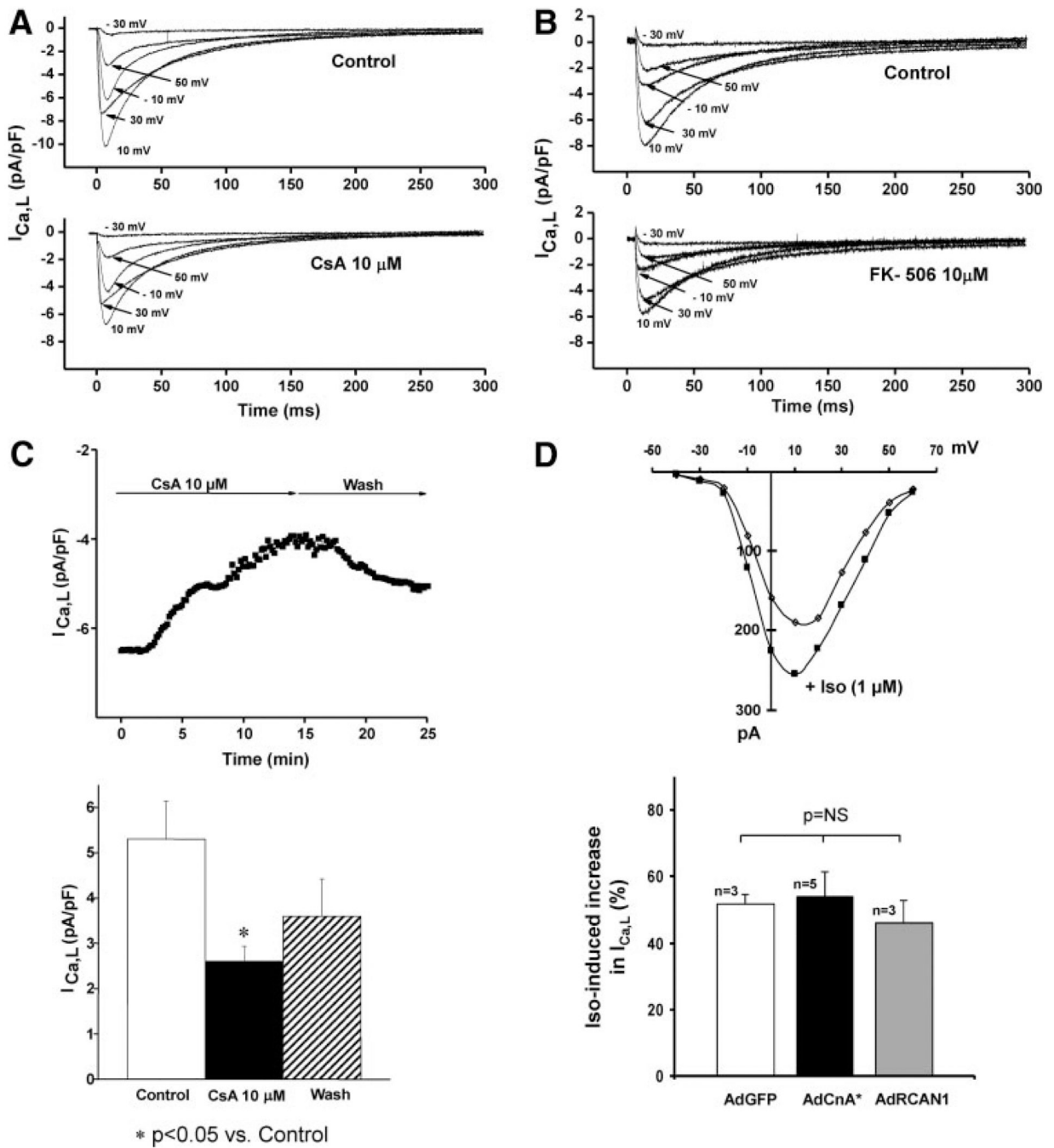
Figure 6.
Acute suppression of calcineurin diminishes $I_{Ca,L}$. Representative whole-cell voltage-clamp measurements of $I_{Ca,L}$ in dissociated ventricular myocytes before and after calcineurin suppression by 2 structurally distinct inhibitors, CsA (10 µmol/L) (A) and tacrolimus (FK-506; 10 µmol/L) (B). C, Representative recording depicting time course of CsA-induced $I_{Ca,L}$ inhibition and partial reversibility on drug washout. Mean data from 4 experiments are shown. D, Representative I–V curve and cumulative (bar graph) data showing equivalent increases in $I_{Ca,L}$ after exposure to Iso (1 µmol/L).
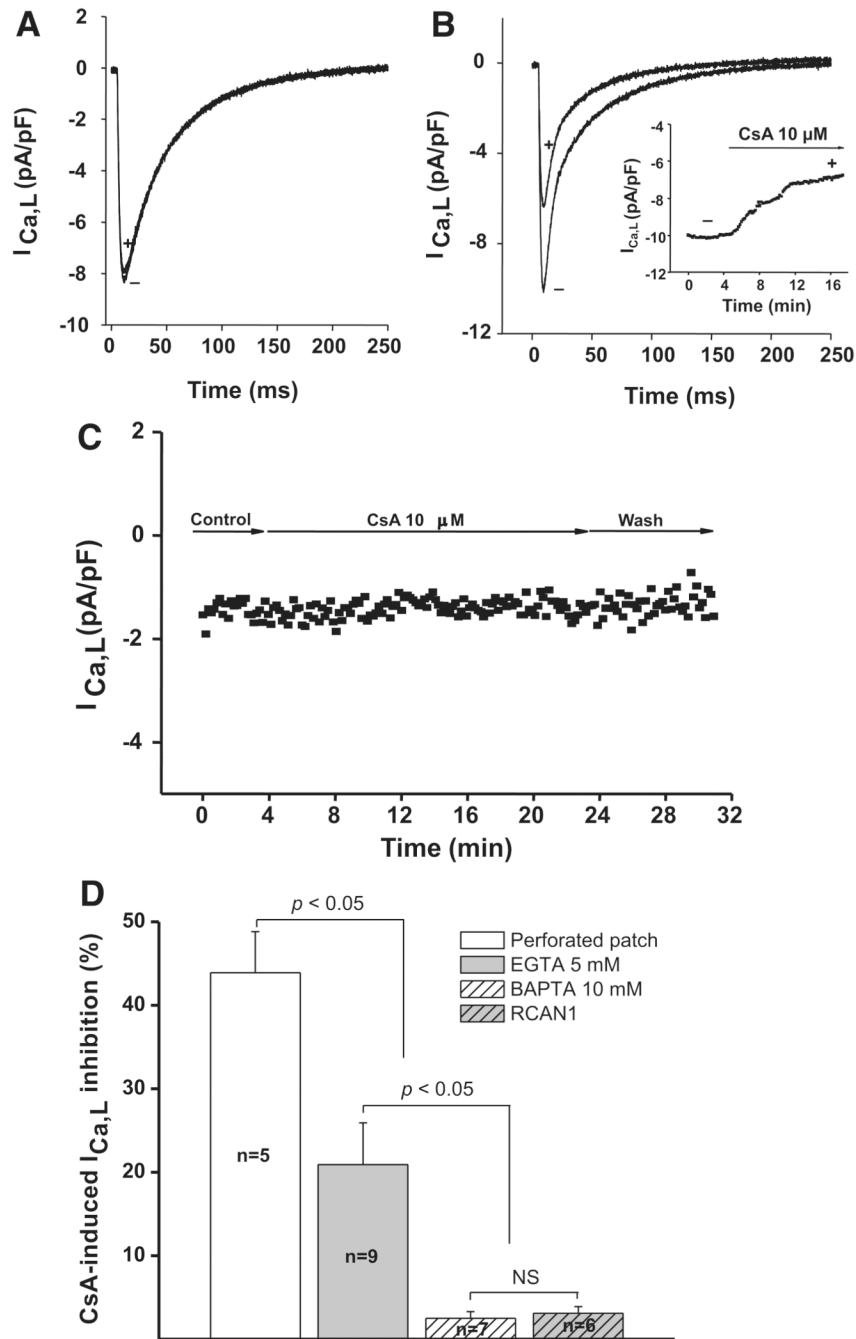
Figure 7.
CsA inhibits $I_{Ca,L}$ in a calcineurin-dependent manner. A, Whole-cell voltage-clamp recordings of $I_{Ca,L}$ in dissociated ventricular myocytes treated with 10 mmol/L BAPTA. In the presence of Ca^{2+} chelation, and consequent calcineurin inactivation, 10 µmol/L CsA had no effect on $I_{Ca,L}$ (10 mmol/L BAPTA [+]; control [−]). B, Representative recordings of $I_{Ca,L}$ density from dissociated ventricular myocytes treated with 5 mmol/L EGTA. With incomplete Ca^{2+} buffering, 10 µmol/L CsA induced a partial reduction in $I_{Ca,L}$ (5 mmol/L EGTA [+]; control [−]). C, Representative time course of $I_{Ca,L}$ recorded from a ventricular myocyte dissociated from cardiomyocyte-overexpressing RCAN1 transgenic mice. D, Mean data of percentage inhibition of $I_{Ca,L}$ induced by 10 µmol/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, $P<0.05$).
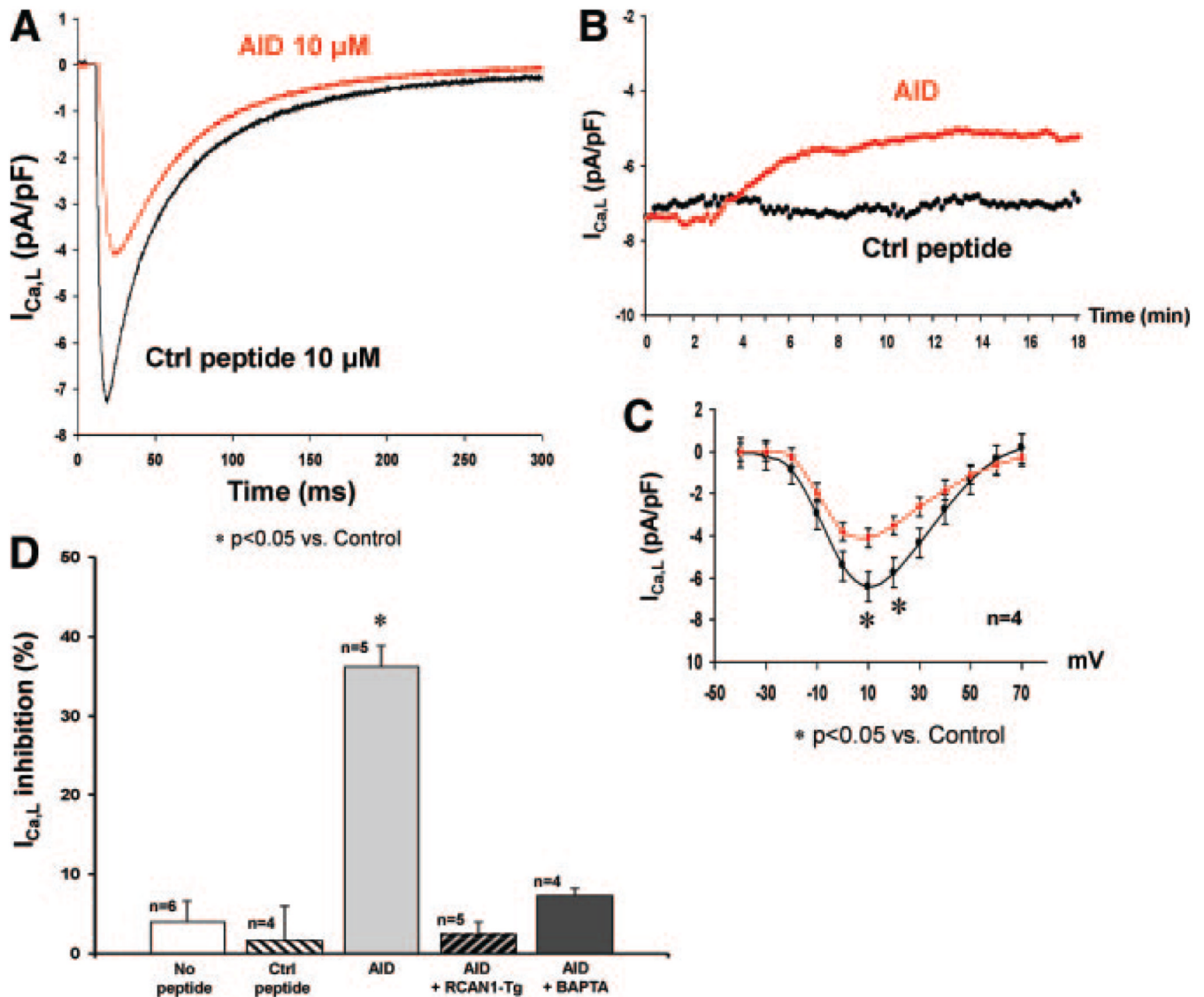
Figure 8.
Targeted inhibition of calcineurin decreases $I_{Ca,L}$. A, Representative peak $I_{Ca,L}$ densities (+10 mV) and time course (B) recorded from ventricular myocytes after peptide dialysis of 10 µmol/L control peptide (2010, black line) or AID (red line) peptide. C, I–V relationship demonstrating 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% ($\pm3\%$, n=5, $P<0.05$) decrease in $I_{Ca,L}$ density vs control peptide ($1.7\pm4.3\%$, n=4, $P<0.05$) or no peptide ($4.1\pm2.5\%$, n=6, $P<0.05$). AID had no effect on $I_{Ca,L}$ density in RCAN1 transgenic myocytes ($2.6\pm1.6\%$, n=5, $P<0.05$) or in the presence of BAPTA ($7.4\pm0.8\%$, n=4, $P<0.05$). All experiments were performed on myocytes isolated from at least 3 different mice of the same strain.