Targeted inhibition of calcineurin prevents agonist-induced cardiomyocyte hypertrophy

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Cardiac hypertrophy is a major predictor of future morbidity and mortality. Recent investigation has centered around identifying the molecular signaling pathways that regulate cardiac myocyte reactivity with the goal of modulating pathologic hypertrophic programs. One potential regulator of cardiomyocyte hypertrophy is the calcium-sensitive phosphatase calcineurin. We show here that calcineurin enzymatic activity, mRNA, and protein levels are increased in cultured neonatal rat cardiomyocytes by hypertrophic agonists such as angiotensin II, phenylephrine, and 1% fetal bovine serum. This induction of calcineurin activity was associated with an increase in calcineurin Aβ (CnAβ) mRNA and protein, but not in CnAα or CnAγ. Agonist-dependent increases in calcineurin enzymatic activity were specifically inhibited with an adenosine expressing a noncompetitive peptide inhibitor of calcineurin known as cain [Lai, M. M., Burnett, P. E., Wolosker, H., Blackshaw, S. & Snyder, S. H. (1998) J. Biol. Chem. 273, 18325–18331]. Targeted inhibition of calcineurin with cain or an adenovirus expressing only the calcineurin inhibitory domain of AKAP79 attenuated cardiomyocyte hypertrophy and atrial natriuretic factor expression in response to angiotensin II, phenylephrine, and 1% fetal bovine serum. These data demonstrate that calcineurin is an important regulator of cardiomyocyte hypertrophy in response to certain agonists and suggest that cyclosporin A and FK506 function to attenuate cardiac hypertrophy by specifically inhibiting calcineurin.

Cardiac hypertrophy is an adaptive response to various intrinsic and extrinsic stimuli. This hypertrophy can be broadly defined as increased two-dimensional cell surface area and re-expression of fetal genes. Prolonged hypertrophy is associated with decompensation, dilated cardiomyopathy, arrhythmia, fibrotic disease, sudden death, or heart failure (1). To elucidate the mechanisms underlying hypertrophy, investigators have used cultured rat neonatal ventricular cardiomyocytes because they respond to various stimuli and undergo hypertrophic growth similar to the adult myocardium (2).

Treatment of cultured cardiomyocytes with hypertrophic agonists such as phenylephrine (PE), angiotensin II (AngII), and endothelin-1 (ET-1) results in activation of G protein-coupled receptors (GPCR) and down-stream pathways such as the mitogen-activated protein kinase (MAPK) signaling cascade (reviewed in ref. 3). Treatment of cultured cardiomyocytes with pharmacologic inhibitors or transient transfection of activated and dominant negative MAPK-signaling factors has implicated an important role for extracellular signal-regulated kinases in cardiomyocyte hypertrophy (reviewed in refs. 4 and 5). However, other studies have disputed the importance of extracellular signal-regulated kinase signaling in cardiomyocyte hypertrophy (6–10) and have instead implicated important roles for p38 MAPK (10–12) or c-Jun N-terminal kinase (9, 13, 14). Despite the differing accounts, it is likely that each of the MAPK-signaling branches plays a role in some aspect of agonist-induced cardiomyocyte hypertrophy (15).

We have recently implicated another intracellular signaling pathway in cardiomyocyte hypertrophy through the calcium-dependent phosphatase calcineurin (16). Cardiac-specific expression of a constitutively active form of calcineurin A in transgenic mice generated hypertrophy that progressed to heart failure (16). The pharmacologic inhibitors of calcineurin, cyclosporin A, and FK506, prevent hypertrophy of cultured neonatal cardiomyocytes induced by AngII or PE (16). Similarly, cyclosporin A and FK506 were shown to inhibit cardiac hypertrophy in transgenic mouse models of hypertrophic cardiomyopathy and in pressure-overloaded rat hearts (17). Although the beneficial effects of these drugs were attributed to calcineurin inhibition, each has multiple biological effects that are independent of calcineurin.

To evaluate the necessity of calcineurin in cardiomyocyte hypertrophy, without the side-effects of cyclosporin A or FK506, we targeted calcineurin by expressing two distinct noncompetitive peptide inhibitors by using adenoviral-mediated gene transfer [Adcain and AdAKAP (A-kinase anchoring protein)]. These constructs attenuated agonist-induced calcineurin activity, cardiomyocyte hypertrophy, and elevated atrial natriuretic factor (ANF) expression.

Materials and Methods

Primary Cardiomyocytes Cultures, Transfections, and Immunocytochemistry. Cardiomyocyte cultures were isolated by enzymatic dissociation of 1- to 2-day-old neonatal rat hearts as described previously (18). After isolation, cardiomyocytes were cultured overnight in M199 media supplemented with 15% fetal bovine serum (FBS), penicillin/streptomycin (100 units/ml), and L-glutamine (2 mM) and thereafter cultured in serum-free media supplemented with Nutridoma (Boehringer Mannheim). Cardiomyocytes were plated on gelatinized 6- and 10-cm plates at a preadherent density of 0.5–1.0 and 1.5–2.5 million cells, respectively.

Cardiomyocytes were prepared for immunocytochemistry as described previously (16). Anti-α-actinin antibody (Sigma) and anti-ANF polyclonal antiserum (Peninsula Laboratories) were added at dilutions of 1:800 and 1:300, respectively. Alternatively, cells were hybridized with anti-flag monoclonal M2 antibody (Sigma) at a dilution of 1:500. Nuclear staining was performed with 0.5 μg/ml bisbenzimide.

Transfections were performed by the calcium phosphate method with 8 μg of a luciferase reporter containing multimeric nuclear factor of activated T cells (NFAT)–binding sites, 2 μg of a plasmid encoding NFAT3, and 2 μg of the mammalian pECE–flag expression vector.

Measurements of cell surface area was performed on α-actinin-labeled cardiomyocytes by using video edge detection and NIH image analysis software, similar to previous descriptions (10, 13).

Plasmid Construction. A cDNA fragment corresponding to the 3′ of cain was obtained as a mouse expressed sequence tag. A 582-bp fragment corresponding to amino acids 1989–2182 was

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Abbreviations: AKAP, A-kinase anchoring protein; AngII, angiotensin II; PE, phenylephrine; ANF, atrial natriuretic factor; CnAα, β, or γ, calcineurin Aα, β, or γ; ET-1, endothelin-1; GPCR, G protein-coupled receptors; MAPK, mitogen-activated protein kinase; RT-PCR, reverse transcription-PCR; NFAT, nuclear factor of activated T cells.

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generated by PCR and subcloned into the SmaI site of the pECE-flag. To generate recombinant adenovirus, the flag-cain cDNA fragment was subcloned into pACCMVpLPa (19). A cDNA fragment encoding amino acids 60–358 of AKAP79 (lacking the protein kinase C- and protein kinase A-binding sites) was generated by PCR and subcloned into pECE-flag, which was then subcloned into pACCMVpLPa (gift from John D. Scott, Vollum Institute, Portland, OR).

Replication-Deficient Adenovirus Production. Adβgal is a replication-deficient, E1A-deleted, recombinant adenovirus expressing a nuclear localized β-galactosidase gene (20). To generate recombinant adenovirus, the pACCMVpLPα-cain or AKAP plasmids were cotransfected with pM17 in HEK293 cells (19). Each adenovirus was plaque purified, expanded, and titered by detection of visible plaques in a HEK293 monolayer by agarose overlays (21). Cardiomyocyte cultures were infected at a multiplicity of infection of 100 plaque-forming units/cell in 2 ml (6-cm dish) or 5 ml (10-cm dish) of DMEM (GIBCO/BRL) supplemented with 2% FBS for 2 hr at 37°C in a humidified, 5% CO2 incubator. Under these conditions, ∼98% of the cells were infected (assessed by flag antibody-directed immunocytochemistry).

Western Blotting. Protein extracts for Western blotting were generated from two, 10-cm plates of cardiomyocytes per lane in extraction buffer (20 mM sodium phosphate/150 mM NaCl/2 mM MgCl2/0.1% Nonidet P-40/10% glycerol/10 mM NaF/0.1 mM sodium orthovanadate/10 mM sodium pyrophosphate/1 mM DTT, 10 μg/ml leupeptin/10 μg/ml aprotinin/10 μg/ml pepstatin/10 μg/ml N-tosylphenylalanyl chloromethyl ketone/10 μg/ml N-tosyllysyl chloromethyl ketone). Samples were subjected to SDS-PAGE, transferred to poly(vinylidene difluoride) membrane, and immuno-detected with the ECF kit (Amer sham). Antibodies used in this study were against calcineurin Aα (CnAα) and CnAβ (Transduction Laboratories, Lexington, KY), or a isoform-specific CnAα, CnAβ, or CnAδ antibody (Santa Cruz Biotechnology), or a glyceraldehyde-3-phosphate dehydrogenase antibody (Research Diagnostics, Flanders, NJ). Blots were quantified for fluorescence with a Storm 860 PhosphorImager (Molecular Dynamics).

Reverse Transcription–PCR (RT-PCR) mRNA Quantitation. Analysis of mRNA levels for CnAα, CnAβ, CnAγ, ANF, and L7 (ribosomal protein, control) were performed with primers designed to detect either mouse or rat gene products. CnAα used primers 5′-CGGCGATGTTGAGCTAGT and 5′-GTCCCGTGTTCT-GTGTTGA, resulting in a 242-bp and 212-bp product (two spliced forms). CnAβ used primers 5′-CCAGGAGTGTGCTACTAGT and 5′-GTCCCGTGTTCT-GTGTTGA, resulting in a 242-bp and 212-bp product. CnAγ used primers 5′-TCCACAGCCACACTCCAC and 5′-CCAGGCTTCTTCTTATGTC, generating a 279-bp fragment. ANF detection used primers 5′-GCCCTGAGCCGACAGGCCG and 5′-GGAGCTTAGGAGGCG, resulting in a 244-bp and 214-bp product (two spliced forms). CnAβ used primers 5′-CACTCCACAGGCCACACTCCAC and 5′-CCAGGCTTCTTCTTATGTC, generating a 279-bp fragment. L7 detection used primers 5′-GGAGCTCATCTATGAGGAGGC and 5′-GAGAGCCAGGACGCT-CAGCAG, generating a 240-bp fragment. RT-PCR reactions were performed with 1 μg of total RNA in the presence of [α32P]dCTP as recommended by the manufacturer (Titan one tube RT-PCR, Boehringer Mannheim), followed by 22 cycles of PCR amplification.

Calcineurin Phosphatase Activity Assay. Cardiomyocytes were collected in 1.5 ml of trypsin-EDTA (GIBCO/BRL), pelleted, and lysed in 100 μl of calcineurin assay buffer (BioMol, Plymouth Meeting, PA). The Quantzyme Assay System AK-804 was performed by using 3 μg of protein according to the manufacturer’s procedure (BioMol). Calcineurin phosphatase activity was measured spectrophotometrically by detecting free-phosphate released from the calcineurin-specific RII phosphopeptide.

Statistical Analysis. Data are presented as means ± SE. The data were evaluated for significance by using a nonpaired Student’s t test or one-way ANOVA followed by Bonferroni multiple comparison test using INSTAT software (Graphpad, San Diego, CA).

Results

Calcineurin Is Activated by Hypertrophic Agonist in Cultured Cardiomyocytes. Because cardiomyocyte hypertrophy is associated with enhanced calcium handling, we investigated the enzymatic activity of calcineurin (catalytic activated) in cultured cardiomyocytes treated with AngII, PE, or 1% FBS. We determined that calcineurin activity was significantly increased by AngII (3.2 ± 0.3 fold), PE (4.1 ± 0.1 fold), and 1% FBS (3.1 ± 0.1 fold) (P < 0.0001). PE-activated phosphatase activity was significantly attenuated by cotreatment with 500 ng/ml of cyclosporin A (P < 0.001) (Fig. 1A).

There are three known genes that encode catalytic calcineurin A protein, CnAα, CnAβ, and CnAγ (22). Isoform-specific RT-PCR demonstrated a hypertrophy-associated induction in only CnAβ mRNA levels (2.5-fold), whereas no changes were
Migration differences due to alternate splicing in CnA.

Cyclosporin A and FK506 inhibit calcineurin (CnA) phosphatase activity, whereas Adcain infection prevented this increase. The data represent two independent experiments performed in triplicate. **P < 0.0001 vs. Adcain control; ***P < 0.0001 vs. Adcain + AngII; †, P < 0.0001 vs. Adcain + PE; ††, P = 0.0001 vs. Adcain + 1% FBS.

Cain attenuates cardiomyocyte hypertrophy in response to AngII, PE, and 1% FBS (Fig. 4E–H). A decrease in NFATx3 luciferase activity was observed in Adcain-infected cardiomyocytes (Fig. 4E and H). Adenoviral infection with either Adβgal (control) or Adββgal induced hypertrophy (Fig. 4E–H). The data demonstrated that Adcain infection significantly reduced the increase in calcineurin activity induced by each agonist (P < 0.0001) (Fig. 3).

Targeted inhibition of calcineurin prevents cardiomyocyte hypertrophy. To establish the necessity of calcineurin in cardiomyocyte hypertrophy, we investigated the effects of Adcain infection on the morphological changes induced by AngII, PE, and 1% FBS over 48 hr. Cardiomyocytes were infected with Adcain (Fig. 4I–L), Adββgal (Fig. 4E–H), or simply left in serum-free media (Fig. 4A–D). The data demonstrated that Adcain infection significantly reduced the increase in calcineurin activity induced by each agonist (P < 0.0001) (Fig. 3).

The Cain Inhibitory Peptide Attenuates Calcineurin Activity and NFAT Transcriptional Responses. Cyclosporin A and FK506 inhibit calcineurin indirectly through immunophilin proteins (22). Because these drug-immunophilin complexes have multiple effects, we designed a unique strategy to diminish calcineurin activity by expressing an inhibitory protein domain. A yeast-two hybrid screen, two groups identified a novel, noncompetitive inhibitor of calcineurin referred to as cain or cabin 1 (23, 24). The calcineurin binding/inhibitory domain was limited to a 38-aa region located in the C terminus of this protein. Interestingly, the calcineurin inhibitory domain of cain contains a conserved motif that is also found in the noncompetitive calcineurin inhibitory domain of AKAP79 and FKBP12 (25, 26) (Fig. 24).

An expression vector encoding the inhibitory domain of cain was transfected into cardiomyocytes along with an NFAT-dependent reporter and a NFAT3 expression vector. CnA expression or cyclosporin extinguished NFAT3 transcriptional activity, a measure of calcineurin activity (Fig. 2C). cDNA fragments encoding a 194-aa fragment of cain or a 299-aa fragment of AKAP79 (lacking the protein kinase C- and protein kinase A-binding sites) were fused to a flag epitope and used to generate replication-deficient adenovirus. Western blotting against the flag epitope demonstrated that each adenovirus generated a stable protein product in neonatal cardiomyocytes (Fig. 2B).
control AdBgal-infected cells (P < 0.001) (Fig. 4M). Adcain infection prevented AngII and PE-stimulated increases in cell surface area, and significantly attenuated the 1% FBS-mediated increase (P < 0.05) (Fig. 4M).

**Targeted Inhibition of Calcineurin Inhibits ANF Expression.** Increased ANF expression is a hallmark of cardiac hypertrophy and is readily detected by immunocytochemistry as perinuclear staining (12, 13). Cardiomyocyte cultures were infected with either Adcain or AdBgal, followed 24 hr later by agonist stimulation for an additional 48 hr. Stimulation with PE, AngII, or 1% FBS induced ANF protein expression in control AdBgal-infected cells (Fig. 5 C, E, and G), whereas Adcain infection blocked ANF expression (Fig. 5 D, F, and H). The ANF panels correspond to the α-actinin-stained panels shown in Fig. 4 E–L. Quantitation of ANF expression demonstrated a significant increase in ANF-positive cells in response to AngII, PE, and 1% serum (P < 0.0001), whereas Adcain abrogated this increase (P < 0.0001) (Fig. 5I). To confirm these results, RT-PCR was performed to quantify ANF mRNA levels (Fig. 5J). The data demonstrate that PE and AngII induced an ∼8-fold increase in ANF mRNA, which was inhibited with Adcain (48 hr).

Adcain infection was performed at a multiplicity of infection of 100 plaque-forming units/cell in 2 ml of media (6-cm dish) for 2 hr. To control for detrimental effects associated with adenoviral infection itself or effects due to nonmyocytes in the cultures, we decreased infectivity to 10 and 25 plaque-forming units/cell, resulting in ∼30% and 70%, respectively, of the cardiomyocytes being infected. Co-immunostaining demonstrated (Fig. 6) that PE-induced ANF expression (green) was abated in cells expressing cain (red), but robustly expressed in neighboring cells void of cain. These data demonstrate that cain specifically inhibits the hypertrophic response in a cell autonomous manner.

**AdAKAP Infection Blocks Cardiomyocyte Hypertrophy.** Although the expressed domain of cain was sufficient to inhibit calcineurin in cardiomyocytes, it is formally possible that this region has other biologic effects. A specific domain in the docking protein...
AKAP79 was previously shown to inhibit calcineurin in a noncompetitive manner (25). We designed an additional adenoviral strategy to inhibit calcineurin and validate the specificity of cain by expressing the calcineurin inhibitory domain of AKAP79 (amino acids 60–358). Interestingly, the calcineurin inhibitory subdomain of AKAP79 is also present in cain and FKBP12 (Fig. 2). Similar to Adcain infection, AdAKAP prevented PE-induced calcineurin activity (data not shown). AdAKAP also blocked agonist-induced cellular hypertrophy and ANF expression (Fig. 6).

Fig. 5. Adcain inhibits ANF expression. The same panels of cardiomyocytes shown in Fig. 4 were colabeled with ANF antibody (green). (A, C, E, and G) Cardiomyocytes infected with AdΔgal showed increased ANF protein expression after agonist stimulation, whereas Adcain-infected cells (B, D, F, and H) did not respond to agonist. The white arrowheads indicate positive perinuclear ANF staining. The arrowheads in F and H represent Adcain cardiomyocytes that were barely expressing ANF. (I) Quantitation of percent of ANF expressing cells demonstrated significant expression in AdΔgal-infected cultures, whereas Adcain blocked agonist-induced expression. (J) RT-PCR quantitation demonstrated that Adcain blocked ANF mRNA expression in agonist stimulated cardiomyoocyte cultures. *, $P < 0.0001$ vs. AdΔgal control; **, $P < 0.0001$ vs. AdΔgal + Ang II; †, $P < 0.0001$ vs. AdΔgal + PE; ††, $P < 0.0001$ vs. AdΔgal + 1% serum.

Fig. 6. Concentration independence of Adcain inhibition. Cardiomyocytes were infected with Adcain at a multiplicity of infection of 25 (A–C) or 10 (D–F) and treated with 50 μM PE for 48 hr. Cardiomyocytes were immunostained with a primary mAb against the flag-epitope (cain) in red and for ANF in green. The data demonstrate that cells expressing cain (arrowheads in A and D) lack ANF expression, and cells expressing ANF (arrowheads in B and E) lack cain expression. (C and F) Overlaid images of both antibodies are shown. The 21-kDa cain-flag peptide fusion was found in both the cytosol and nucleus.

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Fig. 7. AdAKAP (60–358 amino acids) infection attenuates cardiomyocyte hypertrophy. (A and B) AdAKAP infection, but not AdΔgal, attenuated morphologic cardiomyocyte hypertrophy induced by PE. (C) Quantitation of cell surface area demonstrated a significant increase in AdΔgal-infected cells treated with PE, which was prevented by AdAKAP infection. *, $P < 0.001$ vs. AdΔgal; †, $P < 0.001$ vs. AdΔgal + PE.
Adcain and AdAKAP attenuate cardiomyocyte hypertrophy by specifically inhibiting calcineurin.

**Discussion**

**Hypertrophic Agonists Use Calcium and Calcineurin.** It is well established that GPCR ligands such as AngII, PE, and ET-1 are capable of inducing cardiomyocyte hypertrophy through the activation of a wide array of intracellular signaling pathways (3). These agonists have been shown to significantly induce inotropy and calcium transients in cultured cardiomyocytes (27–30), of sufficient magnitude to saturate calmodulin (31). In this report, we establish that calcineurin is also activated by certain GPCR ligands in cultured neonatal cardiomyocytes.

A critical question that remains is the mechanism whereby inotropic increases in calcium might lead to calcineurin activation in cardiomyocytes. Calcineurin activation and subsequent NFAT transcriptional responses have previously been shown to require a sustained elevation of intracellular calcium concentrations (32). Given the oscillatory nature of calcium handling in cardiomyocytes, the aforementioned study might suggest that calcineurin only responds to elevated basal calcium levels and not to increases in transient calcium release. However, a subsequent study revealed that NFAT transcriptional responses can be regulated by cumulative calcium oscillations, demonstrating that calcium transients increase both the efficacy and the information content of calcium signals leading to gene expression and cell differentiation (33). Because AngII, ET-1, and PE are not thought to increase basal calcium levels in cardiomyocytes, it suggests a working model whereby enhanced calcium transients lead to calcineurin activation. Alternatively, subcellular calcium triggers might act as the inducing signal for calcineurin activation. Indeed, targeted disruption of calreticulin, an endoplasmic calcium-binding protein, results in a loss of inducible NFAT3 nuclear translocation in cultured cells (34).

**Targeted Inhibition of Calcineurin Attenuates Cardiomyocyte Hypertrophy.** In a previous study, we reported that cyclosporin A and FK506 could prevent cardiomyocyte hypertrophy in response to AngII and PE (16) and could prevent cardiac hypertrophy in various rodent models of heart disease (17). However, it is becoming increasingly clear that cyclosporin and FK506 have multiple biological effects independent of calcineurin. To directly target calcineurin activity, we used a strategy involving the calcineurin inhibitory protein cain.

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**Multiple Necessary Factors Regulate the Hypertrophic Program.** The observations presented in this study indicate that calcineurin is required for hypertrophic growth of cardiomyocytes in culture. However, this does not exclude the requirement of other intracellular signaling pathways. A number of studies have implicated a requirement for a wide range of signaling factors in cardiac hypertrophy. Hearts of transgenic mice overexpressing a dominant negative G protein fail to efficiently hypertrophy in response to pressure overload, suggesting that GPCR are necessary in cardiac responsiveness (35). Downstream of GPCR, a number of regulatory factors are implicated in cardiac hypertrophy. Pharmacologic inhibition of MAPK-signaling factors has demonstrated a necessary role of p38 and extracellular signal-regulated kinase signaling in mediating hypertrophic growth of cultured cardiomyocytes (10–12, 15). Using adenosine gene transfer, dominant negative SEK1 (a regulator of c-Jun N-terminal kinase activation), prevented the hypertrophic response of cardiomyocytes to ET-1 (14).

Collectively, these studies underscore the multifactorial nature of hypertrophic reactive signaling pathways in cardiomyocytes, suggesting that many regulatory pathways are coordinately required for full responsiveness, but that inhibition of central pathways can short-circuit the entire response. Additional studies will be required to elucidate the interconnectivity between calcineurin and other intracellular signaling pathways to gain a greater understanding of the coordinate regulation of cardiac hypertrophy.

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