Canonical Transient Receptor Potential Channels Promote Cardiomyocyte Hypertrophy through Activation of Calcineurin Signaling*

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The calcium/calmodulin-dependent phosphatase calcineurin plays a central role in the control of cardiomyocyte hypertrophy in response to pathological stimuli. Although calcineurin is present at high levels in normal heart, its activity appears to be unaffected by calcium during the course of a cardiac cycle. The mechanism(s) whereby calcineurin is selectively activated by calcium under pathological conditions has remained unclear. Here, we demonstrate that diverse signals for cardiac hypertrophy stimulate expression of canonical transient receptor potential (TRPC) channels. TRPC consists of a family of seven membrane-spanning nonselective cation channels that have been implicated in the nonvoltage-gated influx of calcium in response to G protein-coupled receptor signaling, receptor tyrosine kinase signaling, and depletion of internal calcium stores. TRPC3 expression is up-regulated in multiple rodent models of pathological cardiac hypertrophy, whereas TRPC5 expression is induced in failing human heart. We demonstrate that TRPC promotes cardiomyocyte hypertrophy through activation of calcineurin and its downstream effector, the nuclear factor of activated T cells transcription factor. These results define a novel role for TRPC channels in the control of cardiac growth, and suggest that a TRPC-derived pool of calcium contributes to selective activation of calcineurin in disease heart.

Cardiac hypertrophy is an adaptive response of the heart to many forms of cardiac disease, including hypertension, mechanical load abnormalities, myocardial infarction, valvular dysfunction, cardiac arrhythmias, endocrine disorders, and genetic mutations in cardiac contractile protein genes. Whereas the hypertrophic response is thought to be an initially compensatory mechanism that augments cardiac performance, sustained hypertrophy is maladaptive and frequently leads to ventricular dilation and the clinical syndrome of heart failure. Accordingly, cardiac hypertrophy has been established as an independent risk factor for cardiac morbidity and mortality (1).

Abnormal calcium handling, characterized by elevated intracellular diastolic calcium levels, is a hallmark of cardiac hypertrophy and heart failure. Elevated intracellular calcium not only impairs the contractile performance of the heart, but also activates calcium-dependent signaling pathways that mediate maladaptive cardiac remodeling (2). One such pathway is regulated by the calcium-calmodulin-dependent phosphatase calcineurin, which has been shown to be sufficient, and in some cases, necessary for pathological hypertrophy (3–5). Activated calcineurin dephosphorylates the transcription factor nuclear factor of activated T-cells (NFAT), facilitating translocation of NFAT to the nucleus where it acts in concert with other proteins to mediate expression of prohypertrophic genes. The activity of calcineurin is tightly regulated in vivo by a negative feedback mechanism; one of the most highly sensitive NFAT target genes encodes a potent calcineurin inhibitor, modulatory calcineurin-interacting protein 1 (MCIP1) (6–8), also known as Down syndrome critical region 1.

How pathological stress is translated into a calcium stimulus capable of activating cardiac calcineurin, and why calcineurin is spared from activation in response to the calcium that drives muscle contraction, remain important unanswered questions. In lymphocytes, influx of extracellular calcium through calcium release-activated calcium channels provides the sustained calcium signal required to activate the calcineurin/NFAT pathway (9). Unlike voltage-gated L-type calcium channels, calcium entry via calcium release-activated calcium channels is triggered by the release of calcium from intracellular stores, a mechanism referred to as store-operated calcium or capacitive calcium entry. Recent work has confirmed that hypertrophic agonists stimulate capacitative calcium entry in cardiac myocytes, leading to activation of the calcineurin/NFAT pathway and cardiac hypertrophy (10, 11). However, the identity of

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4 The abbreviations used are: NFAT, nuclear factor of activated T-cells; MCIP1, modulatory calcineurin-interacting protein 1; TRPC, canonical transient receptor potential channels; PAMH, pyridine activator of myocyte hypertrophy; BTP2, N-[4-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]phenyl]-4-methyl-1,2,3-thiadiazole-5-carboxamide; LV, left ventricle; OAG, oleoyl-2-acetyl-sn-glycerol; 2APB, 2-aminoethoxydiphenylborane; GFP, green fluorescent protein; NRVM, neonatal rat ventricular myocyte; DMEM, Dulbecco’s modified Eagle’s medium; SHHF, spontaneous hypertensive heart failure; TAB, thoracic aortic banding; PBS, phosphate-buffered saline; RT, reverse transcriptase; ANF, atrial natriuretic factor.
the cardiac channel(s) responsible for capacitative calcium entry remains unknown.

Members of the canonical transient receptor potential (TRPC) family of channels have been implicated in the control of capacitative calcium entry in non-cardiac cell types (12). The seven members of the TRPC family (TRPC1–7) are nonselective calcium-permeable cation channels. Most family members can be activated directly by diacylglycerol, a second messenger product of phospholipase C. Furthermore, TRPC channels have recently been shown to function as stretch receptors, admitting calcium influx in response to mechanical stress (13, 14), a pathological stimulus known to contribute to the development of cardiac hypertrophy (15). Other signaling molecules known to regulate TRPC channel activity include the receptor tyrosine kinase Src (16) and the cGMP-dependent protein kinase PKG (17).

Previously, we reported results of microarray studies with RNA from cultured cardiac myocytes exposed to distinct hypertrophic stimuli (18). Among the genes most potently up-regulated by two distinct hypertrophic agonists, the α-adrenergic receptor agonist, phenylephrine (PE), and the serotonin receptor agonist, pyridine activator of myocyte hypertrophy (PAMH), was that encoding TRPC3. Here we demonstrate that cardiac TRPC3 expression is up-regulated through a calcineurin-dependent mechanism by discrete pharmacologic, genetic, and physiologic stimuli that trigger pathological cardiac hypertrophy in rodent models. In contrast, TRPC5 is selectively induced in failing human heart. Gain and loss of function studies demonstrate that TRPC channels positively regulate cardiac hypertrophy through activation of calcineurin/NFAT signaling. These results suggest a role for TRPC channels in the transmission of pathological calcium signals in the heart.

**EXPERIMENTAL PROCEDURES**

**Chemical Reagents, Plasmids, and Adenoviral Constructs**—PE, endothelin-1, oleoyl-2-acetyl-sn-glycerol (OAG), and 2-aminoethoxydiphenylborane (2-APB) were obtained from Sigma. N-[4-[3,5-Bis(trifluoromethyl)-1-phenyl]-4-methyl-1,2,3-thiadiazole-5-carboxamide (BTP2) was obtained from Calbiochem. The cDNA encoding full-length, human TRPC3 (accession number NM_003305) was cloned by reverse transcriptase-PCR with the One Step RT-PCR kit (Invitrogen). For adenovirus production, this cDNA was subcloned into pShuttle-cytomegalovirus (QBiogen) and adenovirus was generated in HEK293 cells according to the manufacturer’s recommendations. Clonal populations of virus were obtained using the agar overlay method and titered with the Adeno-X Rapid Titre Kit (Clontech). The adenovirus encoding GFP-NFATc (NFAT2) was provided by Dr. Martin Schneider (University of Maryland, Baltimore, MD).

**Cardiomyocyte Culture**—Neonatal rat ventricular myocytes (NRVMs) were prepared from 1–2-day-old Sprague-Dawley rats as described previously (19). Cells were plated on gelatin-coated plates in DMEM containing fetal bovine serum (10%), penicillin-streptomycin, and I-glutamine (DMEM complete). After 12 h, plating medium was replaced with serum-free DMEM supplemented with Nutridoma-SP (0.1%; Roche Applied Sciences), which contains albumin, insulin, transferrin, and other defined organic and inorganic compounds the following day. Cells were treated with agonists/inhibitors for 48–72 h.

**Animal Models and Human Heart Samples**—The Institutional Animal Care and Use Committee approved all animal protocols. The spontaneous hypertensive heart failure (SHHF) rat is a well documented genetic model of hypertension, cardiac hypertrophy, and heart failure (20). For the isoproterenol model, male Sprague-Dawley rats were implanted subcutaneously with Alzet Mini-Pumps filled with either vehicle control (0.5 mg/ml sodium bisulfite in 0.9% sterile NaCl) or isoproterenol (dissolved in vehicle sufficient to administer 5 mg/kg/day of isoproterenol for 4 days). For thoracic aortic banding (TAB), male Sprague-Dawley rats (8–12 weeks of age, 200–225 g) were anesthetized with 5% isoflurane (v/v 100% O2), intubated, and maintained at 2.0% isoflurane with positive pressure ventilation. A left thoracotomy through the third intercostal space was performed and the descending thoracic aorta 3–4 mm cranial to the intersection of the aorta and azygous vein was isolated. A segment of 5-0 silk suture was then positioned around the isolated aorta to function as a ligature. A blunted hypodermic needle (gauge determined by weight) was placed between the aorta and the suture to prevent complete aortic occlusion when the suture was tied. When tying was completed, the needle was removed from between the aorta and ligature, re-establishing flow through the vessel. The thorax was then closed and the pneumothorax evacuated. After 7 days of recovery, animals were sacrificed and left ventricular tissue processed for Western blot analysis. Average heart weight to body weight ratios in banded versus sham-operated rats increased 22% at 1 week. The construction and characterization of transgenic mice expressing constitutively active calcineurin have been described elsewhere (3).

Ventricular samples from human patients with end-stage idiopathic dilated cardiomyopathy or nonfailing human cardiac tissue samples were obtained through the University of Colorado Health Sciences Center Cardiac Transplantation Program. End-stage failing hearts came from individuals who underwent heart transplantation because of idiopathic dilated cardiomyopathy (n = 6; 3 male, 3 female, average age 49.8 years). Nonfailing controls were obtained from organ donors whose hearts were unsuitable for donation due to blood type or size incompatibilities (n = 6; 4 male, 2 female; average age 51.8 years). Tissue samples were taken immediately upon explantation and rapidly frozen in liquid nitrogen.

**Immunoblotting**—NRVMs and in vivo tissue samples were homogenized in Tris buffer (50 mM, pH 7.5) containing EDTA (5 mM), Triton X-100 (1%), protease inhibitor mixture (Complete, Roche), phenylmethylsulfonyl fluoride (1 mM), and phosphatase inhibitors (sodium pyrophosphate, 1 mM; sodium fluoride, 2 mM; β-glycerolphosphate, 10 mM; sodium molybdate, 1 mM; and sodium orthovanadate, 1 mM). Homogenates were briefly sonicated and cleared by centrifugation. Protein concentrations were determined by BCA assay (Pierce) and 15 μg of total protein was resolved by SDS-PAGE with gradient gels (4–20% polyacrylamide; Invitrogen). Proteins were transferred to nitrocellulose membranes (Bio-Rad) and probed with TRPC3- or TRPC5-specific antibodies, according to the manufacturer’s instructions (Alomone Labs). Experiments were per-
formed with freshly diluted antibodies, because in our experience we have found that the lyophilized antibodies, once resuspended, lose significant immunoreactivity in storage. Negative controls for specificity were performed by preincubating the antibody with the corresponding antigen peptide (0.75 μg of peptide/1 μg of antibody) for 1 h. Antibodies against MCIP1 and calnexin have been described previously (18). Proteins were visualized using an enhanced chemiluminescence system (Pierce). For immunoprecipitation experiments, 3 μg of antibody was incubated with homogenate in a total volume of 0.5 ml for 1 h. Protein G-Sepharose beads (10 μl, GE Healthcare) were added, and after a 1-h incubation, beads were washed three times in homogenization buffer and prepared for SDS-PAGE.

Hypertrophy Measurements—The atrial natriuretic factor (ANF) competition enzyme-linked immunosorbent assay, measurement of β-myosin heavy chain expression, RNA dot blot assay for hypertrophic marker gene expression, and adenylate kinase release assay have been described previously (19). For cell volume measurements, NRVM cultured in 6-well dishes were harvested by treatment with trypsin (Cellgro). After recovery by centrifugation, cell pellets were washed in PBS, resuspended in 10 ml of IsoFlow electrolyte solution (Beckman-Coulter), and analyzed with a Z2 Coulter Particle Counter and Size Analyzer (Beckman-Coulter).

RT-PCR Analysis—RNA was isolated from NRVMs using TRI Reagent® (Sigma) according to the manufacturer’s instructions. RNA was quantified using both standard spectrophotometry of nucleic acids at 260 nm using a DU64 spectrophotometer (Beckman-Coulter) and fluorescence with a TD700 fluorometer (Turner Designs). RT-PCR of rat TRPC3 (accession number NM_021771) used primers that spanned the first intron (7950 bp): forward primer, 5′-ctggccaacatagagaaggagt-3′; and reverse primer, 5′-caccgattccagatctccat-3′. Rat 18 S ribosomal RNA amplicons were used as input controls: forward primer, 5′-cgaggaattcccagtaagtgc-3′, and reverse primer, 5′-aagttcgaccgtcttctcagc-3′. Reverse transcription and amplification of 0.5 μg of total RNA were done using the SuperScript™ One-step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen), on a I-cycler™ thermocycler (Bio-Rad). Reverse transcription reactions were run on 2% agarose gels containing 0.1 μg/ml ethidium bromide. Imaging and densitometry were performed with an Alpha Innotech gel imaging system and ChemImage 5.5 software. RT-PCR amplified a sin-
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gle 141-bp band of the expected size, which sequencing confirmed to be TRPC3. Negative control reactions excluding RNA template, reverse transcriptase, or either primer did not yield a PCR product. For analysis of human samples, RNA was extracted from left ventricular tissue as described above and subjected to TaqMan real-time quantitative RT-PCR (PerkinElmer Life Sciences) as described by Riccio et al. (21).

Human TRPC5 primers were: forward, 5'-cctctcatagacatgccaa-3', and reverse, 5'-ggttgctgtgtagactcag-3'.

Indirect Immunofluorescence—NRVMs were plated on gelatin-coated 6-well dishes (2.5 × 10^5 cells/well) in DMEM complete and transferred 12 h subsequently to serum-free DMEM supplemented with Nutridoma-SP (0.1%; Roche Applied Sciences). Adenovirus was added to cells at the time of plating. Unless otherwise indicated, virus was used at a multiplicity of infection of 20. Following experimental treatment, cells were washed two times in PBS, fixed with formalin (10%) in PBS, permeabilized, and blocked with PBS containing Nonidet P-40 (0.1%) and bovine serum albumin (3%), and then incubated in the same solution containing primary antibodies specific for either sarcomeric α-actinin (Sigma; 1:200 dilution) or Myc (Santa Cruz; 1:500 dilution) for 1 h at room temperature. Cells were washed 4 times in PBS and incubated in PBS/Nonidet P-40/bovine serum albumin containing either a fluorescein- or CY3-conjugated secondary antibody (Jackson Laboratories; 1:200 dilution) for 30 min at room temperature. Cells were washed 4 times in PBS and then covered with mounting solution (SlowFade, Molecular Probes) and glass coverslips.

Proteins were visualized with an inverted fluorescence microscope (Olympus model BH-2) at ×40 magnification, and images were captured using a digital camera (Photometrics; Roper Scientific).

RESULTS

Calcineurin-dependent Induction of TRPC3 Expression in Cultured Cardiomyocytes—To gain insight into changes in gene expression underlying stress-induced cardiac hypertrophy, RNA microarray studies were performed with cultured NRVMs. NRVMs undergo hypertrophy in response to multiple agonists, including PE, which stimulates the 1-adrenergic receptor, and PAMH, a novel 5-HT2B serotonin receptor agonist. A complete summary of microarray results with RNA from NRVMs stimulated with PE or PAMH for 48 h is found in the NCBI Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE925. Among the genes most potently up-regulated by both agonists were those encoding β-myosin heavy chain, a classical marker of cardiac hypertrophy, and the transient receptor potential channel 3 (TRPC3) calcium channel.

Although calcium is known to play a key role in the regulation of cardiac hypertrophy (2), the relative contribution of distinct calcium pools to this growth response remains unclear. As such, we focus here on elucidating the potential role of TRPC channels in the control of stress-induced cardiac hypertrophy.

To confirm the microarray results, semi-quantitative RT-PCR was performed with RNA from freshly isolated NRVMs treated with PE or two independent hypertrophic agonists, endothelin-1 and fetal bovine serum, for 48 h. Each ago-

![FIGURE 2. Induction of cardiac TRPC3 protein expression in vivo.](http://www.jbc.org/)

Prior studies have suggested that signaling by the calcineurin phosphatase stimulates expression of TRPC3 in skeletal muscle

PE and fetal bovine serum inducing the highest levels (Fig. 1, A and B). Similar results were obtained with RNA from PAMH-treated cells (data not shown).

A, male, 2-month-old Sprague-Dawley (SD) rats were given a sham operation or subjected to thoracic aortic banding to trigger pressure overload hypertrophy of the LV of the heart, as described under “Experimental Procedures.” Seven days following the operation, animals were sacrificed and TRPC3 levels in LV protein lysates analyzed by immunoblotting. Blots were reprobed with calnexin-specific antibodies to control for protein loading. B, parallel samples of LV protein lysates were run to assess MCIP1 protein levels by immunoblot. C, male SHHF rats of the indicated ages were sacrificed and TRPC3 levels in LV protein lysates were run to assess MCIP1 protein levels by immunoblot. D, parallel samples of LV protein lysates were run to assess MCIP1 protein levels by immunoblot. E, male SD rats were implanted with osmotic mini-pumps that release either vehicle or the β-adrenergic receptor agonist isoproterenol (ISO, 4 mg/kg/day) for 4 days. TRPC3 levels in LV preparations were assessed as described in A. F, male mice (~2 months of age) expressing a constitutively active calcineurin transgene (Cn-Tg) and control nontransgenic littermates (WT) were sacrificed and TRPC3 and MCIP1 levels in LV preparations assessed as described in A. F, TRPC3 protein levels in the indicated models were quantified by densitometry. Values represent averages ± S.D. *p < 0.05.
To determine the role of calcineurin in TRPC3 gene regulation in cardiac myocytes, RT-PCR was performed with RNA from NRVMs stimulated with PE in the absence or presence of the calcineurin inhibitors cyclosporine A or FK506. As shown in Fig. 1C, agonist-dependent induction of TRPC3 mRNA transcripts was completely blocked by either of the two calcineurin inhibitors.

To evaluate expression of TRPC3 protein expression in NRVMs and rat and mouse ventricles, we performed immunoblot analysis with a TRPC3-specific antibody. As a control for specificity, the TRPC3 antibody was preincubated with the antigen peptide. The antibody detected immunoreactive bands of the expected molecular mass (110–120 kDa) (Fig. 1D, left panel). The most abundant band observed in NRVM was 115 kDa, with a slightly smaller band of ~110 kDa predominating in rat and mouse ventricular samples. Notably, detection of these immunoreactive bands was efficiently competed by preincubation of the TRPC3 antibody with the antigen peptide. Intentional overexposure of the same immunoblot (Fig. 1D, right panel) revealed a number of non-specific bands that were not competed in the antigen peptide-blocked controls. Immunoblot analysis verified that the observed induction of TRPC3 mRNA correlated with modest (between 1.5- and 2-fold) but significant increases in TRPC3 protein levels in PE-treated NRVMs (Fig. 1, E and F).

**Induction of TRPC3 Expression in Rodent Models of Cardiac Hypertrophy**—Experiments were performed to determine whether TRPC3 expression is elevated in animal models of pathological cardiac hypertrophy. TAB places a pressure overload on the heart and triggers left ventricular (LV) hypertrophy. Immunoblotting studies revealed that TRPC3 protein expression was consistently elevated in LVs of rats subjected to TAB for 7 days (Fig. 2A).

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**FIGURE 3. Ectopic TRPC3 stimulates cardiomyocyte hypertrophy.** A, NRVMs (2 × 10⁶) were cultured in 10-cm dishes, and infected with Myc-tagged TRPC3 adenovirus (Ad-TRPC3) for 48 h. Total protein was prepared and analyzed by immunoblotting with antibodies to TRPC3 (left panel; two independent clones shown) and the Myc epitope tag (right panel). The apparent molecular mass of endogenous and recombinant TRPC3 was ~115 kDa. B, samples prepared as in panel A were immunoprecipitated with either the anti-Myc antibody or the anti-TRPC3 antibody and immunoblotted with the other antibody, confirming that both antibodies immunoprecipitated recombinant TRPC3. C, NRVMs were cultured in 6-well dishes (2.5 × 10⁴/well) and infected with adenoviruses encoding β-galactosidase (Ad-β-GAL) or Myc-tagged TRPC3 (Ad-TRPC3). Cells were cultured for 48 h in serum-free medium in the absence or presence of the TRPC agonist OAG (100 μM). Cells were fixed and sarcomeres were visualized by indirect immunofluorescence with anti-sarcomeric actinin antibodies (top row). Higher magnification images of the same cells are shown (middle row). Cells were co-stained with anti-Myc antibody to reveal the subcellular distribution of TRPC3 (bottom row). D, NRVMs prepared as in panel B were harvested by trypsinization, and mean cell volumes were determined by Coulter analysis. Values represent averages ± S.D. for four independent sets of cells per condition. * p < 0.01 versus uninfected control; # p < 0.05 versus Ad-TRPC3 alone. E, NRVMs (2 × 10⁶) were cultured in 10-cm dishes, infected with either Ad-β-galactosidase or Ad-TRPC3, and maintained in serum-free medium for 72 h. Total RNA was prepared and arterial natriuretic factor (ANF) and α-skeletal actin (α-SK-Actin) mRNA abundance determined by dot blot analysis with radiolabeled probes. Values represent averages ± S.D. for three independent sets of cells per condition. * p < 0.05.
To determine whether induction of TRPC3 expression correlates with stimulation of calcineurin signaling, we quantified expression of the endogenous NFAT target gene, modulatory calcineurin-interacting protein-1 (MCIP1). The MCIP1 protein is a negative-feedback regulator of calcineurin signaling (8), and is expressed as 28- and 38-kDa isoforms (18). Expression of 28-kDa MCIP1 is regulated by an alternative promoter that harbors 15 NFAT binding sites, and thus this lower molecular weight form of the protein serves as a sensitive marker of NFAT activity (23). We observed significantly increased expression of 28-kDa MCIP1 in TAB ventricles, indicating enhanced calcineurin/NFAT activation (Fig. 2B). These observations are consistent with a role of calcineurin in the control of TRPC3 gene expression.

SHHF rats provide a genetic model of pathological cardiac remodeling and heart failure (20). Due to a mutation in the leptin receptor gene, SHHF rats develop hypertension that results in cardiac hypertrophy and eventual heart failure. As shown in Fig. 2C, TRPC3 protein levels were elevated in LVs from failing hearts of ~20-month-old SHHF rats. However, TRPC3 expression was normal in 8–9-month-old rats with compensated LV hypertrophy. TRPC3 protein expression was also increased in LVs of rats infused for 4 days with the α-adrenergic receptor agonist isoproterenol, which stimulates cardiac hypertrophy that is accompanied by fibrosis (Fig. 2D). Similar to the TAB model, increased expression of MCIP1 protein was observed in LV samples from both ISO-infused and ~20-month-old SHHF rats (data not shown).

To address the role of calcineurin signaling in the control of cardiac TRPC3 expression, TRPC3 protein levels were measured in LVs of transgenic mice expressing constitutively active calcineurin in the heart under the control of the α-myosin heavy chain promoter (Cn-Tg) (3). As shown in Fig. 2E, TRPC3 expression was dramatically elevated in Cn-Tg mice compared with wild-type littermates. Levels of TRPC3 protein in LV samples from each model of cardiac hypertrophy were quantified by densitometry (Fig. 2F). Together, the results demonstrate that TRPC3 protein expression is induced by diverse cues for pathological cardiac growth in vivo, and suggest a role for calcineurin signaling in the induction of cardiac TRPC3 expression.

**Regulation of Cardiomyocyte Hypertrophy by TRPC Channels**—Cardiac hypertrophy is associated with enhanced protein synthesis, cell growth in the absence of mitosis, and increased sarcomere assembly. In addition, fetal cardiac genes, including those that encode ANF and α-skeletal actin, and β-myosin heavy chain become reactivated during the hypertrophic response. To begin to address the role of TRPC channels in the regulation of cardiac hypertrophy, we developed an adenovirus that encodes full-length Myc epitope-tagged TRPC3 (Ad-TRPC3) (Fig. 3A). As an additional control for antibody selectivity, we determined that both anti-Myc and anti-TRPC3 antibodies effectively immunoprecipitated recombinant TRPC3.

**FIGURE 4.** Inhibition of hypertrophic gene expression by 2-APB and BTP2. NRVMs were cultured in 96-well dishes and exposed to increasing doses of two different TRPC channel inhibitors, 2-APB or BTP2, in the absence or presence of PE (20 μM). After 48 h of stimulation, ANF abundance in culture supernatants (A and B) and adenylate kinase release (E and F) (cytotoxicity marker) were measured as described under “Experimental Procedures.”
induced expression of ANF and scripts. Of note, OAG did not appear to enhance TRPC3-mediated induction of these genes. Thus, basal TRPC3 activity appears to be sufficient to increase cell volume and induce fetal cardiac genes, whereas full sarcomere altering effects of TRPC3 require activation by OAG. Together, the results suggest that ectopic TRPC3 is capable of stimulating hypertrophy of cultured cardiac myocytes.

As an alternative approach to study the role of TRPC channels in the control of cardiac hypertrophy, we employed two distinct small molecule inhibitors of calcium release-activated calcium channels, 2-APB (25) and the pyrazole derivative BTP2 (28). Whereas these inhibitors block TRPC3-mediated calcium influx, we cannot exclude the possibility that 2-APB and BTP2 may also be acting on other channels including TRPVs and TRPMs. As shown in Fig. 4, A–D, both 2-APB and BTP2 exhibited dose-dependent inhibition of ANF secretion and β-myosin heavy chain expression in NRVMs stimulated with PE. Only cells treated with the highest concentrations of 2-APB and BTP2 showed evidence of cytotoxicity, as determined by assessing the presence of adenylate kinase in culture supernatants (Fig. 4, E and F). Treatment with 2-APB also resulted in suppression of PE-mediated increases in NRVM size and protein synthesis (data not shown). Together, the data from these gain and loss of function experiments suggest a role for TRPC channels in the control of cardiac hypertrophy.

**TRPC3 Activates Calcineurin/NFAT Signaling**—The calcineurin phosphatase plays an essential role in the regulation of cardiac hypertrophy, and has recently been shown to be regulated by TRPC3 in skeletal muscle. As such, we hypothesized that the mechanism whereby TRPC channels stimulate cardiac hypertrophy involves calcineurin. As an initial step to address this hypothesis, we examined the effect of TRPC3 overexpression on the subcellular distribution of the calcineurin target, NFAT. The NFAT transcription factor is a phosphoprotein that resides in the cytosol and, upon dephosphorylation by calcineurin, translocates to the nucleus. NFAT was cytosolic in unstimulated NRVMs, and infection of cells with Ad-β-galactosidase did not alter its subcellular distribution. In contrast, TRPC3 overexpression partially stimulated nuclear import of NFAT (Fig. 5A). Of note, we observed modest induction of NFAT nuclear import in Ad-β-galactosidase-infected cells treated with OAG.
Immunoblotting studies were performed to confirm that TRPC3 stimulates calcineurin-dependent dephosphorylation of NFAT. Hypophosphorylated NFAT migrates more rapidly than hyperphosphorylated NFAT in denaturing polyacrylamide gels. As shown in Fig. 5B, overexpression of TRPC3 in NRVMs triggered dephosphorylation of NFAT, as evidenced by the appearance of the rapidly migrating NFAT species. Consistent with the results presented in Fig. 5A, OAG enhanced TRPC3-mediated dephosphorylation of NFAT.

To determine whether TRPC channels regulate endogenous NFAT target genes, we monitored expression of the 28-kDa isoform of MCIP1, which is dependent on calcineurin/NFAT signaling. As shown in Fig. 5C, exposure of NRVMs to increasing doses of Ad-TRPC3 led to selective up-regulation of 28-kDa MCIP1, indicating that TRPC3overexpression stimulates NFAT-dependent transcription. In contrast, 2-APB blocked PE-mediated induction of 28-kDa MCIP1 (Fig. 5D). Together, the results demonstrate that TRPC channels stimulate cardiac calcineurin/NFAT signaling.

**Induction of TRPC5 Expression in Failing Human Heart**—To begin to address whether TRPC channels play a role in the pathogenesis of human heart failure, quantitative RT-PCR was employed to survey expression of TRPC1, -3, -4, -5, and -6 in nonfailing human heart versus hearts of patients with idiopathic dilated cardiomyopathy. TRPC5 mRNA expression was significantly elevated in failing human heart relative to nonfailing controls (Fig. 6A). Immunoblot analysis confirmed that increased TRPC5 mRNA levels in failing heart correlated with enhanced TRPC5 protein expression (Fig. 6B). We observed two immunoreactive bands in the human heart; both were effectively competed by preincubating the TRPC5 antibody with the corresponding antigen peptide (Fig. 6B). Blots were reprobed with calnexin-specific antibody to control for protein loading (bottom panels).

**DISCUSSION**

The current study suggests that TRPC channels contribute to the maintenance of cardiac hypertrophy via a positive feedback mechanism involving activation of calcineurin/NFAT signaling (Fig. 7). Pathological stress stimuli cause phospholipase C activation and store depletion, which potentiate TRPC activity and
produce a calcium signal sufficient to activate calcineurin/NFAT. Because TRPC channels are mechanosensitive, it is also possible that abnormal mechanical stress in the hypertrophied and failing heart might activate TRPC channels directly. Among the genes modulated by the calcineurin/NFAT pathway are TRPCs themselves, completing a positive feedback circuit that stabilizes a state of hypertrophic gene expression. Calcineurin signaling may also stabilize TRPC3 protein posttranslationally. The interplay between calcineurin and TRPC channels may contribute to the selective activation of cardiac calcineurin by calcium in diseased myocardium.

A similar regulatory mechanism has been hypothesized to operate in skeletal muscle, where calcineurin/NFAT-dependent up-regulation of TRPC3 provides positive feedback reinforcing the slow oxidative muscle fiber phenotype (22). Interplay between TRPC channels and calcineurin was also recently described in a prostate cancer cell line (26). In addition, independent pharmacologic evidence of the central role TRPC channels play in the regulation of calcineurin/NFAT signaling has been provided by recent small molecule screens for NFAT inhibitors. A high-throughput screen for compounds capable of suppressing activation of the NFAT-responsive interleukin-2 promoter (27) identified a series of 3,5-bistrifluoromethyl pyrazole store-operated calcium inhibitors (including BTP2) (28), which have subsequently been shown to be TRPC antagonists (29). An alternative high-throughput screen for compounds that block nuclear translocation of NFAT also produced a series of store-operated calcium inhibitors (30).

Aberrant TRPC expression or activity is thought to contribute to the pathogenesis of a number of human diseases (31). Recent studies indicate that TRPC channels mediate the abnormal calcium influx and overload observed in skeletal myotubes from Duchenne muscular dystrophy patients (32). The pathologic hyperplasia of pulmonary arterial smooth muscle cells that occurs in idiopathic pulmonary hypertension is driven, in part, by increased calcium influx through TRPC channels (33). Bosentan, a non-selective endothelin receptor antagonist used for the treatment of idiopathic pulmonary arterial hypertension, may exert antiproliferative effects via down-regulation of TRPC6 (34).

We observed that TRPC expression is up-regulated in animal models of cardiac hypertrophy and human heart failure, and that pharmacologic inhibition of channel activity suppressed hypertrophy in vitro. However, the relative contribution of this channel family to the abnormal calcium handling seen in the hypertrophied and failing human heart remains to be further elucidated. Intriguingly, down-regulation of cardiac SERCA2 in vitro (a hallmark of hypertrophy in vivo) is associated with coordinate up-regulation of TRPC channels and calcineurin activation, suggesting a link between the regulation of calcium reuptake and extracellular calcium influx (35). Because TRPC family members can form heteromultimers with distinct electrophysiological properties, it will be particularly important to assess the relative contributions of individual family members to the activation of cardiac calcineurin/NFAT signaling. Our own observations indicate that TRPC5, rather than TRPC3, is up-regulated in the failing human heart, perhaps revealing a crucial species difference. In addition, cardiac-specific overexpression of TRPC6 has been shown to induce heart failure in mice.5

As regulators of pro-hypertrophic calcium signaling in cardiomyocytes, TRPC channels may represent novel pharmacologic targets for modulating pathologic calcineurin/NFAT signaling in the heart. Future work focused on the development of selective inhibitors of TRPC channel activity or expression will provide essential tools for the functional characterization of these channels in cardiomyocytes, and may yield therapeutically useful compounds for the treatment of cardiac hypertrophy and heart failure.

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