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Research Article **Cardiology**

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Local $InsP_3$-dependent perinuclear Ca^{2+} signaling in cardiac myocyte excitation-transcription coupling

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Introduction

Ca^{2+}, calmodulin (CaM), and Ca^{2+}-CaM–dependent protein kinase II (CaMKII) signaling have been implicated in the regulation of gene expression in cardiac hypertrophy and heart failure (HF) (1–8). CaMKII expression is elevated in human HF (9) and animal models of HF (10), and overexpression of either CaM or CaMKII can induce hypertrophy and HF (4, 5, 7, 8). CaMKII inhibition protects against structural heart disease (11). However, the mechanisms by which CaMKII alters gene expression in heart and the way in which cardiac myocytes distinguish Ca^{2+} transients that occur at every heartbeat from those meant to regulate transcription are enigmas that are addressed here.

CaMK can phosphorylate type II histone deacetylases (HDACs) (12). These HDACs (HDAC4, 5, 7, and 9) normally repress transcriptional activation (e.g., activation driven by myocyte enhancer factor-2 [MEF2]) and favor condensed DNA. When HDAC is phosphorylated in response to neurohumoral stimuli, it is exported from the nucleus (in association with the chaperone protein 14-3-3), MEF2 is derepressed, and a hypertrophic program of cardiac gene expression is activated (13). Indeed, genetic knockout of these HDACs results in marked cardiac hypertrophy (14). Nonetheless, it is unknown how CaMKII is activated by neurohumoral stimuli that induce hypertrophy or whether this causes HDAC nuclear export in adult ventricular myocytes.

Inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) are ubiquitous intracellular Ca^{2+} release channels. They are present in cardiac myocytes, albeit at lower levels than the related ryanodine receptor (RyR), which is the main source of Ca^{2+} in excitation-contraction coupling (ECC) (15). The type 2 $InsP_3R$ ($InsP_3R2$) is the predominant subtype in cardiac myocytes (16), but its role in heart is poorly understood. In atrial myocytes, some $InsP_3R$ are located near RyRs at sarcoplasmic reticulum (SR) Ca^{2+} release sites in the cell periphery, and these may contribute to altered ECC and arrhythmogenesis in atria (17, 18). In ventricular myocytes, $InsP_3R2$ are mainly in the nuclear envelope where they complex with CaMKII (19), and evidence for ventricular $InsP_3R$ in ECC or myocyte Ca^{2+} transients is mainly negative (17, 18). Thus, the role of $InsP_3R2$ in ventricular myocytes and their functional relationship with CaMKII colocalized at the nucleus are unknown. Here we explore whether $InsP_3R$, CaMKII, and HDAC signaling pathways converge.

We tested the hypothesis (Figure 1) that stimulation of adult ventricular myocytes by the physiological hypertrophic agonist endothelin-1 (ET-1) activates HDAC5 nuclear export by a local Ca^{2+}-dependent pathway at the nuclear envelope. To accomplish this, $InsP_3$ produced by ET-1–induced activation of phospholipases causes very local Ca^{2+} release via $InsP_3R2$ in the nuclear envelope, which in turn activates CaMKII, phosphorylates HDAC5, and causes HDAC5 nuclear export. A corollary hypothesis is that the Ca^{2+} involved in this activation is relatively insulated from the Ca^{2+} transients associated with each heartbeat (i.e., that responses to neurohumoral stimuli and cytosolic Ca^{2+} transients are distinct). This paradigm is analogous to local control involved in Ca^{2+}-induced Ca^{2+} release during ECC and to Ca^{2+}-dependent inactivation of Ca^{2+} current (20).

Nonstandard abbreviations used: AIP, autocamtide inhibitory peptide; 2-APB, 2-aminoethoxydiphenyl borate; Bis I, bisindolylmaleimide I; CaM, calmodulin; CaMKII, Ca^{2+}-CaM–dependent protein kinase II; DAG, diacylglycerol; ECC, excitation-contraction coupling; ET-1, endothelin-1; ETC, excitation-transcription coupling; HDAC, histone deacetylase; HF, heart failure; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3R$, $InsP_3$ receptor; MEF2, myocyte enhancer factor-2; NFAT, nuclear factor of activated T cells; PKD, protein kinase D; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; TG, thapsigargin.

Conflict of interest: The authors have declared that no conflict of interest exists.

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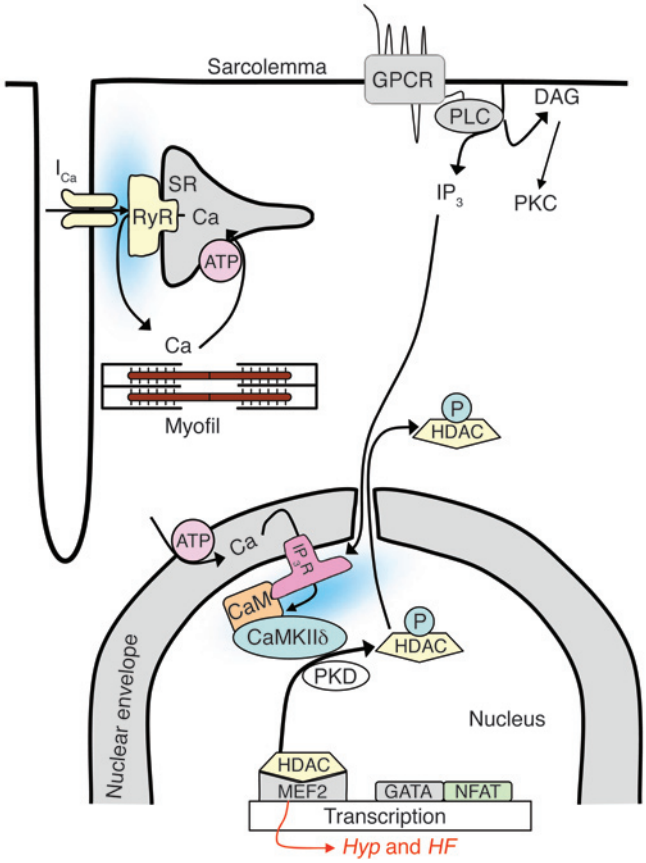

Figure 1
Working hypothesis. ET-1 induces HDAC5 nuclear export via a pathway involving $InsP_3$ (IP_3) and $InsP_3R$ (IP_3R) with associated CaM and CaMKII, leading to HDAC5 phosphorylation and nuclear export, thereby derepressing the transcription factor MEF2 (see text for additional details). Hyp, hypertrophy.

Results

Figure 2A shows a resting adult rabbit ventricular myocyte in which HDAC5 tagged with GFP (HDAC5-GFP) was expressed by adenoviral infection. HDAC5 was initially largely confined to the nucleus. ET-1 exposure induced a time-dependent export of HDAC5 from the nucleus (Figure 2, A–C). Phenylephrine also caused HDAC5 nuclear export (data not shown). Notably, basal $[Ca^{2+}]_i$ (assessed using Rhod-2) was not significantly altered (Figure 2D), nor was there any detectable local or global Ca^{2+} transients or $[Ca^{2+}]_i$ elevation in or around the nucleus or elsewhere (see below and refs. 18, 21).

Role of $InsP_3$ and nuclear envelope Ca^{2+} stores in ET-1–induced HDAC5 export. Phospholipase C produces both $InsP_3$ and diacylglycerol (DAG), an activator of PKC. Either second messenger could mediate ET-1 effects on HDAC export. However, we found that ET-1–induced HDAC5 nuclear export was completely blocked by the $InsP_3R$ inhibitor 2-aminoethoxydiphenyl borate (2-APB) but unaffected by the PKC inhibitor bisindolylmaleimide I (Bis I) (Figure 3A). These data suggest that $InsP_3$ rather than PKC is the critical second messenger in ET-1–induced HDAC5 nuclear export.

Complementary experiments indicate (Figure 3B) that the selective SR/ER Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin (TG) (which depletes both SR and ER Ca^{2+} stores) completely abolished ET-1–induced HDAC5 nuclear export. To confirm that TG depleted Ca^{2+} in the nuclear envelope, parallel studies were done using the low affinity Ca^{2+} sensor Fluo-5N, which can be trapped in the SR and nuclear envelope to sense free $[Ca^{2+}]$ inside Ca^{2+} stores (22). Because of the low Ca^{2+} affinity of Fluo-5N ($K_d \sim 400$ μM), fluorescence is only bright in organelles like SR and nuclear envelope, where free $[Ca^{2+}]$ is approximately equal to 1 mM. The inset in Figure 3B shows that 1 μM TG preincubation for 10 minutes depleted the nuclear envelope Ca^{2+} stores.

In addition, direct $InsP_3R$ activation in permeabilized myocytes by the selective $InsP_3R$ agonist adenophostin could fully mimic the effect of ET-1 (Figure 3C). These data further demonstrate that $InsP_3$-dependent Ca^{2+} stores can induce HDAC5 translocation.

To more directly test the involvement of $InsP_3R2$ in ET-1–induced HDAC5 nuclear export, we used mouse ventricular myocytes in

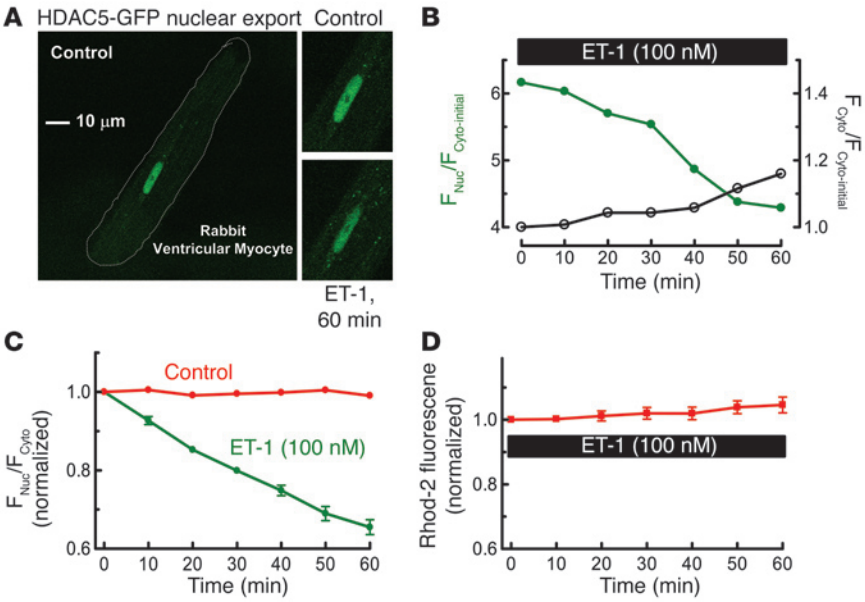

Figure 2
ET-1 induces nuclear export of HDAC5. (**A**) ET-1 (100 nM) was applied for 60 minutes to quiescent rabbit ventricular myocyte expressing the fusion protein HDAC5-GFP. (**B**) Individual traces of nuclear and cytosolic fluorescence (F_{Nuc}/F_{Cyto}) for a single myocyte, where each trace is normalized to the F_{cyto} before ET-1 exposure ($F_{cyto\text{-}initial}$). (**C**) HDAC5 nuclear export was analyzed as decrease of F_{Nuc}/F_{Cyto}, normalized to the initial ratio (6.3 ± 0.4; $n = 12$). The control group was treated the same, except without ET-1 application ($n = 10$). (**D**) $[Ca^{2+}]_i$ measured by Rhod-2 fluorescence upon exposure to 100 nM ET-1 ($n = 7$). Long-term exposure to ET-1 induced further HDAC5 export, such that after 24 hours the nucleus was virtually depleted of HDAC5 (not shown), analogous to effects in cultured neonatal rat myocytes (31).

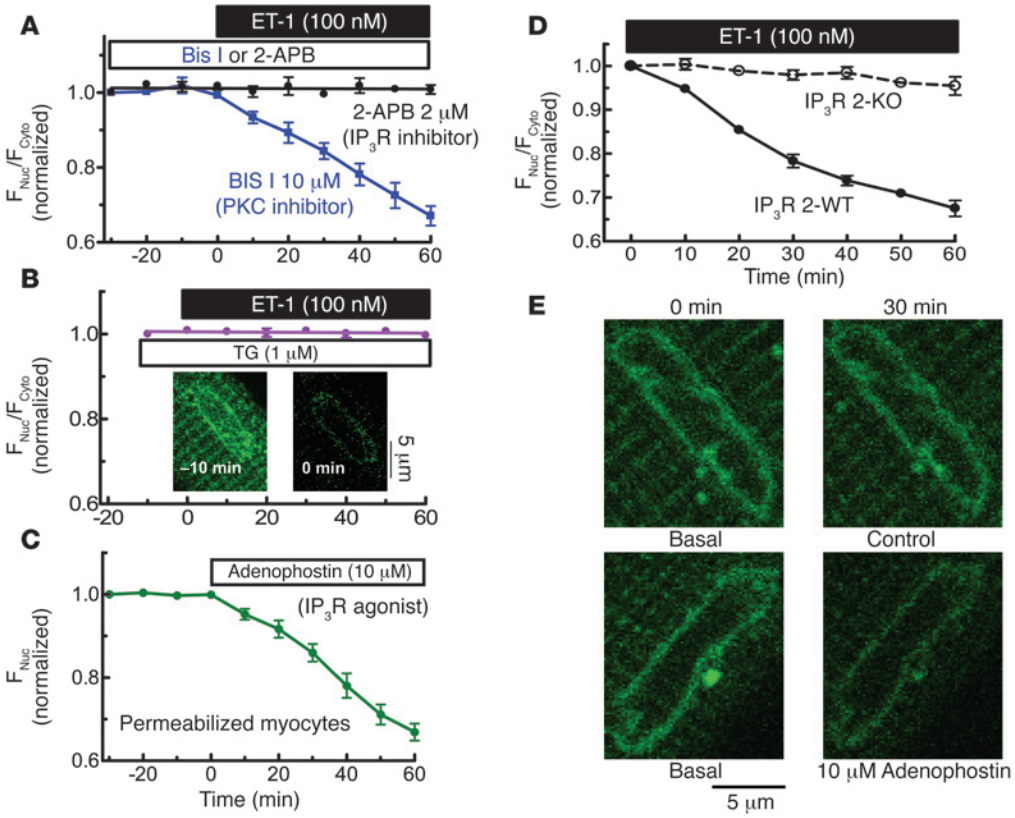

Figure 3
ET-1–induced HDAC5 nuclear export is dependent on $InsP_3R$ and Ca^{2+} in $InsP_3$-sensitive stores. (**A**) HDAC5-GFP–expressing myocytes were pretreated with 2 μM 2-APB ($n = 6$) or 10 μM Bis I ($n = 5$) for 30 minutes, followed by application of 100 nM ET-1. (**B**) SR and nuclear envelope Ca^{2+} stores were depleted by preincubation with SR/ER Ca^{2+}-ATPase (SERCA) inhibitor TG 1 μM ($n = 7$) for 10 minutes. Insets are the Fluo-5N images, showing that this TG treatment depleted nuclear envelope Ca^{2+} stores. (**C**) In permeabilized HDAC5-GFP expressing myocytes, 10 μM adenophostin was applied after 30 minutes at 100 nM $[Ca^{2+}]_i$ ($n = 8$). HDAC5 nuclear export was measured as the decrease of nuclear fluorescence. In the permeabilized cell, cytosolic concentration is irrelevant as HDAC5-GFP can readily diffuse to the bath. (**D**) ET-1–induced HDAC5 nuclear export assessed in mouse ventricular myocytes that lack $InsP_3R2$ ($InsP_3R$ 2 KO) or WT littermates. (**E**) Fluo-5N–loaded myocytes were permeabilized and treated without or with 10 μM adenophostin for 30 minutes. Nuclear envelope Ca^{2+} release was indicated by decreased Fluo-5N fluorescence.

which the *$InsP_3R2$* gene had been knocked out ($InsP_3R2$ KO) (23). In myocytes from WT mice, ET-1 induced HDAC5 export to an extent similar to that seen in rabbit myocytes (Figure 3D versus Figure 2C). However, in $InsP_3R$ 2 KO mice, ET-1–induced HDAC5 nuclear export was abolished (Figure 3D).

Using Fluo-5N to assess $[Ca^{2+}]$ in the nuclear envelope (as in Figure 3B), we found that adenophostin (or $InsP_3$; not shown) mobilized Ca^{2+} from the nuclear envelope (Figure 3E), consistent with activation of $InsP_3Rs$ at that site (19). As in prior studies using Fluo-3 or Rhod-2 in adult ventricular myocytes (18), we did not detect ET-1– or adenophostin-induced Ca^{2+} mobilization near the nuclear envelope. This may reflect the small number of $InsP_3R2$, the low unitary Ca^{2+} flux rate (versus RyRs), nonsynchronized openings (versus coactivation of RyRs in Ca^{2+} sparks), or diffusion and dilution of Ca^{2+}. Measuring $[Ca^{2+}]$ inside the nuclear envelope obviated these limitations. Thus, Figure 3 shows that activation of $InsP_3R2$ and Ca^{2+} release from nuclear envelope stores is necessary and sufficient to trigger HDAC5 nuclear export initiated by ET-1.

Local versus global Ca^{2+} signals in ET-1–induced HDAC5 export. Next, we assessed the ability of the normal global Ca^{2+} transients that accompany each heartbeat to trigger HDAC5 nuclear export. Figure 4A shows simultaneous measurements of nuclear HDAC5 and Ca^{2+} transients in the cytosol and nucleus. Electrically stimulated Ca^{2+} transients (at either 0.5 or 1 Hz) failed to induce detectable nuclear HDAC5 export (Figure 4B). In permeabilized myocytes where $[Ca^{2+}]_i$ is controlled, HDAC5 remained nuclear for at least 60 minutes at resting $[Ca^{2+}]_i$ equal to 100 nM, and only when $[Ca^{2+}]_i$ was chronically elevated to 500 nM (which does not happen in the living cell) was HDAC5 slowly exported (to half the extent induced by ET-1; Figure 4C). Activation of HDAC5 nuclear export was thus isolated from the physiological swings of bulk cytosolic and nuclear $[Ca^{2+}]$. This is consistent with results of Schneider and his colleagues (24), who showed in skeletal muscle that pacing did not cause HDAC5 nuclear export or CaM nuclear import (24). However, they did show that pacing (10 Hz train stimulation) activated HDAC4 nuclear export and nuclear import of nuclear factor of activated T cells (NFAT) (24, 25). This may be due to the different local Ca^{2+} signal decoding in translocation of HDAC4 and NFAT versus HDAC5 and CaM (and skeletal muscle coding may differ from heart).

This failure of HDAC5 export to respond to global pulsatile $[Ca^{2+}]_i$ elevations is reminiscent of the well-accepted local control of SR Ca^{2+} release during cardiac ECC. There, local Ca^{2+} current activation is required at each junction where sarcolemma couples with SR, allowing each junction to function independently (15, 20). The local $[Ca^{2+}]_i$ near the Ca^{2+} channel mouth (>20 μM) can activate local SR Ca^{2+} release, but $[Ca^{2+}]_i$ declines as it diffuses from the source such that global $[Ca^{2+}]_i$ (even at the Ca^{2+} transient peak, approximately 1 μM) is not at the threshold for triggering SR Ca^{2+} release at junctions where no Ca^{2+} channel opens (see Figure 4D) (20, 26). The relative insensitivity of HDAC5 export to global Ca^{2+} transients observed here would be expected if high local $[Ca^{2+}]_i$ extremely close to the $InsP_3R$ is likewise needed to trigger the cascade of events leading to HDAC5 nuclear export.

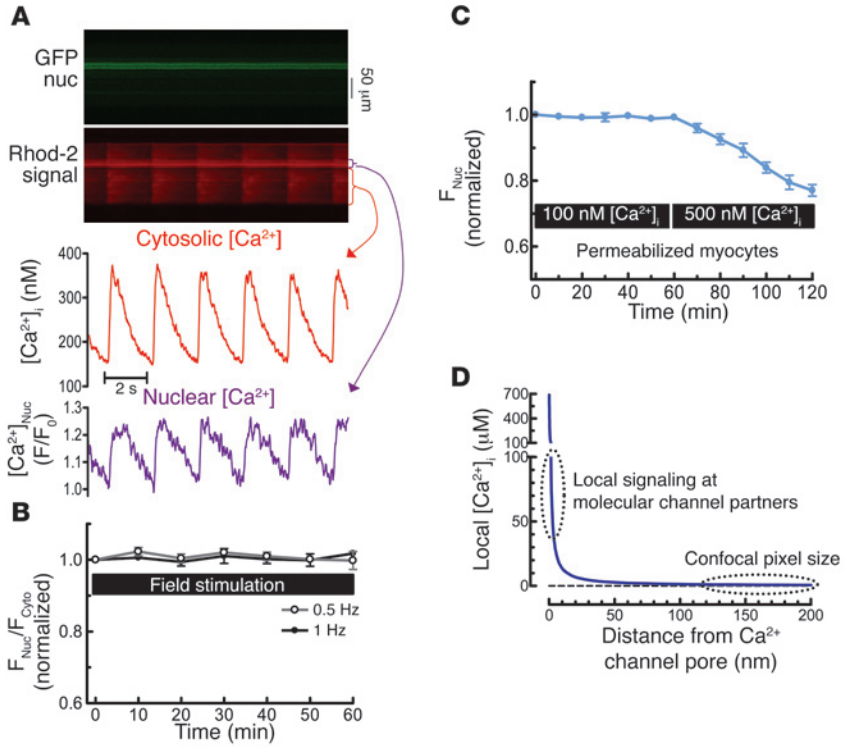

Figure 4
HDAC5 nuclear export is Ca^{2+} dependent but not activated by global Ca^{2+} transients. (**A**) Adenoviral HDAC5-GFP–infected myocytes were also loaded with Rhod-2 to measure $[Ca^{2+}]_i$. Rhod-2 is more concentrated in the nucleus (nuc), complicating Ca^{2+} transient calibration there. (**B**) HDAC5 remained nuclear when myocytes were field stimulated at 0.5 Hz ($n = 5$) or 1 Hz ($n = 7$) for 60 minutes or even at 2 Hz (not shown). (**C**) In permeabilized myocytes, internal $[Ca^{2+}]$ was increased from 100 nM to 500 nM (at 60 minutes) ($n = 7$). 2,3 butanedione monoxime (5 mM) was used to prevent contraction. (**D**) Expected local $[Ca^{2+}]$ gradient around the mouth of an $InsP_3R$ Ca^{2+} channel: $[Ca^{2+}]_j = [Ca^{2+}]_{Init} + q/(2\pi Dr) \times \text{erfc}\{r/(2\sqrt{Dr})\}$, where q = single channel current (0.1 pA), D = diffusion coefficient (600 μm²/s), $[Ca^{2+}]_{Init}$ = 100 nM, erfc = complementary error function, and r = radial distance from the channel mouth for hemispheric diffusion. This is the steady state, achieved in approximately 10 μs without buffering and much less than 1 ms when local Ca^{2+} buffering is included (26).

HDAC5 phosphorylation is required for this effect because the broad spectrum serine/threonine protein kinase inhibitor K-252a completely abolished HDAC5 export (Figure 5A) despite the fact that PKC inhibition had no effect (Figure 3A). Furthermore, the nonphosphorylatable mutant HDAC5-S/A-GFP (where both regulatory phosphorylation sites on HDAC5 were mutated to alanine) was not exported from the nucleus in response to ET-1 stimulation (Figure 5B).

CaMKII inhibition by KN-93 inhibited HDAC5 export, but only by approximately 50% (Figure 5, C and D). To further test the role of nuclear CaMKII, we used mouse myocytes isolated from transgenic mice in which a CaMKII autocamtide inhibitory peptide (AIP) was targeted to the nucleus via a nuclear localization signal (AIP-NLS; Figure 5D). In the WT littermates, ET-1–induced HDAC5 nuclear export was similar to that in the other WT mice and rabbits used here. However, in the AIP-NLS mice, ET-1–induced HDAC5 nuclear export was inhibited approximately 50% (Figure 5D). This is comparable in extent to the KN-93 effect in rabbit and is most consistent with the critical CaMKII action being inside the nucleus. Collectively, these data implicate CaM and nuclear CaMKII in HDAC export.

While CaMK is an HDAC kinase (12), it was recently reported that protein kinase D (PKD) was the primary mediator of HDAC5 phosphorylation in cultured neonatal rat ventricular myocytes stimulated with ET-1 (31). Figure 5C shows that inhibition of PKD with Gö6976 also inhibited HDAC5 by 50% (comparable to CaMKII inhibition), and when combined with KN-93, completely prevented HDAC5 nuclear export. This suggests that CaMKII and PKD both contribute to the ET-1–induced HDAC5 nuclear export in adult rabbit myocytes. Notably, PKD and CaMKII show similarities in terms of active sites and substrate specificity (32, 33); thus, these kinases may both contribute to HDAC5 phosphorylation in vivo.

Importantly, the volume sensed in a single confocal pixel ($150 \times 150 \times 1000$ nm = 2.3×10^7 nm³) is more than 10,000 times larger than a 20 nm hemisphere (2100 nm³) around the Ca^{2+} channels involved in either ECC or nuclear $InsP_3R2$ (where the Ca^{2+}-dependent proteins are). This means that saturation of Ca^{2+} indicator in this small volume can be impossible to see using optical methods (0.01% change in signal), although it physically must occur.

CaM and protein kinases are involved in ET-1–induced HDAC5 export. We also explored downstream links between Ca^{2+} release from $InsP_3R$ and HDAC phosphorylation. CaM and CaMKII associate with the $InsP_3R$ (19, 27), and prior work has focused on how they regulate the $InsP_3R$ (19, 27–30) (e.g., CaMKII phosphorylates the $InsP_3R2$, which feeds back on channel gating; ref. 19). We hypothesize that Ca^{2+} released by the $InsP_3R$ and consequent activation of CaM and CaMKII mediate local Ca^{2+} signaling to other downstream targets such as HDAC. In support of this possibility, pretreatment of myocytes with the CaM antagonist W-7 completely prevented ET-1–induced HDAC5 nuclear export (Figure 5A), implicating CaM as a local mediator.

CaMKII activated directly by Ca^{2+}-CaM can then be autophosphorylated, resulting in sustained CaMKII activation, even when $[Ca^{2+}]_i$ declines (34). Exposure of resting myocytes to ET-1 (Figure 6A) induced CaMKII autophosphorylation, and adenophostin (the $InsP_3R$ agonist) had the same effect in permeabilized myocytes (again, without detectable global or local $[Ca^{2+}]_i$ elevation). Both effects were completely blocked by the $InsP_3R$ inhibitor 2-APB. Thus, CaMKII activation, like HDAC export, appears to be mediated through Ca^{2+} release from $InsP_3$-sensitive stores.

Figure 5D summarizes HDAC5 export data, obtained at 1 hour of ET-1 exposure in the presence of various inhibitors (or activators alone) in rabbit and mouse myocytes. Most aspects of our working hypothesis (Figure 1) are supported.

Does ET-1 stimulate Ca^{2+} store–dependent activation of MEF2? To test whether Ca^{2+}-dependent HDAC5 nuclear export activates transcription, we used a MEF2-luciferase reporter adenovirus

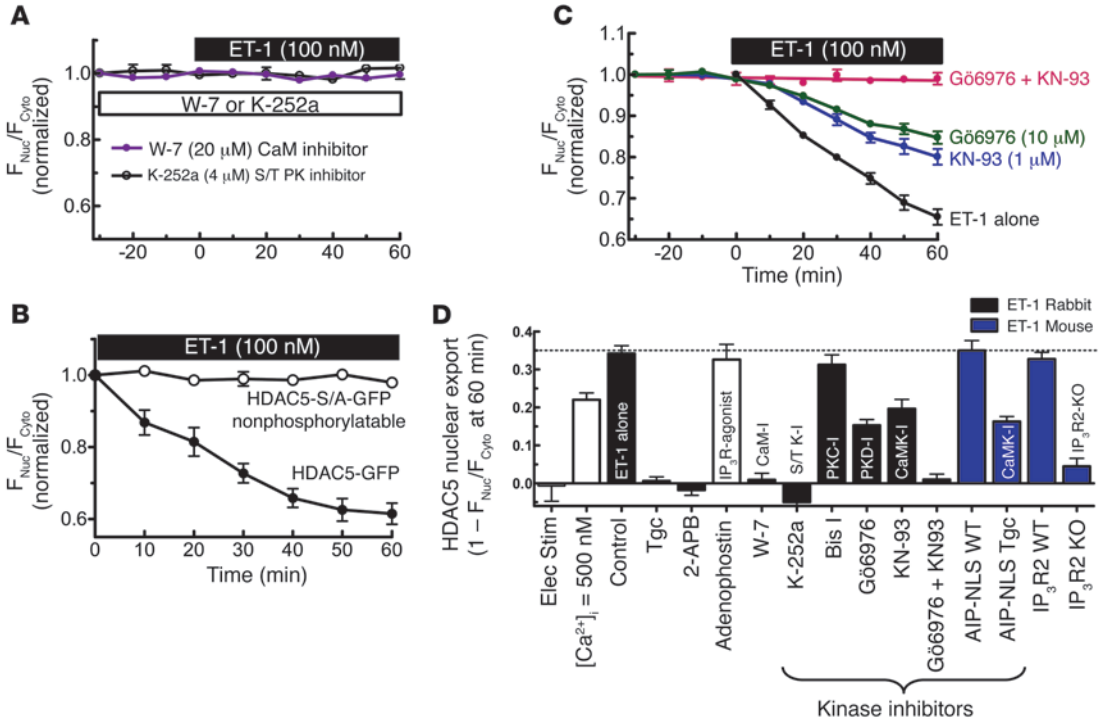

Figure 5
Role of CaM and protein kinase in ET-1–induced HDAC5 nuclear export. (**A**) Myocytes were preincubated for 30 minutes with 4 μM K-252a ($n = 8$) or 20 μM W-7 ($n = 7$) followed by ET-1 (100 nM) application. (**B**) ET-1–induced nuclear export of HDAC5-GFP versus mutant nonphosphorylatable HDAC5 (HDAC5-S/A-GFP; $n = 4$). (**C**) Adv-GFP-HDAC5–infected myocytes were pretreated with 1 μM KN-93 ($n = 6$) or 10 μM Gö6976 ($n = 8$), separately or simultaneously for 20–30 minutes followed by ET-1 application. (**D**) Summary of HDAC5 nuclear export under different conditions. Elec stim, electrical stimulation; S/T PK, serine/threonine protein kinase; Tgc, transgenic.

expressed in adult rabbit ventricular myocytes (Figure 6B). ET-1 increased luciferase expression after 1 hour of exposure (and 24 hours after incubation to allow accumulation of gene product). This effect was completely prevented by pretreatment with TG, demonstrating that intracellular Ca^{2+} stores are required. Coexpression with HDAC5-GFP reduced the MEF2 reporter expression overall, but the TG-sensitive stimulation by ET-1 to approximately 300% was unaltered. Thus, basal MEF2 activation is repressed when we express exogenous HDAC5-GFP. This is also a useful internal control experiment, that is, it means that the HDAC5-GFP we express is (a) functional (repressing MEF2) and (b) not excessive enough to shut down the control under study. These results again suggest that Ca^{2+} release from stores is required for potent ET-1–induced MEF2 transcriptional activation and that it is HDAC5 sensitive.

CaM also translocates to the nucleus upon ET-1 exposure. In neurons, CaM can also be translocated to the nucleus, where it can participate in transcriptional regulation (35). In our case, CaM translocation to the nucleus could synergize with the ET-1–induced activation (e.g., by enhancing nuclear CaMKII activity). We measured CaM movements in adult rabbit ventricular myocytes after ET-1 treatment or electrical pacing (Figure 6C). After 30 minutes of ET-1 exposure in intact resting myocytes, there was a significant shift of CaM from cytosol to nucleus (measured by immunofluorescence), but pacing at 1 Hz for 30 minutes or even 60 minutes (not shown) did not cause significant CaM movement. Thus, CaM may also move to the nucleus in response to ET-1, and this could synergize with the Ca^{2+}-, $InsP_3R$-, and CaMKII-dependent signals involved in activating HDAC5 nuclear export.

Discussion

The present study provides novel compelling evidence for a working model of ET-1–induced transcriptional regulation in adult ventricular myocytes as illustrated in Figure 1. That is, ET-1 stimulates $InsP_3$ production, which activates $InsP_3R2$ on the nuclear envelope to release Ca^{2+}, causing local Ca^{2+}-dependent activation of nuclear CaMKII, HDAC5 phosphorylation, consequent HDAC5 nuclear export, and activation (derepression) of MEF2-dependent transcription. This provides a clear and unifying mechanistic explanation for how Ca^{2+}, CaM, CaMKII, and $InsP_3R$ can regulate transcription in adult ventricular myocytes in response to neurohumoral signals and potentially be involved in altered gene expression in hypertrophy and HF.

$InsP_3$ and $InsP_3R$ in ET-1–induced HDAC5 export. Many G protein–coupled receptors are known to activate phospholipases and cause production of both $InsP_3$ and DAG, the latter of which activates several PKC isoforms. While PKC activation causes many downstream effects, some of which alter transcription (36), the ET-1–induced HDAC5 nuclear export studied here was entirely attributable to $InsP_3$-dependent signaling. This is especially salient because the functional role of $InsP_3$ and $InsP_3R$ in ventricular myocytes has been a puzzle for some time. Indeed, while $InsP_3$ in SR of atrial myocytes may modulate ECC and arrhythmogenesis, in ventricu-

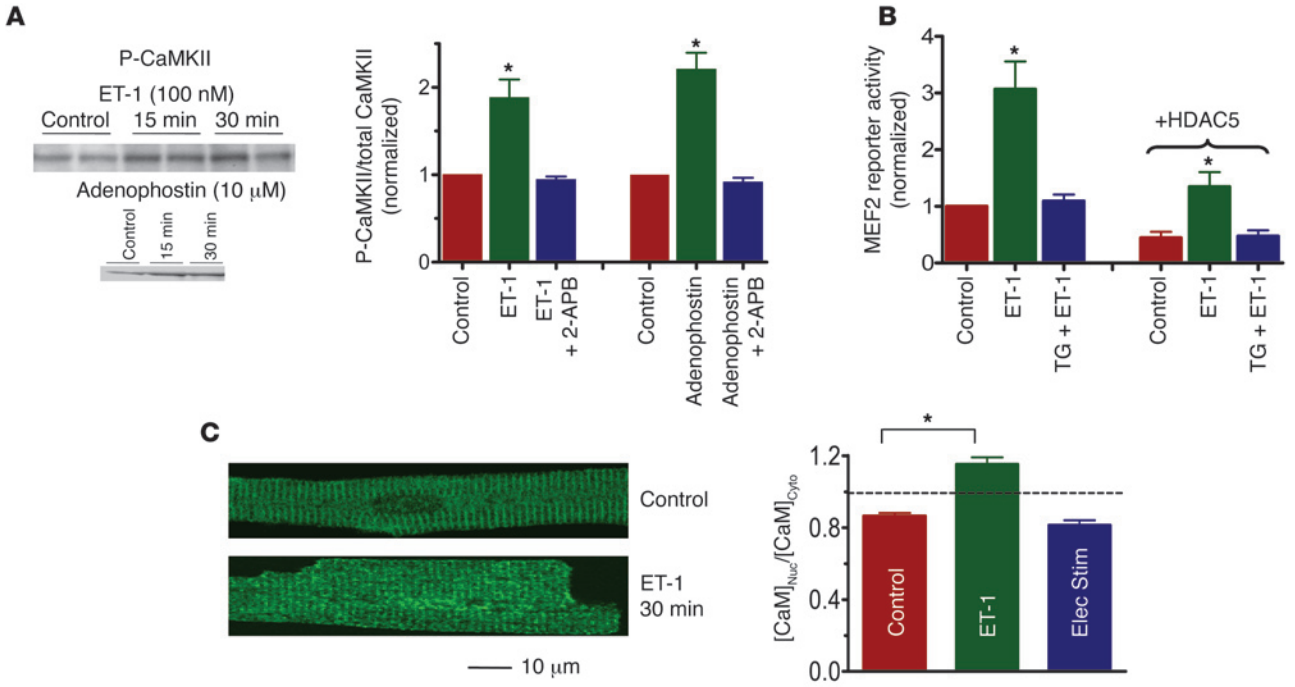

Figure 6

CaMKII autophosphorylation, MEF2 activity, and CaM translocation. (**A**) Adult rabbit myocytes were treated with 100 nM ET-1 or 10 μM adenophostin as in Figures 2B and 3C, with cells quickly frozen for CaMKII autophosphorylation measurement by Western blot ($n = 3$ for each group). (**B**) Adenoviral MEF2-luciferase reporter was expressed in rabbit ventricular myocyte (± Adv-HDAC5-GFP) and challenged for 1 hour with 100 nM ET-1 (± pretreatment for 10 minutes with 1 μM TG). Luciferase activity was normalized to control (without HDAC5-GFP). (**C**) Rabbit myocytes ± ET-1, with CaM movement detected by immunocytochemistry during rest (control), exposure to 100 nM ET-1, or electrical stimulation at 1 Hz ($n = 8$ for each group). *$P < 0.05$.

lar myocytes, $InsP_3R$ are localized to the nuclear envelope (19) and do not normally alter ECC (17, 18). We have drawn the $InsP_3R2$ facing the inside of the nucleus in Figure 1. While consistent with our data and working model and results in skeletal muscle (37), this still requires confirmation in cardiac myocytes.

One might question whether $InsP_3$ can diffuse from the plasma membrane to the nucleus. However, it should be borne in mind that ventricular myocytes have an intense transverse tubule network such that no place in the cell is far from the sarcolemma. Additionally, Remus et al. (38), using a novel fluorescent $InsP_3$ sensor, demonstrated that ET-1, angiotensin II, and phenylephrine all induce rapid increases in [$InsP_3$] in adult ventricular myocytes. They also showed that $InsP_3$ could diffuse from one end of the cell to the other (including the nucleus) and that [$InsP_3$] in the nucleus lagged only slightly behind the rise in the cytosol. Thus, it makes sense that $InsP_3$ produced at the sarcolemma can diffuse to nuclear $InsP_3R$. Indeed, Allbritton et al. (39) showed that in cells, $InsP_3$ (via diffusion) serves as a much better long-distance Ca^{2+}-signal controller than Ca^{2+} itself.

CaMKII is a local partner of nuclear $InsP_3R$. In ventricular myocytes, the $InsP_3R2$ associates with CaMKII (19), and CaM is also known to interact with $InsP_3R$ (27, 28). Moreover, activation of $InsP_3R$-associated CaMKII can phosphorylate the $InsP_3R2$ and decrease its open probability (19). This may be a negative feedback mechanism, such that Ca^{2+} released from the $InsP_3R$ activates local CaMKII, which feeds back to shut off further Ca^{2+} release from the nuclear envelope. The activated CaMKII could then go on to phosphorylate other targets. In the present study we have identified 1 downstream target of this local $InsP_3R$-dependent Ca^{2+} release and CaMKII activation (HDAC5). We also show that $InsP_3R$ activation is necessary and sufficient to trigger ET-1–induced CaMKII autophosphorylation and HDAC5 nuclear export.

Signaling may differ between neonatal and adult ventricular myocytes. The control of HDAC5 nuclear export reported here in adult rabbit and mouse ventricular myocytes differs somewhat from reports in Cos-1 cells or cultured neonatal rat ventricular myocytes (31). In those systems, PKD was dominant over CaMKII, and there was little evidence of Ca^{2+} dependence. These differences emphasize that there may be important developmental and/or cell type–specific changes in how this signaling system is controlled. This regulatory pathway might also be modified in pathophysiological states (e.g., in HF, where there is intense neurohumoral activation, and both CaMKII and $InsP_3R$ expression are greatly increased; refs. 9, 10, 40).

Our data show that ET-1–induced HDAC5 nuclear export is entirely Ca^{2+} and CaM dependent. This Ca^{2+}-CaM dependence is normal for CaMKII, but there is no prior work that shows that PKD is activated by Ca^{2+}. It is possible that Ca^{2+} (and/or CaM) is involved in this particular pathway for PKD activation or translocation, but the mechanism might still be indirect. The possible role of Ca^{2+} or CaM in the activation of PKD may require additional study (and in the appropriate cellular context).

Local versus global [Ca^{2+}] is important in activating HDAC5 translocation. It is remarkable that the activation of HDAC5 nuclear export here is entirely Ca^{2+} dependent but that global Ca^{2+} transients (rising to 0.5–1 μM), even in the nucleus, are unable to

significantly activate HDAC5 translocation. However, this is entirely consistent with very local Ca^{2+} signaling to proteins that are physically associated with the $InsP_3R$ channel (e.g., CaM and CaMKII). Indeed, very high local [Ca^{2+}] (>> 10 μM; Figure 4D) must exist around the $InsP_3R$, and the associated CaM and CaMKII must be exposed to it (note that Ca^{2+} binds to CaM with K_d values in the 1–10 μM range). This scenario is expected for CaMKII, as in other systems where CaMKII is activated by high local [Ca^{2+}] and the CaMKII activation state may sense local Ca^{2+} spikes in an integrating manner (41). This may also relate to the relatively modest affinity of Ca^{2+}-CaM for CaMKII (~200-fold lower than for calcineurin, which would be expected to sense [Ca^{2+}] differently than CaMKII).

The data here reveal a novel local Ca^{2+} control system for excitation-transcription coupling (ETC) in adult ventricular myocytes. This pathway is expected to be important in cardiac transcriptional regulation since both the initiating stimulus (ET-1) and end points studied here (HDAC5 nuclear export and MEF2 activation) have accepted roles in this process. This pathway demonstrates a long sought for and important signaling role for $InsP_3R$ in ventricular myocyte which is independent of ECC and centered at the nuclear envelope. Indeed, the local Ca^{2+} control features of this ETC pathway resemble that in ECC. A remarkable feature of ETC here is that the local Ca^{2+} signaling is relatively insulated from the large swings of cytosolic and nuclear $[Ca^{2+}]_i$ that occur at every heartbeat. This may be an extremely important way by which myocytes can use neurohumoral signaling via Ca^{2+}-dependent mechanisms without being distracted by the "noise" of beat-to-beat Ca^{2+} transients. It may also complement other Ca^{2+}- and CaM-dependent transcriptional factors, such as the calcineurin/NFAT pathway (42), which may respond to very different local or global Ca^{2+} signals than shown here for CaMKII/ HDAC5. Likewise, CaMKII may activate transcription via phosphorylating cAMP response element-binding protein (CREB), as seen in neurons, but this is not yet apparent in heart (35, 43, 44).

These experiments mechanistically explain how $InsP_3$, $InsP_3R$, CaM, and CaMKII work in concert to activate HDAC5 translocation and thus transcription in cardiac myocytes. They also provide a functional role for the robust physical association between $InsP_3R2$ and CaMKII on the nuclear envelope (19). CaMKII in this complex can thus signal transcriptional activation but also feed back by phosphorylating the $InsP_3R2$ to inhibit further Ca^{2+} release (19), creating an elegant fine tuning system. Since both CaMKII and $InsP_3R$ are upregulated in HF, this may also constitute a pathway that contributes to either the development or reinforcement of the hypertrophic or HF phenotype.

Methods

Myocyte isolation and adenoviral infection. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Loyola University Chicago Institutional Animal Care and Use Committee. Myocytes were isolated as previously described (45). Myocyte superfusate for experiments contained 140 mM NaCl; 4 mM KCl; 1 mM $MgCl_2$; 2 mM $CaCl_2$ (rabbit) or 1 mM $CaCl_2$ (mouse); 10 mM HEPES; and 10 mM glucose; pH 7.4; 23°C.

Myocytes were seeded on laminin-coated culture inserts. Culture media for rabbit were supplemented with serum-free medium 199 (M199) or in mouse with minimum essential medium containing 1% FBS. Some mouse experiments were done without 1% FBS, and results were similar. Myocytes were infected for 2 hours at MOI equal to 10 with recombinant replication-deficient adenovirus expressing HDAC5-GFP (or HDAC5-S/A-GFP, a mutant in which the critical phosphorylation sites (12) at serine 259 and 498 are mutated to alanine) under the cytomegalovirus promoter, with subsequent culture for 24–36 hours. GFP fluorescence indicated infection and localization. For MEF2-luciferase reporter experiments, cultured rabbit ventricular myocytes were infected with adenovirus encoding MEF2-luciferase gene for 48 hours total (with or without coexpression with GFP-HDAC5). After the first 24 hours, myocytes were exposed (or not) for 1 hour to 100 nM ET-1 with or without TG 1 μM pretreatment for 10 minutes before ET-1 exposure. Then, ET-1 and TG were removed and cells were incubated for another 24 hours to allow the effects of ET-1 on MEF2-luciferase expression to occur.

Genetically modified mice. The $InsP_3R2$ gene was ablated as described to generate $InsP_3R2$ KO mice (23). Transgenic mice with nuclear-targeted AIP (a specific and potent CaMKII inhibitor) were generated similarly to those in Ji et al. (46). A synthetic gene encoding 4 repeats of AIP, KKALRRQEAVDAL, was placed between a FLAG epitope, DYKDDDDK (3′), and an SV40 large T antigen nuclear location signal, PKKRKV (5′). This expression unit was cloned into a pBluescript vector between the 5.5-kb murine α-myosin heavy chain promoter (from J. Robbins, Cincinnati Children's Hospital, Cincinnati, Ohio, USA) and an SV40 polyadenylation signal. Purified linearized transgene was injected into pronuclei of fertilized mouse oocytes by the University of Cincinnati Transgenic Core Facility. Founders were bred with FVB/N WT mice for 4 generations.

Reagents. Rhod-2 and Fluo-5N were from Invitrogen Corp., ET-1 and TG from Sigma-Aldrich. W-7, 2-APB, adenophostin A, and K-252a were from Calbiochem. KN-93 was from Seikagaku Corp. and Bis I and Gö6976 from A.G. Scientific Inc. CaMKII phosphorylation–specific antibody was from Affinity BioReagents, and Western blots were as described previously (6). CaM monoclonal antibody was from Upstate USA Inc., with immunocytochemistry as described (35).

MEF2-luciferase assay. The cultured myocytes were lysed in lysis buffer (Promega). Luciferase activity was determined with the luciferase assay kit (Promega).

Ca^{2+} indicators loading and confocal imaging. Myocytes were loaded with 10 μM Rhod-2 AM for 30 minutes (15 minutes for deesterification) or with 10 μM Fluo-5N AM for 90 minutes (60 minutes for deesterification). GFP-HDAC5 signals were measured by confocal microscopy with argon laser excitation at 488 nm and emitted fluorescence (F) at 500–530 nm. Rhod-2 was excited with a HeNe laser at 543 nm with emission at greater than 570 nm, and cytosolic $[Ca^{2+}]_i$ was calibrated as follows: $[Ca^{2+}]_i = K_d(F/F_0)/(K_d/[Ca^{2+}]_{dias} + 1 - F/F_0)$, assuming K_d=710 nM, $[Ca^{2+}]_{dias}$=150 nM, and F_0=diastolic F. Fluo-5N excited at 488 nm, with emission greater than 500 nm (22).

Image-J software (http://rsb.info.nih.gov/ij/) was used for image analysis, with the intensity of regions of interest normalized to area. Confocal line scan (1-dimensional x-t) and 2-dimensional imaging were performed with a ×40 oil immersion objective with temporal resolution of 166 lines s^{-1}.

Myocyte permeabilization. Myocytes, in relaxation solution for 2 minutes, were permeabilized by adding saponin (50 μg/ml) for 20 seconds and then exposed to internal solution containing 0.5 mM EGTA; 10 mM HEPES; 120 mM K-aspartate, 1 mM free Mg; 100 nM free Ca^{2+}; 5 mM ATP; 10 mM reduced glutathione; 5 mM phosphocreatine di-tris; 5U/ml creatine phosphokinase; 8% dextran; pH 7.2. Relaxing solution was the same except EGTA was 0.1 mM and Ca^{2+} was omitted. When required, 10 μM adenophostin was added to the internal solution or free [Ca^{2+}] was changed. Low laser power (4%) was used to minimize Fluo-5N photobleaching.

Statistics. Results are mean ± SEM with significance ($P < 0.05$) determined using unpaired 2-tailed Student's *t* test.

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1. Frey, N., McKinsey, T.A., and Olson, E.N. 2000. Decoding calcium signals involved in cardiac growth and function. *Nat. Med.* **6**:1221–1227.
2. Crabtree, G.R., and Olson, E.N. 2002. NFAT signaling: choreographing the social lives of cells. *Cell.* **109**:S67–S79.
3. Zhu, W., et al. 2000. Ca^{2+}/calmodulin-dependent kinase II and calcineurin play critical roles in endothelin-1-induced cardiomyocyte hypertrophy. *J. Biol. Chem.* **275**:15239–15245.
4. Gruver, C.L., DeMayo, F., Goldstein, M.A., and Means, A.R. 1993. Targeted developmental overexpression of calmodulin induces proliferative and hypertrophic growth of cardiomyocytes in transgenic mice. *Endocrinology.* **133**:376–388.
5. Colomer, J.M., and Means, A.R. 2000. Chronic elevation of calmodulin in the ventricles of transgenic mice increases the autonomous activity of calmodulin-dependent protein kinase II, which regulates atrial natriuretic factor gene expression. *Mol. Endocrinol.* **14**:1125–1136.
6. Ramirez, M.T., Zhao, X.L., Schulman, H., and Brown, J.H. 1997. The nuclear deltaB isoform of Ca^{2+}/calmodulin-dependent protein kinase II regulates atrial natriuretic factor gene expression in ventricular myocytes. *J. Biol. Chem.* **272**:31203–31208.
7. Zhang, T., et al. 2003. The deltaC isoform of CaMKII is activated in cardiac hypertrophy and induces dilated cardiomyopathy and heart failure. *Circ. Res.* **92**:912–919.
8. Zhang, T., et al. 2002. The cardiac-specific nuclear delta(B) isoform of Ca^{2+}/calmodulin-dependent protein kinase II induces hypertrophy and dilated cardiomyopathy associated with increased protein phosphatase 2A activity. *J. Biol. Chem.* **277**:1261–1267.
9. Hoch, B., Meyer, R., Hetzer, R., Krause, E.G., and Karczewski, P. 1999. Identification and expression of delta-isoforms of the multifunctional Ca^{2+}/calmodulin-dependent protein kinase in failing and nonfailing human myocardium. *Circ. Res.* **84**:713–721.
10. Ai, X., Curran, J.W., Shannon, T.R., Bers, D.M., and Pogwizd, S.M. 2005. Ca^{2+}/calmodulin-dependent protein kinase modulates cardiac ryanodine receptor phosphorylation and sarcoplasmic reticulum Ca^{2+} leak in heart failure. *Circ. Res.* **97**:1314–1322.
11. Zhang, R., et al. 2005. Calmodulin kinase II inhibition protects against structural heart disease. *Nat. Med.* **11**:409–417.
12. McKinsey, T.A., Zhang, C.L., Lu, J., and Olson, E.N. 2000. Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. *Nature.* **408**:106–111.
13. Olson, E.N., and Schneider, M.D. 2003. Sizing up the heart: development redux in disease. *Genes Dev.* **17**:1937–1956.
14. Zhang, C.L., et al. 2002. Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. *Cell.* **110**:479–488.
15. Bers, D.M. 2002. Cardiac excitation-contraction coupling. *Nature.* **415**:198–205.
16. Perez, P.J., Ramos-Franco, J., Fill, M., and Mignery, G.A. 1997. Identification and functional reconstitution of the type 2 inositol 1,4,5-trisphosphate receptor from ventricular cardiac myocytes. *J. Biol. Chem.* **272**:23961–23969.
17. Mackenzie, L., et al. 2002. The role of inositol 1,4,5-trisphosphate receptors in Ca^{2+} signalling and the generation of arrhythmias in rat atrial myocytes. *J. Physiol.* **541**:395–409.
18. Zima, A.V., and Blatter, L.A. 2004. Inositol-1,4,5-trisphosphate-dependent Ca^{2+} signalling in cat atrial excitation-contraction coupling and arrhythmias. *J. Physiol.* **555**:607–615.
19. Bare, D.J., Kettlun, C.S., Liang, M., Bers, D.M., and Mignery, G.A. 2005. Cardiac type 2 inositol 1,4,5-trisphosphate receptor: interaction and modulation by calcium/calmodulin-dependent protein kinase II. *J. Biol. Chem.* **280**:15912–15920.
20. Bers, D.M. 2001. *Excitation-contraction coupling and cardiac contractile force.* 2nd edition. Kluwer Academic Publishers. Dordrecht, Holland. 427 pp.
21. Lipp, P., et al. 2000. Functional InsP3 receptors that may modulate excitation-contraction coupling in the heart. *Curr. Biol.* **10**:939–942.
22. Shannon, T.R., Guo, T., and Bers, D.M. 2003. Ca^{2+} scraps: local depletions of free $[Ca^{2+}]$ in cardiac sarcoplasmic reticulum during contractions leave substantial Ca^{2+} reserve. *Circ. Res.* **93**:40–45.
23. Li, X., Zima, A.V., Sheikh, F., Blatter, L.A., and Chen, J. 2005. Endothelin-1-induced arrhythmogenic Ca^{2+} signaling is abolished in atrial myocytes of inositol-1,4,5-trisphosphate(IP3)-receptor type 2-deficient mice. *Circ. Res.* **96**:1274–1281.
24. Liu, Y., Randall, W.R., and Schneider, M.F. 2005. Activity-dependent and -independent nuclear fluxes of HDAC4 mediated by different kinases in adult skeletal muscle. *J. Cell Biol.* **168**:887–897.
25. Liu, Y., Cseresnyes, Z., Randall, W.R., and Schneider, M.F. 2001. Activity-dependent nuclear translocation and intranuclear distribution of NFATc in adult skeletal muscle fibers. *J. Cell Biol.* **155**:27–39.
26. Bers, D.M., and Peskoff, A. 1991. Diffusion around a cardiac calcium channel and the role of surface bound calcium. *Biophys. J.* **59**:703–721.
27. Yamada, M., et al. 1995. The calmodulin-binding domain in the mouse type 1 inositol 1,4,5-trisphosphate receptor. *Biochem. J.* **308**:83–88.
28. Cardy, T.J., and Taylor, C.W. 1998. A novel role for calmodulin: Ca^{2+}-independent inhibition of type-1 inositol trisphosphate receptors. *Biochem. J.* **334**:447–455.
29. Michikawa, T., et al. 1999. Calmodulin mediates calcium-dependent inactivation of the cerebellar type 1 inositol 1,4,5-trisphosphate receptor. *Neuron.* **23**:799–808.
30. Missiaen, L., et al. 1999. The bell-shaped Ca^{2+} dependence of the inositol 1,4, 5-trisphosphate-induced Ca^{2+} release is modulated by Ca^{2+}/calmodulin. *J. Biol. Chem.* **274**:13748–13751.
31. Vega, R.B., et al. 2004. Protein kinases C and D mediate agonist-dependent cardiac hypertrophy through nuclear export of histone deacetylase 5. *Mol. Cell. Biol.* **24**:8374–8385.
32. Valverde, A.M., Sinnett-Smith, J., Van Lint, J., and Rozengurt, E. 1994. Molecular cloning and characterization of protein kinase D: a target for diacylglycerol and phorbol esters with a distinctive catalytic domain. *Proc. Natl. Acad. Sci. U. S. A.* **91**:8572–8576.
33. Doppler, H., Storz, P., Li, J., Comb, M.J., and Toker, A. 2005. A phosphorylation state-specific antibody recognizes Hsp27, a novel substrate of protein kinase D. *J. Biol. Chem.* **280**:15013–15019.
34. MacNicol, M., Jefferson, A.B., and Schulman, H. 1990. Ca^{2+}/calmodulin kinase is activated by the phosphatidylinositol signaling pathway and becomes Ca^{2+}-independent in PC12 cells. *J. Biol. Chem.* **265**:18055–18058.
35. Deisseroth, K., Heist, E.K., and Tsien, R.W. 1998. Translocation of calmodulin to the nucleus supports CREB phosphorylation in hippocampal neurons. *Nature.* **392**:198–202.
36. Dorn, G.W., 2nd, and Force, T. 2005. Protein kinase cascades in the regulation of cardiac hypertrophy. *J. Clin. Invest.* **115**:527–537. doi:10.1172/JCI200524178.
37. Cardenas, C., et al. 2005. Nuclear inositol 1,4,5-trisphosphate receptors regulate local Ca^{2+} transients and modulate cAMP response element binding protein phosphorylation. *J. Cell Sci.* **118**:3131–3140.
38. Remus, T.P., et al. 2006. Biosensors to measure $InsP_3$ concentration in living cells with spatio-temporal resolution. *J. Biol. Chem.* **281**:608–616.
39. Allbritton, N.L., Meyer, T., and Stryer, L. 1992. Range of messenger action of calcium ion and inositol 1,4,5-trisphosphate. *Science.* **258**:1812–1815.
40. Go, L.O., et al. 1995. Differential regulation of 2 types of intracellular calcium release channels during end-stage heart failure. *J. Clin. Invest.* **95**:888–894.
41. De Koninck, P., and Schulman, H. 1998. Sensitivity of CaM kinase II to the frequency of Ca^{2+} oscillations. *Science.* **279**:227–230.
42. Molkentin, J.D., et al. 1998. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell.* **93**:215–228.
43. Zhang, T., and Brown, J.H. 2004. Role of Ca^{2+}/calmodulin-dependent protein kinase II in cardiac hypertrophy and heart failure. *Cardiovasc. Res.* **63**:476–486.
44. Passier, R., et al. 2000. CaM kinase signaling induces cardiac hypertrophy and activates the MEF2 transcription factor in vivo. *J. Clin. Invest.* **105**:1395–1406.
45. Bassani, J.W., Bassani, R.A., and Bers, D.M. 1995. Calibration of indo-1 and resting intracellular $[Ca^{2+}]i$ in intact rabbit cardiac myocytes. *Biophys. J.* **68**:1453–1460.
46. Ji, Y., et al. 2003. Targeted inhibition of Ca^{2+}/calmodulin-dependent protein kinase II in cardiac longitudinal sarcoplasmic reticulum results in decreased phospholamban phosphorylation at threonine 17. *J. Biol. Chem.* **278**:25063–25071.