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Crosstalk between CaMKII and ERK in the regulation of Cardiac Hypertrophy

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AC2 Autocamtide-2
ANF Atrial Natriuretic Factor
Ang II Angiotensin II
AP-1 Activation Protein 1
AT1 Angiotensin II Receptors type 1
ATF Activating Transcription Factor
ATP Adenosine Triphosphate
BSA Bovine Serum Albumin
BP Blood Pressure
BW Body Weight
Ca^{2+} Calcium
$CaCl_2$ Calcium Chloride
CaM Calmodulin
CaMK Ca^{2+}/Calmodulin-Dependent Protein Kinase
CaM-KNtide CaMKinase II Inhibitor
C/EBP CAAT-Enhancer Binding Protein
CH cardiac hiperthophy
CH_3CN Acetonitrile
CREB cAMP-Response Element Binding Protein
Ctr Control
DAG Diacyl Glycerol
DBP Diastolic Blood Pressure
DCM Dichloromethane
DIEA N,N-diisopropylethylamine
DMEM Dulbecco's Minimal Essential Medium
DMF N,N-dimethylformamide
DTT Dithiothreitol
ECM Extracellular Matrix
EDTA Ethylenediaminetetraacetic Acid
EF-2 Elongation Factor 2
EGF Epidermal Growth Factor
EGFR Epidermal Growth Factor Receptor
EGTA Ethylene Glycol Tetraacetic Acid
ERK Extracellular Regulated Kinase
ESI-MS Electrospray Ionization Mass Spectrometry
FAK Focal Adhesion Kinase
FGF Fibroblast Growth Factor
Fmoc 9-fluorenyl-methoxycarbonyl
gp 130 glycoprotein 130
HB-EGF Heparin-Binding Epidermal Growth Factor
HBTU 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro phosphate
HDAC Histone Deacetylases
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF Heart Failure
HOBt N-hydroxy-benzotriazole
HW Heart Weight
IGF Insulin-Like Growth Factor
IL-6 Interleukin 6
IL-6R Interleukin 6 Receptor
$Ins(1,4,5)P_3$ Inositol-1,4,5-Trisphosphate
IVS Interventricular Septum
KO Knockout
LIF Leukemia Inhibitory Factor
LVEDD Lelf Ventricular end-diastolic diameters
LVEF Lelf Ventricular Ejection Fraction
LVES Lelf Ventricular fractional shortening
LVESD Lelf Ventricular end-systolic diameters
LVH Left Ventricular Hypertrophy
LVM Left Ventricular Mass
MEF-2 Myocyte Enhancer Factor 2
MLCK Myosin Light Chain Kinase

NLS Nuclear Localization Signals
PBS Phosphate Buffer Saline
PE Phenylephrine
PLCβ phospholipase Cβ
PKA Protein Kinase A
PKC Protein Kinase C
PP Protein Phosphatase
PW Posterior Wall
RP-HPLC Reversed-Phase High Performance Liquid Chromatography
RT-PCR Real-Time Polymerase Chain Reaction
SBP Systolic Blood Pressure
SDS sodium dodecyl sulfate
SHR Spontaneously Hypertensive
SRF Serum Response Factor
TAC Transverse Aortic-Constricted
Tat 35-amino-acid sequence from the HIV Tat protein
TGF-β Transforming Growth Factor-Beta
TIS Triisopropylsilane
t_R Retention Time
WB Western Blot
WT Wild Type
WKY Wistar Kyoto

ABSTRACT

Aims: Activation of Ca2+/Calmodulin protein kinase II (CaMKII) is an important step in signaling of cardiac hypertrophy. The molecular mechanisms by which CaMKII integrates with other pathways to develop cardiac hypertrophy are incompletely understood. We hypothesize that CaMKII association with extracellular regulated kinase (ERK), promotes cardiac hypertrophy through ERK nuclear localization.

Methods and results: In H9C2 cardiomyoblasts, the selective CaMKII peptide inhibitor AntCaNtide, "its penetratin" conjugated minimal inhibitory sequence analog tat-CN17β, and the MEK/ERK inhibitor UO126 all reduce phenylephrine (PE)-mediated ERK and CaMKII activation and their interaction. Moreover, AntCaNtide or tat-CN17β pretreatment prevented PE induced CaMKII and ERK nuclear accumulation in H9C2s and reduced the hypertrophy responses. To determine the role of CaMKII in cardiac hypertrophy in vivo, spontaneously hypertensive rats were subjected to intramyocardial injections of AntCaNtide or tat-CN17β. Left ventricular hypertrophy was evaluated weekly for 3 weeks by cardiac ultrasounds. We observed that the treatment with CaMKII inhibitors induced similar but significant reduction of cardiac size, left ventricular mass, and thickness of cardiac wall. The treatment with CaMKII inhibitors caused a significant reduction of CaMKII and ERK phosphorylation levels and their nuclear localization in the heart.

Conclusion: These results indicate that CaMKII and ERK interact to promote activation in hypertrophy; the inhibition of CaMKII-ERK interaction offers a novel therapeutic approach to limit cardiac hypertrophy.

1. BACKGROUND

1.1 Cardiovascular Hypertrophy

Cardiovascular disease remains the number one cause of mortality in the Western world, with heart failure representing the fastest growing subclass over the past decade (Kannel et al. 2000; Hobbs 2004; Levy et al. 2002; Zannad et al. 1999).

Heart failure is defined as a deficiency in the capability of the heart to adequately pump blood in response to systemic demands, which results in premature fatigue, dyspnoea, and/or oedema. It is typically induced by a number of common disease stimuli, including: long-standing hypertension; myocardial infarction or ischemia associated with coronary artery disease; valvular insufficiency and stenosis; myocarditis due to an infectious agent; congenital malformations; familial hypertrophic and dilated cardiomyopathies; and diabetic cardiomyopathy (Klein, L. et al. 2003; Lips et al. 2003). Most of these stimuli first induce a phase of cardiac hypertrophy in which individual myocytes grow in length and/or width as a means of increasing cardiac pump function and decreasing ventricular wall tension (inducing a state of 'compensated hypertrophy') (Berenji et al. 2005; Haider et al. 1998). However, in the long term, myocardial hypertrophy predisposes individuals to heart failure, arrhythmia and sudden death.

Cardiac hypertrophy is an adaptive response to pressure or volume stress, mutations of sarcomeric (or other) proteins, or loss of contractile mass from prior infarction. Hypertrophic growth accompanies many forms of heart disease, including ischemic disease, hypertension, heart failure, and valvular disease. In these types of cardiac pathology, pressure overload-induced concentric hypertrophy is believed to have a compensatory function by diminishing wall stress and oxygen consumption (Sandler and Dodge 1963; Rackley and Rolett 1968; Grossman et al. 1975). At the same time, ventricular hypertrophy is associated with significantly increased risk of heart failure and malignant arrhythmia (Levy D et al. 1990; Koren et al. 1991).

In the 1960s, Meerson and colleagues (Meerson 1961) divided hypertrophic transformation of the heart into 3 stages: (1) developing hypertrophy, in which load exceeds output, (2) compensatory hypertrophy, in which the workload/mass ratio is normalized and resting cardiac output is maintained, and (3) overt heart failure, with ventricular dilation and progressive declines in cardiac output despite continuous activation of the hypertrophic program. The late-phase “remodeling” process that leads to failure is associated with functional perturbations of cellular Ca2+ homeostasis (Bers 2002) and ionic currents, (Armoundas et al. 2001; Hill 2003) which contribute to an adverse prognosis by predisposing to ventricular dysfunction and malignant arrhythmia. Significant morphological changes include increased rates of apoptosis (Haunstetter and Izumo 2000), fibrosis, and chamber dilation. Even though the dichotomy between adaptive and

maladaptive hypertrophy has been appreciated for more than a century, (Osler 1892) mechanisms that determine how long-standing hypertrophy ultimately progresses to overt heart failure are poorly understood.

At the cellular level, cardiomyocyte hypertrophy is characterized by an increase in cell size, enhanced protein synthesis, and heightened organization of the sarcomere. Classically, 2 different hypertrophic phenotypes can be distinguished: (1) concentric hypertrophy due to pressure overload, which is characterized by parallel addition of sarcomeres and lateral growth of individual cardiomyocytes, and (2) eccentric hypertrophy due to volume overload or prior infarction, characterized by addition of sarcomeres in series and longitudinal cell growth (Dorn et al. 2003). At the molecular level, these changes in cellular phenotype are accompanied by reinduction of the so-called fetal gene program, because patterns of gene expression mimic those seen during embryonic development.

Hypertrophy that occurs as a consequence of pressure overload is termed “compensatory” on the premise that it facilitates ejection performance by normalizing systolic wall stress. Recent experimental results, however, call into question the necessity of normalization of wall stress that results from hypertrophic growth of the heart. These findings, largely from studies in genetically engineered mice, raise the prospect of modulating hypertrophic growth of the myocardium to afford clinical benefit without provoking hemodynamic compromise.

To accomplish this goal, it is essential to identify molecular events involved in the hypertrophic process (Frey and Olson 2003; Akazawa and Komuro 2003), and to identify commonalities and differences in the signaling systems that promote pathological hypertrophy versus physiological hypertrophy (Katz 1990). Especially critical is elucidation of mechanisms underlying the maladaptive features of hypertrophy, such as arrhythmogenicity and transformation to heart failure.

Biomechanical stress can be produced in the myocardium by means of 2 types of noxious events: mechanical stretch and the release of neurohormonal factors (Díez et al. 2001). The mechanical stress induced by the physical stretching of adult and neonatal cardiomyocytes is sufficient to induce a phenotypic and genetic hypertrophic response, even in the absence of humoral and neuronal factors (Sadoshima and Izumo 1997). Despite its pathophysiological importance, little is known about how this biomechanical stress is perceived by the cardiomyocyte and how this is translated into prohypertrophic intracellular signals. Although the mechanical stimulus activates multiple messengers and intracellular signaling pathways, we do not know for certain which are activated directly and which are stimulated through other agents. Nor is the time sequence of the events clear since the initial moment of second messenger activation has not been determined systematically.

It is traditionally assumed that, in order for a mechanosensitive molecule to quantify plasma membrane stress, there should be some type of

interaction between the two. Stress has long been recognized as a biochemical regulator of the activity of many types of cells such as, for example, the skeletal myocyte (Huxley 1974), in which the force generated on the membrane is sufficient to alter a number of enzyme activities (Sheetz 2001). As a result, several types of molecules have been proposed as candidates to fulfill this role, in particular the ion channels (Matsuda et al. 1996; Sasaki et al. 1994), integrins (Ross and Borg 2001), and tyrosine kinases (Sadoshima et al. 1996). The sarcomeric Z disc has been the object of increasing interest as a candidate to carry out the task of mechanotransduction for several years now (Epstein and Davis 2003; Knoll et al. 2002). Mouse cardiomyocytes lacking the MLP protein (muscle LIM protein), which is normally present in the Z disc, exhibit a selective absence of the response to mechanical stretch, but respond normally to G protein-coupled receptor agonists; on the other hand, in humans, a MLP gene mutation results in telethonin/ T-cap bond breakage, which leads to dilated cardiomyopathy (Knoll et al. 2002; Frey et al. 2000) recently reported a novel family of proteins specific for sarcomeric Z-disc of striated muscle, referred to as calsarcins, which are capable of interacting with both telethonin/T-cap and calcineurin, a circumstance that indicates a possible role as a link between the capture of the mechanical stimulus and hypertrophic signaling.

Despite our limited knowledge concerning its receptors and transduction pathways, the importance of the mechanical stimulus in triggering the hypertrophic process is supported by a large body of evidence from studies of different types. In addition, it is now accepted that mechanical stress acts as the initial stimulus in the series of events that results in in vivo myocyte growth (Komuro 2001). This has led to the search for better experimental models that enable us to faithfully mimic the in vivo situation of the cardiomyocyte, resulting in the availability of a considerable number of methodological approaches for the mechanical stimulation of these cells.

The changes in the myocardium of a hypertensive patient occur not only in left ventricle, which is directly exposed to the hemodynamic overload, but in the interventricular septum and right ventricle, as well (Díez et al. 2001). On the other hand, the infarcted myocardium presents phenotypic alterations in regions far from the necrosed tissue. This evidence indicates that, together with the mechanical component that acts in the myocardial region that is directly subjected to stress, humoral factors intervene, exerting their action in a more diffuse manner (Díez et al. 2001). There are a number of humoral factors that can act as a hypertrophic stimulus in the cardiomyocyte, and the consequences of their activation differ. A first group consists of the growth factors (transforming growth factor-beta [TGF-β , fibroblast growth factor [FGF], and insulin-like growth factor [IGF], among others) (Oudit et al. 2004; Lupu et al. 2001) which, through their action in membrane receptors with tyrosine kinase activity, activate an intracellular second messenger cascade which ultimately leads to a normal growth pattern, characteristic of postnatal myocyte growth

(also referred to as eutrophy) and of physiological or adaptive hypertrophic growth.

A second type of humoral stimulus involved in cardiomyocyte growth comes from the stimulation of heptahelical G-protein-coupled receptors, particularly the beta 1-adrenergic receptors for epinephrine and norepinephrine, the AT1 receptor for angiotensin II (Ang II) and the ET receptor for endothelin-1, all of which have been related to the development of pathological CH and its eventual progression to heart failure (Molkentin and Dorn 2001; Hunter and Chien 1999). In experimental models and clinical syndromes of heart failure, antagonists and synthesis inhibitors of these receptors have modulated the hypertrophic response and improved the prognosis (Mehra et al. 2003). Of these receptors, that most widely studied has been Ang II. In fact, it is now accepted that, together with its regulation of the vasomotor tone and extracellular fluid, the interaction of Ang II with its type 1 receptors (AT1) mediates the response of the myocardial cells to biomechanical stress, involving both those that lead to hypertrophy and those implicated in remodeling (Kajstura et al. 1995). It should be pointed out that, while the different heptahelical receptor agonists share a common signaling pattern, there are considerable differences in the cardiac response to these agents, and individual roles for catecholamines versus renin-angiotensin in the cardiac hypertrophic response still remain to be defined (Dorn and Force 2005).

Both the cardiomyocytes and the blood vessels possess mineralocorticoid receptors, the stimulation of which can activate important physiological and pathological mechanisms. Peripheral infusion of aldosterone in rats receiving a sodium-rich diet leads to cardiac hypertrophy and fibrosis, an effect that is independent of the increased arterial pressure (Young et al. 1995). The mechanism responsible for CH secondary to aldosterone administration is being investigated. Aldosterone has been reported to increase AT1 receptor density, involving both the mRNA and cardiovascular tissue protein (Robert et al. 1999), and that treatment with losartan partially prevents aldosterone-induced CH and myocardial fibrosis in rats (Takeda et al. 2002). On the other hand, aldosterone also appears to be related to an increase in the activity of the phosphatase, calcineurin, which, as will be dealt with later on, is implicated in the hypertrophic process; the treatment of rats with FK506, a calcineurin inhibitor, partially prevents the prohypertrophic effects of aldosterone. Moreover, a number of studies indicate that aldosterone has a direct profibrotic effect, which would be related to the activation of inflammatory mediators and necrotic changes (Takeda 2004; Rocha and Funder 2002; Brilla et al. 1990). These events appear to be responsible, at least in part, for the clinical effect of eplerenone, a selective mineralocorticoid receptor antagonist, in patients with heart failure and left ventricular dysfunction secondary to acute myocardial infarction (Pitt et al. 2003).

Inflammatory cytokines have been the object of increasing interest in the field of cardiovascular research. Through their local action, they play a role not only in the pathogenesis of atherosclerosis, but in the cardiac dysfunction

that accompanies systemic sepsis, viral myocarditis and other diseases, as well (Blum and Miller 2001; Wollert and Drexler 2001).

One of the cytokines that has received the most attention is interleukin-6 (IL-6) (also known as cardiotropin 1), a 185-amino acid polypeptide that has a number of biological functions, both proinflammatory and antiinflammatory, mediated by its binding to its receptor (IL-6R) and to glycoprotein 130 (gp 130) (Kanda and Takahashi 2004). Results relating IL-6 to the development of CH have been published for a number of years. In this regard, it has been reported that neonatal cardiomyocytes cultured in vitro increase in size when exposed to increasing doses of IL-6, and that overactivation of the gp 130 signaling pathway leads to CH in mice (Hirota et al. 1995). These findings have been extended in later works in which an increase in IL-6 expression has been reported in the myocardium of patients with CH who died from myocardial infarction, an increase which would appear to be related to the pathogenesis of the observed hypertrophy (Kaneko et al.1997).

It is essential to stress the fact that, here, the different stimuli have been described separately for explanatory reasons, but that this separation does not exist in the real pathophysiological situation, in which there is an intricate network of interconnections among these elements, a circumstance that complicates their study considerably and requires caution when interpreting the reported phenomena. On the other hand, we should not overlook the fact that two thirds of the cells of the normal heart are nonmuscle cells, mostly fibroblasts (Maisch 1995; Weber 2004), whose role in the pathogenesis of several pathological processes, such as CH and myocardial remodeling, appears to be crucial (Creemers et al. 2001; Weber et al.1989). In response to different types of stress, these cells present phenotypic changes, with the expression of markers characteristic of smooth muscle cells. For this reason, they are referred to as myofibroblasts, which produce and release substances such as growth factors, cytokines, extracellular matrix (ECM) proteins and proteases (Tomasek et al. 2002; Powell et al.1999). Both fibroblasts and myofibroblasts play an important role in regulating the synthesis and degradation of the ECM, the composition and amount of which is the result of a fine balance between collagen synthesis (and, to a lesser extent, the synthesis of other proteins) and the activity of metalloproteases and tissue inhibitors of metalloproteases (Manabe et al. 2002). The disturbance of this delicate balance can lead to fibrotic changes that, together with CH, fibroblast proliferation and cell death, constitute the phenomenon of remodeling (Swynghedauw 1999).

The "dialogue" between fibroblasts and cardiomyocytes appears to play a role in both the physiological situation and the pathogenesis of CH and other diseases. For example, the mechanical stretch of cardiomyocytes induces Ang II release which, in turn, provokes the release of a number of growth factors and cytokines on the part of fibroblasts and myofibroblasts, which affect themselves through autocrine mechanisms, and other cell types, including cardiomyocytes, in a paracrine manner. Thus, the available evidence indicates that, just as the paracrine agents released by cardiac fibroblasts are relevant in

the induction of the hypertrophic response of the cardiomyocyte, the paracrine activities of the latter are also important for the regulation of fibroblast function (Vatner et al. 2000; Matsusaka et al. 1999). Moreover, the heart has other cell types, like endothelial cells, pericytes, smooth muscle cells and immune system cells, which also participate in this communications network and probably play a role in the pathogenesis of myocardial remodeling, which, in turn, can affect the course and progression of CH to heart failure (Braunwald and Bristow 2000).

1.2 Intracellular Signaling pathway

1.2.1 Activation of G-protein-coupled receptors.

Angiotensin II,endothelin-1 and catecholamines (α-adrenergic) bind to specific seven-transmembrane-spanning receptors that are coupled to heterotrimeric G proteins of the Gαq/α11 subclass. These G proteins are coupled to phospholipase Cβ (PLCβ). This coupling induces the generation of diacyl glycerol (DAG), which functions as an intra cellular ligand for protein kinase C (PKC), leading to PKC activation, and production of inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$). Accumulation of $Ins(1,4,5)P_3$ leads to the mobilization of internal Ca^{2+} by directly binding to the $Ins(1,4,5)P_3$ receptor located in the endoplasmic reticulum or the nuclear envelope. Recently, the generation of $Ins(1,4,5)P_3$ and the associated liberation of internal Ca^{2+} stores was shown to mediate hypertrophic signaling through calcineurin–NFAT activation or calmodulin dependent kinase (CaMK)–HDAC inactivation (Wilkins and Molkentin 2004; Wu et al. 2006). A landmark study showing that a compartmentspecific pool of Ca^{2+} near the nuclear envelope regulates CaMK activity and HDAC nuclear cytoplasmic shuttling through Ins(1,4,5)P3-receptor-regulated Ca^{2+}release. Activation of Gαq/α11 is also a potent inducer of MAPK signalling in cardiac myocytes, although the exact mechanism of MAPK coupling has not been described (Clerk and Sugden 1999). Studies in genetically modified mouse models have confirmed that Gαq/α11 coupling is a necessary event in the induction of pathological cardiac hypertrophy, whereas IGF-I signalling regulates physiological cardiac hypertrophy, as well as the growth of the heart during normal development (see below). The overexpression of wild-type Gαq (or an activated form of Gαq) in the heart induced a uniform profile of cardiac hypertrophy that resulted in heart failure and showed increased propensity towards apoptosis (Adams et al. 1998; D'Angelo et al. 1997). Shows that simple overexpression of the Gαq subunit of the heterotrimeric G protein was sufficient to drive pathological cardiac hypertrophy in the mouse (Fan et al. 2005; Mende et al. 1998). In support of these results, transgenic mice that express a 54-amino-acid Gαq/α11-inhibitory peptide in the heart showed specific attenuation of pressure-overload hypertrophy and downregulation of MAPK signaling (Akhter et al. 1998). First study showing that Gαq/α11 protein function is necessary for mediating cardiac

hypertrophy in an animal model of pressure-overload-induced hypertrophy (Esposito et al. 2002).

Shows that inhibition of pressure-overload hypertrophy through two separate means in genetically altered mice did not lead to decompensation and heart failure, indicating that hypertrophy might not be necessary for cardiac compensation. The combined disruption of the genes that encode the Gαq - and Gα11-subunits also compromised the capability of the myocardium to undergo hypertrophy following pressure overload (Wettschureck et al. 2001). Heart specific and combined disruption of the genes that encode Gαq and Gα11 inhibited the cardiac hypertrophic response following pressure-overload stimulation.]. Similarly, the overexpression of the GTPase-activating protein RGS4 (regulators of G-protein signalling) in the heart, which inactivates Gαi - and Gαq –subfamily members, reduced pressure-overload-induced cardiac hypertrophy (Rogers et al. 1999). Inhibition of the hypertrophic response in these models (with the exception of the RGS4 transgenic mice) did not impair cardiac function, indicating that Gαq -dependent hypertrophy is not required for functional compensation (Akhter et al. 1998). First study showing that Gαq/α11 protein function is necessary for mediating cardiac hypertrophy in an animal model of pressure-overload-induced hypertrophy (Esposito et al. 2002). Shows that inhibition of pressure-overload hypertrophy through two separate means in genetically altered mice did not lead to decompensation and heart failure, indicating that hypertrophy might not be necessary for cardiac compensation (Wettschureck et al. 2001). Heart specific and combined disruption of the genes that encode Gαq and Gα11 inhibited the cardiac hypertrophic response following pressure-overload stimulation.Therefore, inhibiting specific GPCRs or the Gαq/α11-subunits themselves are attractive therapeutic strategies for treating pathological cardiac hypertrophy. GPCR agonists were also shown to induce cardiac hypertrophy through the shedding of heparin-binding epidermal growth factor (HB-EGF) by the metalloprotease ADAM12 (Asakura et al. 2002). In this manner, EGF could then signal through the EGF receptor (EGFR) to induce hypertrophy in conjunction with downstream signaling effectors. Similar to this paradigm, the receptor tyrosine kinase ERBB2 heterodimerizes with ERBB3 or ERBB4 and binds to neuregulin, whereby it transmits a signal by itself or through transactivation by EGFR, gp130, and GPCRs (Gschwind et al. 2001). Treatment with Herceptin, a recombinant antibody that blocks ERBB2 signalling (Garratt et al. 2003), as well as the cardiac-specific deletion of ERBB2 both lead to cardiomyopathy, showing the critical importance of this signaling effector in the maintenance of myocyte integrity and growth (Crone et al. 2002; Ozcelik et al. 2002).

1.2.2 Mek1 And 2 Extracellular Signal-Regulated Kinase1 And 2 Pathway

Two separate ERK isoforms have been described, ERK1 and ERK2, that are coordinately phosphorylated and activated by a wide array of mitogenic stimuli (Garrington and Johnson 1999).

The major upstream activators of ERK1 and 2 MAPKs are two MAPK kinases (MAPKKs), MEK1 and MEK2, which directly phosphorylate the dual site in ERK1 and 2 (Thr-Glu-Tyr). Directly upstream of MEK1 and 2 in the MAPK-signaling cascade are the MAPKK kinase (MAPKKK) Raf-1, A-Raf, B-Raf, and MEKK1–3 (Garrington and Johnson 1999).

In response to agonist stimulation or cell stretching, ERK1 and 2 become activated both in cultured cardiac myocytes and in isolated perfused hearts (Yamazaki et al. 1993; Bogoyevitch et al. 1994.)

Endothelin-1 and fibroblast growth factors stimulate the mitogen-activated protein kinase signaling cascade in cardiac myocytes: the potential role of the cascade in the integration of two signaling pathways leading to myocyte hypertrophy (Clerk et al. 1994; Post et al. 1996; Zou et al. 1996; Bogoyevitch et al. 1996; Clerk et al. 1998). These observations have implicated ERK1- and 2-signaling factors as regulators of the hypertrophic response. In support of this notion, transfection of a constitutively active MEK1-encoding construct (immediate upstream activator of ERK1 and 2) augmented ANF promoter activity in cultured cardiomyocytes, whereas a dominant-negative MEK1-encoding construct attenuated activity (Gillespie-Brown et al. 1995). Using antisense oligonucleotides, Glennon et al demonstrated that ERK signaling is necessary for PE-induced cardiomyocyte hypertrophy in culture (Glennon et al. 1996). Similarly, using the MEK1 inhibitor PD98059, Clerk et al reported that the ERKs were required for sarcomeric organization induced by hypertrophic agonists (Clerk et al. 1998). However, this same study also concluded that PD98059 did not prevent cellular hypertrophy in response to agonist stimulation, suggesting that ERKs play a more specialized role in cardiomyocyte hypertrophy.

Although the Ras-Raf-1-MEK1/2-ERK1/2-signaling pathway may regulate certain intracellular responses to a hypertrophic agonist, a large number of studies have disputed the importance of this pathway in the regulation of cardiac hypertrophy. Thorburn et al demonstrated that, although Ras-Raf-1-ERK activation was suffi- cient to augment c-Fos and ANF promoter activity in cardiomyocytes, inhibition of these signaling factors did not antagonize hypertrophic morphology or cytoskeletal organization in response to the agonist (Thorburn et al. 1994; Thorburn et al. 1994). However, Post et al reported that neither dominant-negative ERK1 and 2 nor PD98059 were sufficient to block PEinduced ANF promoter activity in cultured cardiomyocytes, suggesting that ERKs are not even important for inducible gene expression. In a subsequent study, transfection of an activated MEK1 encoding expression plasmid was shown to induce c-Fos, but not ANF or

myosin light-chain-2V promoter activity in cultured cardiomyocytes (Thorburn et al. 1995). More recent studies with the MEK1 inhibitor PD98059 also suggest a minimal role for ERKs in cardiac hypertrophy (Ramirez et al. 1997; Zechner et al. 1997; Choukroun et al. 1998) Intriguingly, one study has even suggested that ERK activation in response to ANF treatment was associated with prevention of cardiomyocyte hypertrophy (Silberbach et al 1999). Although there is clearly a lack of consensus regarding the necessity of ERK signaling as a hypertrophic mediator, overwhelming evidence implicates ERKs as immediate downstream effectors of the hypertrophic response. However, the effector functions of activated ERKs have yet to be fully explored in cultured cardiomyocytes, nor have conclusive genetic or in vivo approaches been used to date. In the future, it will be interesting to examine the association between ERK signaling and cardiac hypertrophy using transgenic or knockout model systems in the mouse.

1.3 CaMKII

CaMKs are serine/threonine kinases that are regulated by Ca^{2+} liganded calmodulin (CaM). Myosin light chain kinase (MLCK) and phosphorylase kinase are Ca^{2+}/CaM dependent protein kinases which are dedicated to a particular substrate (Hudmon et al. 2002). Elongation factor-2 (EF-2) kinase (originally termed CaMKIII) is also a kinase dedicated to the phosphorylation of a single substrate (Nairn AC et al 1985). In contrast, CaMKI, CaMKII and CaMKIV are multifunctional CaMKs. CaMKI and CaMKIV are monomeric enzymes which are activated through phosphorylation by an upstream CaMK kinase (CaMKK) following their binding to Ca^{2+}/CaM (Lee and Edelman 1994; Tokumitsu et al. 1995). CaMKI has broad tissue distribution and is cytoplasmic in mammalian cells (Soderling 1999), although a C-terminally truncated form of CaMKI can go to nucleus and phosphorylate and activate transcription factors when expressed in mammalian cells (Sun et al. 1996; Passier et al. 2000). While CaMKI has been shown to be present in the heart, its function in this tissue remains to be determined since it is not upregulated during hypertrophy (Uemura et al. 1998; Colomer et al. 2003). CaMKIV has restricted expression being found largely in neuronal tissues, T lymphocytes and testis (Soderling 1999), and undetectable in the heart (Colomer et al. 2003; Miyano et al. 1992). CaMKIV, while present in the cytoplasm, is predominantly localized in the nucleus (Jensen et al. 1991), implying a role for CaMKIV in transcriptional regulation and gene expression. In contrast to CaMKI and CaMKIV, the ubiquitously expressed CaMKII is a homo- or heteromultimer of 6–12 subunits consisting of α, β, γ or δ subunits, each encoded by a separate gene (Braun and Schulman 1995; Kanaseki et al. 1991). The α and β subunits are mainly restricted to neuronal tissues while the γ and δ subunits are ubiquitous (Tobimatsu and Fujisawa 1989). Alternative splice variants of the CaMKII subunits have been identified and several of them (i.e. α_B, γ_A and δ_B) contain nuclear localization signals (NLS) which target them to

the nucleus. The subcellular localization of heteromultimers of CaMKII is determined by the ratio of the cytoplasmic to nuclear targeted subunits (Srinivasan et al. 1994).

Many signals have risen and fallen in the tide of investigation into mechanisms of myocardial hypertrophy and heart failure (HF). In our opinion, the multifunctional Ca and calmodulin-dependent protein kinase II (CaMKII) has emerged as a molecule to watch, in part because a solid body of accumulated data essentially satisfy Koch's postulates, showing that the CaMKII pathway is a core mechanism for promoting myocardial hypertrophy and heart failure. Multiple groups have now confirmed the following: (1) that CaMKII activity is increased in hypertrophied and failing myocardium from animal models and patients; (2) CaMKII overexpression causes myocardial hypertrophy and HF and (3) CaMKII inhibition (by drugs, inhibitory peptides and gene deletion) improves myocardial hypertrophy and HF. Patients with myocardial disease die in equal proportion from HF and arrhythmias, and a major therapeutic obstacle is that drugs designed to enhance myocardial contraction promote arrhythmias. In contrast, inhibiting the CaMKII pathway appears to reduce arrhythmias and improve myocardial responses to pathological stimuli.

CaMKII is a serine–threonine kinase that exists as an elaborate holoenzyme complex consisting of a pair of hexameric stacked rings . There are four CaMKII gene products (α, β, γ, δ). These CaMKII isoforms have different tissue distribution and may have subtle differences in Ca^{2+}/CaM sensitivity and activation kinetics, but, at present, understanding of the potentially specific roles for various CaMKII isoforms is incomplete. The predominant, though not exclusive, form in myocardium appears to be CaMKIIδ. Two major splice variants of CaMKIIδ are expressed in the adult heart, CaMKIIδB (Zhang et al. 2002; Zhang et al. 2007) and CaMKIIδC (Zhang et al. 2007; Zhang et al. 2003; Sag et al. 2009), the former containing an 11 amino acid nuclear localization sequence (Edman and Schulman 1994). It appears that CaMKIIδ is functionally significant for myocardial pathology, as it was recently shown that targeted deletion of CaMKIIδ is sufficient to prevent adverse consequences of transaortic banding, a surgical model of pathological afterload augmentation (Backs et al. 2009; Ling et al. 2009). Each of the dozen CaMKII monomers that compose the holoenzyme consists of three domains. Under basal conditions CaMKII activity is submaximal because the N-terminus catalytic domain is constrained by the pseudosubstrate region within the regulatory domain. CaMKII indirectly senses increases in intracellular Ca^{2+} by binding calcified calmodulin (Ca^{2+}/CaM) at the CaM-binding region in the regulatory domain, which is adjacent to the pseudosubstrate region. Ca^{2+}/CaM binding reorders CaMKII so that the catalytic domain is not constrained by the pseudosubstrate domain. During brief, low frequency increases in intracellular [Ca^{2+}], CaMKII deactivates after Ca^{2+}/CaM unbinds from the regulatory domain. Under resting conditions, the regulatory and catalytic domains are closely associated with one another,

blocking substrate binding and resulting in autoinhibition of the kinase. Inspection of the crystal structure of the autoinhibited kinase reveals that neighboring regulatory domains form dimeric coiled-coil pairs that block substrate and ATP binding (Rosenberg et al. 2005). If intracellular calcium concentration rises (halfmaximal activation requires [Ca^{2+}] 1.0 M) (Rostas and Dunkley 1992) calcified calmodulin (Ca^{2+}/CaM) binds to CaMKII at the regulatory domain. Ca^{2+}/CaM binding disrupts the association of the regulatory and catalytic domains, causing a conformational shift that exposes the catalytic domain for substrate binding and relieves autoinhibition (189). If CaMKII activity is sustained by lengthy or frequent calcium transients in the presence of ATP, CaMKII undergoes intersubunit autophosphorylation at Thr-287. The addition of a phosphate group at this residue within the regulatory domain increases the affinity of Ca^{2+}/CaM for CaMKII over 1,000-fold (Meyer et al. 1992). Moreover, phosphorylation at Thr-287 prevents reassociation with the catalytic domain. As a result, Thr-287 phosphorylation can permit persistent CaMKII activity even after the dissociation of Ca^{2+}/CaM from the kinase. Autophosphorylation at Thr-287 is a critical feature of CaMKII function, as it allows the kinase to translate changes in calcium concentration or transient frequency into sustained enzyme activity. Indeed, CaMKII is known as the "memory molecule" in part because recent calcium conditions within the cell are reflected in the shift between Ca^{2+}/CaM-dependent and Ca^{2+}/CaM-independent activity. Interestingly, new evidence from computer modeling and CaMKII crystal structure indicates that activation via autophosphorylation may be cooperative (Chao et al. 2010), a function of intersubunit capture of regulatory domains rather than simple coincidence detection. To return to basal inactivation, Thr-287 phosphorylation must be removed by the action of phosphatases, including protein phosphatase (PP)1 and PP2A (220). As protein phosphatases are themselves subject to complex regulation, for instance, by oxidative stress (Howe et al. 2004) it becomes clear that a number of mechanisms work in tandem to modulate CaMKII activity in vivo.

1.4 Role of Ca^{2+} Signaling in Gene Expression and Cardiac Hypertrophic Growth

Ca^{2+} is a well-established regulator of transcriptional changes in gene expression (Hardingham and Bading 1998). Changes in intracellular Ca^{2+} also have been suggested to mediate cardiac hypertrophic responses (Frey et al. 2000). Modulations in Ca^{2+} levels would need to be transmitted to the nucleus to affect transcriptional regulation of genes associated with cardiac hypertrophy. The nuclear isoform of CaMKII (CaMKIIδ_B) would, therefore, be the isoform predicted to play a predominant role in Ca^{2+}-mediated transcriptional gene regulation. Both Ca^{2+} and CaM can translocate into the nucleus and could activate nuclear localized CaMKII (Heist and Schulman 1998). There also is evidence for independent regulation of nuclear Ca^{2+} via IP3 receptors localized in the cell nucleus (Malviya and Rogue 1998). CaMK

and calcineurin have been shown to play critical and often synergistic roles in transcriptional regulation in cardiomyocytes (Passier Zeng et al. 2000). There is growing evidence that the amplitude, frequency, source, and subcellular localization (spatial and/or temporal modes) of Ca^{2+} signals are determinants of distinct transcriptional responses (Berridge 1997; Dolmetsch et al. 1997, allowing regulation of diverse cellular processes in response to the same second messenger (Ca^{2+}). In this regard, it may be important to note that CaMK is activated by high and transient Ca^{2+} spikes and its activity is dependent on Ca^{2+} spike frequency (Hudmon and Schulman 2002), whereas calcineurin responds to low, sustained Ca^{2+} plateaus (Dolmetsch et al. 1997).

1.4.1 The Effect of CaMK on Gene Transcription

CaMK has been suggested to regulate gene expression via activation of several different transcription factors, including activation protein 1 (AP-1) (Gullberg and Chatila 1996), CAAT-enhancer binding protein (C/EBP) (Wegner et al. 1992), activating transcription factor (ATF-1) (Sun et al. 1996), serum response factor (SRF) (Misra et al. 1994), cAMP-response element binding protein (CREB) (Sheng et al. 1991), and myocyte enhancer factor 2 (MEF2) (Passier Zeng et al. 2000). CaMKII and CaMKIV have been shown to activate CREB by phosphorylation of Ser133 (Matthews et al. 1994). Surprisingly, CREB phosphorylation does not appear to be altered in transgenic mice expressing CaMKIIB (Zhang et al. 2002) or CaMKIV (Passier Zeng et al. 2000), both of which localize to the nucleus. Therefore, phosphorylated CREB cannot account for the long-term changes in cardiac function in these mice. Another transcription factor, MEF2, is upregulated during cardiac hypertrophy (Kolodziejczyk et al. 1999; Lu McKinsey and Nicol 2000) and has been suggested to act as a common endpoint for hypertrophic signaling pathways in the myocardium (Kolodziejczyk et al. 1999; Lu McKinsey and Nicol 2000). Studies using transgenic overexpression of CaMKIV demonstrate that MEF2 is a downstream target for CaMKIV (Passier Zeng et al. 2000). The mechanism by which CaMK signaling activates MEF2 in vivo remains to be determined but recent studies have suggested that MEF2 interacts with class II histone deacetylases (HDACs) and other repressors that normally limit expression of MEF2-dependent genes. CaMKI and CaMKIV activate MEF2 by dissociating HDACs and other repressors (Lu McKinsey et al. 2000). Most recently, SRF has been shown to be activated by CaMKIV in a similar manner (i.e., by dissociating HDACs) (Davis et al. 2003). Both MEF2 and SRF activation have been demonstrated to occur in response to activation of the noncardiac CaMKI and IV isoforms.

1.5 The Role of CaMK in Hypertrophic Growth

Studies from a variety of in vivo preparations — including hypertensive rat hearts, coronary artery-ligated rabbit hearts, and transverse aortic-

constricted (TAC) mouse hearts — have demonstrated increased CaMKII expression and activity in hypertrophied myocardium (Currie and Smith 1999; Boknik et al. 2001; Hagemann et al. 2001; Zhang et al. 2003). Studies using isolated cardiomyocytes and CaMK inhibitors KN-62 or KN-93 also suggested that CaMKII was involved in cardiomyocyte hypertrophy induced by agonists such as endothelin-1, leukemia inhibitory factor (LIF), and phenylephrine (Sei et al. 1991; Ramirez et al. 1997; Kato et al. 2000; Zhu et al. 2000). Several transgenic mouse models subsequently confirmed a role for CaMK in activation of the hypertrophic gene program and development of hypertrophy. Transgenic mice overexpressing calmodulin were generated nearly 10 years ago and shown to develop severe cardiac hypertrophy (Gruver et al. 1993; Hagemann et al. 2000). This subsequently was demonstrated to be associated with an increase in the autonomous activity of CaMKII in vivo (Colomer and Means 2000). Pronounced hypertrophy also develops in transgenic mice that overexpress CaMKIV (Passier et al., 2000). This is associated with specific changes in gene expression. However, CaMKIV knockout (KO) mice still are able to develop hypertrophy after TAC (Colomer et al. 2003), presumably because CaMKIV is not one of the major CaMK isoforms present in the heart (Edman and Schulman 1994; Colomer et al. 2003). Since hypertrophic growth is associated with a specific program of altered gene expression, and CaMKII is implicated in this response, It has been hypothesized that CaMKII isoforms expressed in the nucleus would selectively regulate hypertrophic transcriptional responses. In support of this, we reported that transient expression of the nuclear δ_B isoform of CaMKII in neonatal rat ventricular myocytes induced expression of the atrial natriuretic factor (ANF) gene, an established indicator of cardiomyocyte hypertrophy, as indicated by enhanced transcriptional activation of an ANF-luciferase reporter gene and increased ANF protein (Ramirez et al. 1997). The nuclear localization signal of CaMKIIδ_B was shown to be required for this response, as transient expression of CaMKIIδ_C did not result in enhanced ANF expression. Indeed, CaMKII heteromultimers formed predominantly of δC subunits were excluded from the nucleus and failed to induce ANF expression (Ramirez et al. 1997). These findings are consistent with the observation that constitutively active CaMKI and CaMKIV, which enter the nucleus, induce a hypertrophic response in cardiomyocytes in vitro (Passier Zeng et al. 2000). It has also been reported that hypertrophic growth occurs in transgenic mice that overexpress the CaMKIIδ_B isoform, which is highly concentrated in cardiomyocyte nuclei (Zhang et al. 2002).

2. Aims of the study

Activation of Ca^{2+}/Calmodulin protein kinase II (CaMKII) is an important step in signaling of cardiac hypertrophy.

The molecular mechanisms by which CaMKII integrates with other pathways to develop cardiac hypertrophy are incompletely understood.

We hypothesize that CaMKII association with extracellular regulated kinase (ERK), promotes cardiac hypertrophy through ERK nuclear localization.

3. Materials and Methods

3.1. Peptide synthesis

The synthesis of CaM-KNtide β analogs was performed according to the solid phase approach using standard Fmoc methodology in a manual reaction vessel (Atherton et al. 1989). $N\alpha$-Fmoc-protected amino acids, Rinkamide-resin, HOBt, HBTU, DIEA, piperidine and trifluoroacetic acid were purchased from Iris Biotech (Germany). Peptide synthesis solvents, reagents, as well as CH_3CN for HPLC were reagent grade and were acquired from commercial sources and used without further purification unless otherwise noted. The corresponding first amino acid, NαFmoc-Xaa-OH, was linked on to the resin previously deprotected by a 25% piperidine solution in DMF for 30 min. The following protected amino acids were then added stepwise. Each coupling reaction was accomplished using a 3-fold excess of amino acid with HBTU and HOBt in the presence of DIEA (6 eq.). The Nα-Fmoc protecting groups were removed by treating the protected peptide resin with a 25% solution of piperidine in DMF (1 $\times$ 5 min and 1 $\times$ 25 min). The peptide resin was washed three times with DMF, and the next coupling step was initiated in a stepwise manner. The peptide resin was washed with DCM (3 $\times$ 25), DMF (3$\times$), and DCM (3$\times$), and the deprotection protocol was repeated after each coupling step.

The N-terminal Fmoc group was removed as described above, and the peptide was released from the resin with TFA/TIS/H_2O (90:5:5) for 3 h. The resin was removed by filtration, and the crude peptide was recovered by precipitation with cold anhydrous ethyl ether to give a white powder and then lyophilized.

3.2. Peptides: purification and characterization

All crude cyclic peptides were purified by RP-HPLC on a semipreparative C18-bonded silica column (Phenomenex, Jupiter, 250 $\times$ 10 mm) using a Shimadzu SPD 10A UV/VIS detector, with detection at 210 and 254 nm.

The column was perfused at a flow rate of 3 ml/min with solvent A (10%, v/v, water in 0.1% aqueous TFA), and a linear gradient from 10 to 90% of solvent B (80%, v/v, acetonitrile in 0.1% aqueous TFA) over 40 min was adopted for peptide elution. Analytical purity and retention time (t_R) of each peptide were determined using HPLC conditions in the above solvent system (solvents A and B) programmed at a flow rate of 1 ml/min using a linear gradient from 10 to 90% B over 25 min, fitted with C-18 column Phenomenex, Jupiter C-18 column (250 $\times$ 4.60 mm; 5 μm).

All analogs showed >97% purity when monitored at 215 nm. Homogeneous fractions, as established using analytical HPLC, were pooled and lyophilized.

Peptides molecular weights were determined by ESI mass spectrometry. ESI-MS analysis in positive ion mode, were made using a Finnigan LCQ Deca ion trap instrument, manufactured by Thermo Finnigan (San Jose, CA, USA), equipped with the Excalibur software for processing the data acquired. The sample was dissolved in a mixture of water and methanol (50/50) and injected directly into the electrospray source, using a syringe pump, which maintains constant flow at 5 μl/min. The temperature of the capillary was set at 220°C.

3.3. CaMKII activity assay

In all CaMKII assays, purified or endogenous CaMKII was incubated with Ca^{2+}/CaM; EGTA was used as a negative control. Active recombinant full-length alpha CaMKII (Signal Chem, La Jolla) was incubated for 30 min at 30 $^{\circ}$ C with 1 mmol/L $CaCl_2$ and 1 μmol/L CaM in 10 μl of a reaction mixture (50 mmol/L HEPES pH 7.5, 10 mmol/L $MgCl_2$, 0.5 mmol/L dithiothreitol (DTT), 100 nmol/L microcystin, 0.1 mmol/L non-radiolabeled ATP) (Chin et al. 1997). In a second reaction step, an aliquot from the first reaction was incubated with 200 μM of Autocamtide-2 as a substrate for CaMKII and the different peptides at concentration of 5 μM, in presence of 0.2 μCi/μl of Easy Tides Adenosine 5′-triphosphate [γ 32P]-ATP (Perkin Elmer) for 30 min at 30 $^{\circ}$ C; EGTA was added to quantify CaMKII autonomous activity. 32P-incorporation was determined by spotting 20 μl of the reaction to Whatman P-81 phosphocellulose paper, and subsequently washing in 75 mM phosphoric acid. Dried filters were counted on a Beckman LS 6000 scintillation counter.

3.4. Isolation of ventricular cardiomyocytes for in vitro experiments.

Adult rat ventricular myocytes were isolated from 12 week old Wistar/Kyoto rat hearts by a standard enzymatic digestion procedure as previously described (Ciccarelli et al. 2011; O'Connell et al. 2007). Briefly, animals were anesthetized with isofluorane (2% v/v) and 0.5 ml of 100U.I./ml heparin injected (i.p.). After 5 min, chest was opened and heart removed by a transverse cut between aorta and carotid arteries, then cannulated to the perfusion system. Heart was initially perfused (NaCl 120 mmol/L, KCl 14.7 mmol/L, KH2PO4 0.6 mmol/L, Na2PO4 0.6 mmol/L, MgSO4 1.2 mMol/L, HEPES 10mmol/L, Glucose 5.5 mmol/L, butendionemonoxime 10 mmol/L) for 4 min followed by enzymatic digestion with Collagenase type II (Worthington, 1 mg/ml of perfusion buffer) for 12 min. At the end of

perfusion, a spongy, flaccid heart was detached from the cannula, atria and right ventricle removed and then cut in small pieces with scissors. Digested prep was filtered and a cell pellet obtained by centrifugation (300 rpm, 2 min.), which has been then resuspended in lysis buffer for western blot.

3.5. Cell culture

Cardiomyoblasts H9C2 were cultured in Dulbecco's minimal essential medium (DMEM, GIBCO) supplemented with 0.1 g/L fetal bovine serum (FBS, GIBCO) 200 mg/mL L-glutamine, 100 units/mL penicillin, and 10 mg/mL streptomycin (Sigma-Aldrich), at 37°C in 0.95 g/L air-0.05 g/L CO2. The H9C2 were studied between passages 4 and 10. Serum-starved cells were exposed to PE (100 nmol/L, Sigma Aldrigh) at different time points, in the presence and absence of the CaMKs inhibitor KN93 (10 µmol/L, methossibenensulphonamide, Seikagaku), in the presence and absence of the selective CaMKII inhibitor, AntCaNtide (5 µmol/L) and the tat-CN17 β (5 µmol/L). Next, in order to study the effect of ERK inhibition on PE mediated CaMKII activation, we pre-treated the cells for 30 min with the MAP Kinase inhibitor UO126 (10 µmol/L, Promega).

3.6 Cell Infection

H9C2 cells at ≈ 70% confluence were incubated 1 h at 37°C with 5 mL DMEM containing purified adenovirus at a multiplicity of infection (moi) of 100:1, encoding either the kinase-dead (CaMKII-DN, rCaMKIIalpha, K42M, impaired ATP binding pocket), or the wild type (CaMKII-WT,rCaMKIIalpha) variant of CaMKII or the empty virus as a negative control (Ctr) (Cipolletta et al. 2010). 24 h after the infection, the cells were used for the experiments. These adenoviruses were expanded using the AdEasy system from Qbiogene as described previously (Kahl et al. 2004).

3.7. Nuclear Extracts Preparation

H9C2 were washed in ice-cold phosphate buffer (PBS) and resuspended in hypotonic buffer [10 mmol/L Hepes pH 7.9; 10 mmol/L KCl; 0.1 mmol/L EDTA; 0.1 mmol/L EGTA; 0.1 mmol/L NaVO3; 1 mmol/L DTT; 0.5 mM PMSF]. Cells were lysed by adding 0.1 g/L Nonidet P-40 and vortexing vigorously. Nuclei were pelleted by centrifugation at 12000 rpm for 30 min. Supernatant (cytosol) was saved for analysis. The nuclei were resuspended in hypertonic buffer [20 mmol/L Hepes pH7.9; 400 mmol/L NaCl; 1 mmol/L EDTA; 1 mmol/L EGTA; 0.2 g/L Glycerol; 0.1 mmol/L NaVO3; 1 mmol/L DTT; 0.5 mmol/L PMSF] and rocked 30 min on a shaking platform at 4°C. The samples were centrifuged at 14000 rpm for 5 min and the supernatants (nuclear extracts) were saved. Protein concentration was determined by using

the Bradford method (Bio-Rad). Cytosol and nuclear extracts were confirmed by WB by using anti-Actin and anti-Histone 3 antibodies.

3.8 Western Blot and Immunoprecipitation Analysis

H9C2, where indicated, were stimulated with PE 100 nmol/L at different times as indicated in each experiment. Alternatively, the cells were pre-treated with the appropriate inhibitor (KN93 10 µmol/L, and AntCaNtide 5 µmol/L,) for 30 min before stimulation with PE. Rat isolated cardiomyocytes and H9C2 cells were lysed in ice-cold RIPA/SDS buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCL, 0.01 g/L NP-40, 0.0025 g/L deoxycholate, 2 mmol/L Na3VO4, 0.2 g/L sodium, 2 mmol/L EDTA, 2 mmol/L PMSF, 5 µg/mL leupeptin, 5 µg/mL pepstatin]. Protein concentration was determined using BCA assay kit (Pierce). Endogenous total CaMKII or CaMKII specific isoforms were immunoprecipitated with 5 µL of anti-CaMKII antibody or anti-CaMKII isoforms specific antibodies (α, □β,□ δ,□ γ□□) and 25 µL of protein G plus/protein A agarose beads/1 mg total cell extract (Santa Cruz) for three hours at 4°C. The samples were then washed twice with lysis buffer, twice with 1×phosphate-buffered saline, and resuspended in 1×SDS gel loading buffer. The immunoprecipitated kinases were either used to assay activity, or resolved on SDS-PAGE in order to visualize the associated proteins by WB and specific antibodies. Equal amounts of total cellular extracts or immunocomplexes were electrophoresed on 4-12% SDS-PAGE gel (NOVEX) and transferred to a nitrocellulose filter (Immobilon P; Millipore Corporation, Bedford, MA). The membranes were blocked in Tris buffered saline containing 0.002 g/L Tween 20 (TBST) and 0.05 g/L nonfat dry milk. After blocking, the membranes were washed three times in TBST and then incubated overnight at 4°C in TBST containing 5% BSA with primary specific antibody: total ERK1/2 (1:1000; Millipore, CA), total CaMKII (1:1000, Santa Cruz, CA), CaMKIIα, δ, γ (1:1000 Santa Cruz, CA), CaMKIIβ (Zymed, Invitrogen Inc, CA□), phospho-tyrosyne p44/p42 ERK (1:1000 Santa Cruz, CA), Actin (1:1000; Santa Cruz, CA), Histone 3 (1:1000 Santa Cruz, CA), and phospho-CaMKII antibody (pT286) (Zymed, Invitrogen Inc, CA). The blots were washed three times in TBST incubated in appropriate HRP-conjugated secondary antibodies (1:2000, Santa Cruz, CA) diluited in TBST containing 5% nonfat dry milk and incubated for 1 h at room temperature. After 3 additional washes with TBST, immunoreactive bands were visualized by enhanced chemiluminescence using the ECL-plus detection kit (Amersham Biosciences) and quantified by using ImageQuant software (Amersham Biosciences).

3.9 Real Time PCR

Total RNA from H9C2 cell line and isolated ventricular cardiomyocytes was isolated using Trizol reagent (Invitrogen) and cDNA was

synthetized by means of Thermo-Script RT-PCR System (Invitrogen), following the manufacturer instruction. After reverse transcription reaction, real-time quantitative polymerase chain reaction (PCR) was performed with the SYBR Green real time PCR master mix kit (Applied Biosystems, Foster City, CA, USA). The reaction was visualized by SYBR Green Analysis (Applied Biosystem) on StepOne instrument (Applied Biosystem). Primers for gene analysis were as follows:

- ANF: For 5'cgtgccccgacccacgccagcatgggctcc3', Rev 5'ggctccgagggccagcgagcagagccctca3';
- 18S: For 5'gtaacccgttgaacccatt3', Rev 5'ccatccaatcggtagtagcg3';
- CaMKII α: For 5'cctgtatatcttgctggttggg3'; Rev 5'ttgatcagatcctt-ggcttcc3';
- CaMKII β: For 5'tcaagccccagacaaacag3'; Rev 5'ttccttaatgccgt-ccactg3';
- CaMKII γ: For 5'aaacctgtggatatctgggc3'; Rev 5'ctggtgatggg-aaatcgtagg3';
- CaMKII δ: For 5'atagaagttcaaggcgaccag3'; Rev 5'cagcaaga-tgtagaggatgacg3';
- Actin: For 5'ggcatcgtgatggactccg3'; Rev 5'gctggaaggtg-gacagcga3'.

3.10 In vivo study

3.10.1 Animals

13-weeks-old normotensive Wistar Kyoto (WKY, n=7) and spontaneously hypertensive (SHR, n=20) male rats (Charles River, Calco, LC, Italy) which had access to water and food ad libitum were used in these experiments. The animals were anesthetized by vaporized isoflurane (4%). After the induction of anesthesia, rats were orotracheally intubated, the inspired concentration of isoflurane was reduced to 2% and lungs were mechanically ventilated (New England Medical Instruments Scientific Inc., Medway, MA, USA) as previously described and validated (Kirchhefer et al. 1999; Kato et al. 2000; Colomer et al. 2000). SHRs were divided into three groups: SHR-AntCaNtide (n=8) SHR- tat-CN17β (n=7) and SHR-Control (n=5). The untreated WKY rats (n=7) were used as the control. The chest was opened under sterile conditions through a right parasternal mini-thoracotomy, to expose the heart. Then, we performed three injections (50 µl each) of AntCaNtide (50 µg/kg), tat-CN17β (50 µg/kg) or NaCl 0.9%, as control, into the cardiac wall (anterior, lateral, posterior, apical) (Kirchhefer et al. 1999; Colomer et al. 2000). Finally, the chest wall was quickly closed in layers using 3-0 silk suture and animals were observed and monitored until recovery. This

procedure was performed once week for 3 times. One week after last treatment, rats were weighed and then euthanized. Hearts were immediately harvested, rinsed 3 times in cold PBS and blotted dry, weighed, divided in left and right ventricles, and then rapidly frozen in liquid nitrogen and stored at -270°C until needed for biochemical studies. Animal procedures were performed in accordance with the guidelines of the Federico II University of Naples Institutional Animal Usage Committee. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by US National Institute of Health (NIH Publication 85-23, rev. 1996) and approved by the Ethics Committee of the Federico II University.

3.10.2 Echocardiography

Transthoracic echocardiography was performed at day 0, 7, 14 and 21 after surgery, using a dedicated small-animal high-resolution ultrasound system (VeVo 770, Visualsonics Inc. Toronto, ON, Canada) equipped with a 17.5 MHz transducer (RMV-716). The rats were anaesthetized by isoflurane (4%) inhalation and maintained by mask ventilation (isoflurane 2%). The chest was shaved by appling a depilatory cream (Veet, Reckitt Benckiser, Milano, Italy) (Liang et al. 2000). LV end-diastolic and LV end-systolic diameters (LVEDD and LVESD, respectively) were measured at the level of the papillary muscles from the parasternal short-axis view as recommended (Lu et al. 2009). Intraventricularseptal (IVS) and left ventricular posterior wall thickness (PW) were measured at end diastole. LV fractional shortening (LVFS) was calculated as follow: LVFS = (LVEDD – LVESD)/LVEDD x 100. Left ventricular ejection fraction (LVEF) was calculated using a built-in software of the VeVo 770 (Kato et al. 2000). Left ventricular mass (LVM) was calculated according to the following formula, representing the M-mode cubic method: LVM=1.05×[(IVS+LVEDD+LVPW)3-(LVEDD)3]; LVM was corrected by body weight. All measurements were averaged on 5 consecutive cardiac cycles and analyzed by two experienced investigators blinded to treatment (G.S. and A.A.).

3.10.3 Blood Pressure measurement and evaluation of cardiac performance

Blood pressure (BP) was measured as previously described (Kato et al. 2000; Illario et al. 2013) and the record of both systolic (SBP) and diastolic (DBP) was taken using a pressure transducer catheter (Mikro-Tip, Millar Instruments, Inc.; Houston, TX, USA). The catheters already placed in the ascending aorta were then advanced further into LV to record the maximal and minimal first derivatives of pressure over time (dP/dtmax and dP/dtmin), as indexes of global cardiac contractility and relaxation10. To take into account the effects of differences of SBP on cardiac contractility, we also corrected

dP/dtmax by peak systolic pressure (dP/dtmax/SBP) as previously described (Colomer et al. 2003).

3.10.4 Histology

Four weeks after AntCaNtide (50 µg/kg), tat-CN17β (50 µg/kg) or NaCl 0.9%, intra-cardiac injection, the hearts were immersion fixed in 10% buffered paraformaldehyde. The tissues were embedded in paraffin, cut at 5 µm, and processed. For Masson trichrome staining of collagen fibers, slides were stained with Weigert Hematoxylin (Sigma-Aldrich, St. Louis, MO) for 10 minutes, rinsed in PBS (Invitrogen) and then stained with Biebrich scarlet-acid fuchsin (Sigma-Aldrich) for 5 minutes. Slides were rinsed in PBS and stained with phosphomolybdic/phosphotungstic acid solution (Sigma-Aldrich) for 5 minutes then stained with light green (Sigma-Aldrich) for 5 minutes. Slides were rinsed in distilled water, dehydrated with 95% and absolute alcohol and a coverslip was placed. For the analysis of cardiomyocites size, Masson trichrome staining sections were used (Kato et al. 2000). The areas (µm2) of ~100 cardiac myocites per heart were measured with the public domain Java image processing program Image (Cipolletta et al. 2010).

3.11 Statistical Analysis

One-way ANOVA was performed to compare different groups followed by a Bonferroni post-hoc analysis. Two-way analysis of variance was applied to analyze different parameters among the different groups. A significance level of $p<0.05$ was assumed for all statistical evaluations. Statistics were computed with dedicated software (GraphPad Prism, San Diego, California). All values in figures are presented as mean±SEM of at least 3 independent experiments. Data from immunoblots were quantified by densitomentric analysis.

4. Results

4.1 Synthesis, purification and identification of peptide

Peptides were synthesized according to the solid-phase approach using standard Fmoc methodology in a manual reaction vessel (Atherton et al. 1989). The purification was achieved using a semipreparative RP-HPLC C18 bonded silica column. The purified peptides were 98% pure as determined by analytical RP-HPLC. The correct molecular weight of the peptides was confirmed by mass spectrometry and amino acid analysis. The synthesized analogs and their physicochemical characteristics are summarized in Tables 1 e 3. The ability of these compounds to inhibit the CaMKII activity was assayed in vitro using the autocamtide-2 (AC2) as phosphorylation substrate. AC2 is a highly selective synthetic peptide substrate for CaMKII derived from the autophosphorylation site of a subunit of CaMKII (Hanson et al. 1989; Chin et al. 1997). In this assay the reference compound CaM-KNtideb, showed a maximal inhibitory activity at concentration in the range 1 e 5 mM (Figure 1). Accordingly, we evaluated the inhibitory potencies for all analogs at a concentration of 5 mM. These data are summarized in Tables 1e3. As first series, we synthesized a scramble analog of the CaM-KNtideb sequence and seven peptides that cover the entire CaM-KNtideb sequence. Each short peptide had the sequence shifted of five residues, both from N- and C-terminal side (compounds 1e7). The shortest peptide that retained good inhibition of CaMKII (>75%) was the fragment containing amino-acids 1e17 (peptide 5, named now CN17b). All further truncations at the C-terminus by 4 amino acids or more (peptides 6 and 7) and any truncation at the Nterminus by 5 amino acids or more (peptides 1e3) showed minimal or no inhibition. CN17b seems to be slightly more potent than the corresponding analog S12 (KRPPKLGQIGRSKRVVI, termed CN17a), described by Vest et al., which inhibit <70% CaMKII activity at 5 mM. These results confirmed that CaM-KNtideb sequence containing an Ala residue at position 12 was an effective starting point to develop novel agents with CaMKII inhibitor activity.

Table 1
Structure and inhibitory activity of compounds CaM-KNtide, and peptides **1**–**7**.

Frag.	Peptide*	Sequence	**Inhib. (%±SD)**	HPLC k'	ESI MS Found	ESI MS Calculated
1-27	CaM-KNtideβ	KRPPKLGQIGRAKRVVIEDDRIDDVLK	**94.8± 5.0**	3.27	3115.30	3115.70
	Scramble	IDGVIAQGDLPVDKEPKRLRKDIRKRV	**13.3±6.2**	3.27	3115.82	3115.70
6-27	**1**	LGQIGRAKRVVIEDDRIDDVLK	**< 5**	2.69	2509.22	2508.90
11-27	**2**	RSKRVVIEDDRIDDVLK	**< 5**	2.49	2057.23	2056.30
17-27	**3**	VIEDDRIDDVLK	**13.0±9.1**	2.52	1429.70	1429.50
1-22	**4**	KRPPKLGQIGRAKRVVIEDDRI	**56.0±14.0**	2.78	2544.62	2544.04
1-17	**5**	KRPPKLGQIGRAKRVVI	**76.0±4.1**	3.12	1916.64	1915.40
1-12	**6**	KRPPKLGQIGRA	**< 5**	2.75	1321.04	1320.60
1-7	**7**	KRPPKLG	**< 5**	2.08	796.21	795.01

k' [(peptide retention time – solvent retention time)/solvent retention time]. Peptide concentration 5 μM. *Peptides are named by the nomenclature that adhere to the reasoning behind their design

Table 2
Structure and inhibitory activity of peptides CN19, **5** and **8**–**16**.

Frag.	Peptide*	Sequence	**Inhib. (%±SD)**	HPLC k'	ESI MS Found	ESI MS Calculated
1-19	**CN19**	KRPPKLGQIGR**S**KRVVIE D	**54.0±1.0**	3.17	2176.23	2175.28
1-17	**CN17β**	KRPPKLGQIGR**A**KRVVI	**76.0±4.1**	3.12	1916.64	1915.40
2-17	**8**	RPPKLGQIGRAKRVVI	**< 5**	3.01	1788.40	1788.23
3-17	**9**	PPKLGQIGRAKRVVI	**< 5**	3.08	1632.72	1632.04
4-17	**10**	PKLGQIGRAKRVVI	**< 5**	2.57	1535.03	1534.93
5-17	**11**	KLGQIGRAKRVVI	**< 5**	2.54	1437.98	1437.81
6-17	**12**	LGQIGRAKRVVI	**< 5**	2.58	1309.72	1309.60
1-16	**13**	KRPPKLGQIGRAKRVV	**10.2±2.0**	2.61	1803.77	1803.25
1-15	**14**	KRPPKLGQIGRAKRV	**18.0±9.0**	2.60	1704.30	1704.13
1-14	**15**	KRPPKLGQIGRAKR	**< 5**	2.09	1605.11	1604.98
1-13	**16**	KRPPKLGQIGRAK	**< 5**	2.34	1449.99	1448.79

k' [(peptide retention time – solvent retention time)/solvent retention time]. Peptide concentration 5 μM. *Peptides are named by the nomenclature that adhere to the reasoning behind their design.

Table 3
Structure and inhibitory activity of peptides CN17β and Ala scan derivatives.

Peptide	Sequence	Inhib.	HPLC	ESI MS	
		(%±SD)	*k'*	Found	Calculated
CN17β	KRPPKLGQIGRAKRVVI	76.0 ± 44	3.15	1916.64	1915.40
K1A	ARPPKLGQIGRAKRVVI	13.5 ± 2	3.20	1859.32	1858.30
R2A	KAPPKLGQIGRAKRVVI	<5	3.17	1832.72	1830.29
P3A	KRAPKLGQIGRAKRVVI	78.4 ± 4	3.13	1890.09	1889.36
P4A	KRPAKLGQIGRAKRVVI	13.2 ± 2	3.13	1890.75	1889.36
K5A	KRPPALGQIGRAKRVVI	7.0 ± 2	4.71	1859.96	1858.30
L6A	KRPPKAGQIGRAKRVVI	<5	3.35	1876.40	1874.33
G7A	KRPPKLAQIGRAKRVVI	26.1 ± 4	3.18	1931.16	1929.43
Q8A	KRPPKLGAIGRAKRVVI	34.4 ± 3	3.16	1860.22	1858.35
I9A	KRPPKLGQAGRAKRVVI	<5	3.15	1874.58	1873.32
G10A	KRPPKLGQIARAKRVVI	21.6 ± 2	3.15	1931.22	1929.43
R11A	KRPPKLGQIGAAKRVVI	13.0 ± 2	3.19	1831.12	1830.29
K13A	KRPPKLGQIGRAARVVI	33.7 ± 1	3.17	1860.14	1859.31
R14A	KRPPKLGQIGRAKAVVI	69.0 ± 2	3.16	1830.95	1831.30
V15A	KRPPKLGQIGRAKRAVI	24.2 ± 2	3.16	1889.21	1888.35
V16A	KRPPKLGQIGRAKRVAI	74.2 ± 1	3.14	1888.16	1888.30
I17A	KRPPKLGQIGRAKRVVA	48.7 ± 3	4.71	1875.52	1874.32

k' [(peptide retention time − solvent retention time)/solvent retention time]. Peptide concentration 5 μM.

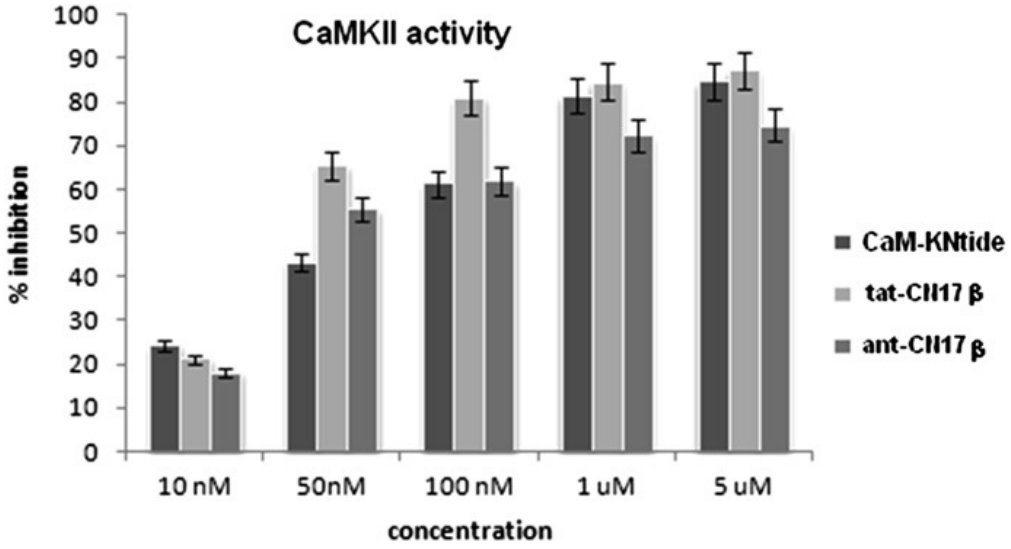

Figure 1. Dose/response curves for CaM-KNtide and peptides tat-CN17b and ant-CN17b. Data are indicated as percent of inhibition over untreated; error bars represent ±standard deviation (SD).

4.2 Analisys of signal transduction

CaMKII is involved in the activation of the most important signaling pathway regulating cell proliferation: the Ras/Raf/MEK/ERK signaling pathway. Illario et al. demonstrated the existence of a cross-talk between Ca2+/CaM/CaMKII and Ras/Raf/MEK/ERK pathways (Illario et al. 2003; Illario et al. 2005). Accordingly, ant-CaNtide is able to significantly affect CaMKII-mediated cell proliferation, by down regulating ERK activation. As tat-CN17b affected cell proliferation, we investigated whether it is also able to interfere with ERK activation.With this aim, we evaluated the expression of Erk, phospho(p)Erk, CaMKII, and phospho(p)CaMKII in cardiomyoblast (H9C2) and colon cancer (HT-29) cell lines by western blot (Figure 2A and B). Cells were serum starved over night in 0.5% DMEM/BSA and treated for 30 min with 10% of FBS, with or without CaMKII inhibitor tat-CN17b. As shown in Fig. 4A and B, serum stimulation induced both CaMKII and ERK activation, which was altered by treatment with different concentrations of tat-CN17b. Densitometry measurements of immunoblots analyzing the levels of depicting pCaMKII and pERK in H9C2 and HT29 cells were performed using Image J software (NIH) (Figure 2C and D). In H9C2 cells (Figure 2A and C), ERK phosphorylation was down-regulated in a dose-dependent manner, whereas in HT-29 cells tat-CN17b at 100 nM induced the maximal down-regulation of ERK phosphorylation (Figure 2B and D). These results suggest that the peptide interferes with cell proliferation by regulating, at least, the Ras/Raf/MEK/ERK signaling pathway.

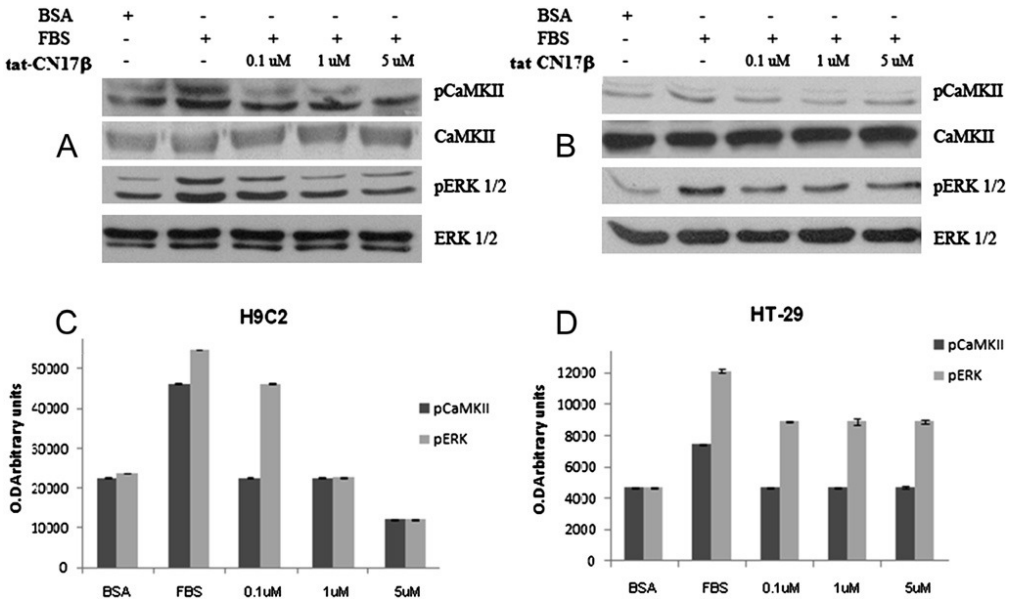

Figure 2. Immunoblots for Erk, phospho(p)Erk, CaMKII, and phospho(p)CaMKII in (A) H9C2 and (B) HT-29 cells stimulated with serum and treated with 0.1, 1, and 5 mM tat-CN17b for 30 min. The respective densitometry analysis (C) and (D) are shown. Signal densities of bands represent individual and average values ±SD from 3 experiments.

4.3 Expression of isoforms of CaMKII in H9C2 cells and cardiomyocytes

The kinase Ca^{2+}/ calmodulin dependent are crucial transducers of Ca^{2+}; signaling, I therefore, examined the expression of CaMKII isoforms in cells systems used. H9C2 cells express CaMKII α, β and γ isoforms, similar to adult ventricular myocytes. Interestingly, the β isoform is the most abundant, where as CaMKIIα, γ and δ are only detectable after immunoprecipitates from H9C2 cells or purified rat cardiomyocytes (Figure 3A). These results were confirmed by RT-PCR analysis (Fig. 3B).

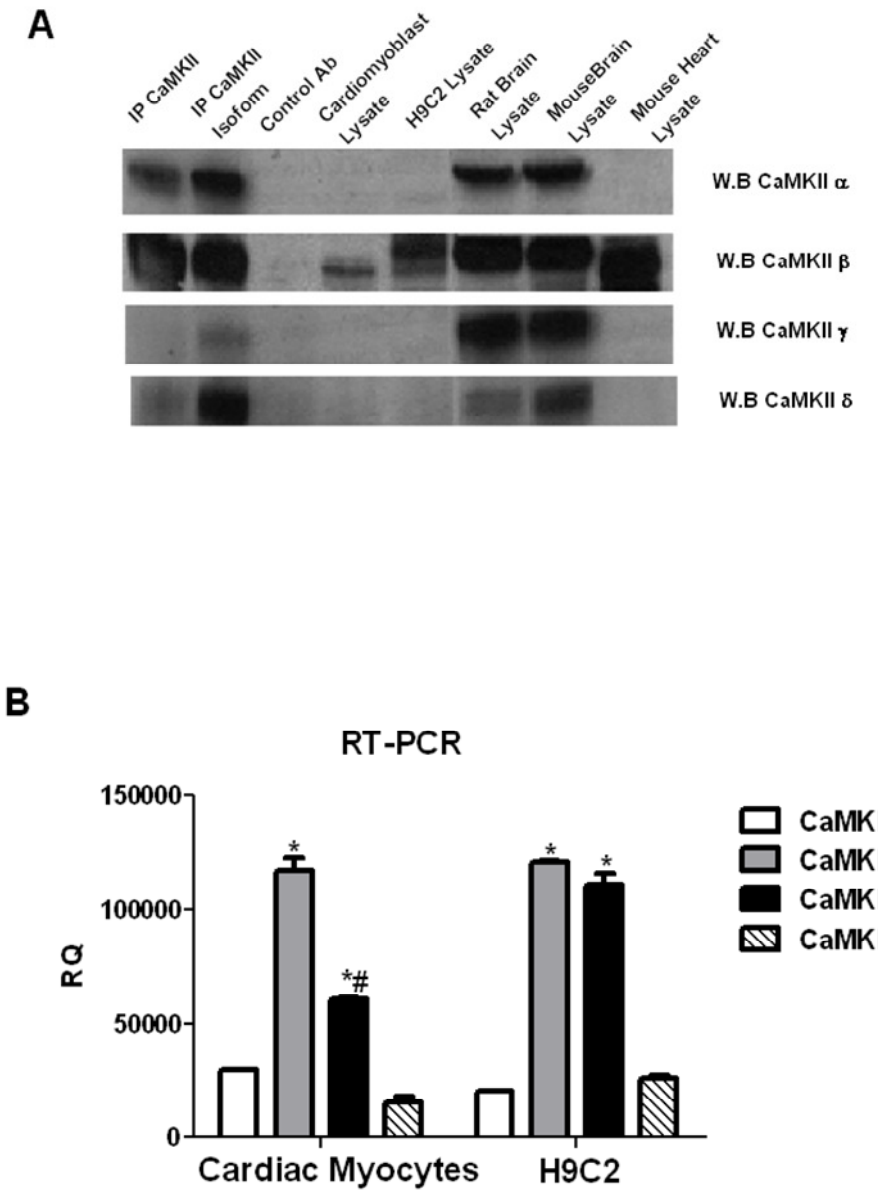

Figure 3. CaMKII isoforms

A. To evaluate the expression of CaMKII isoforms in H9C2s, a cell line of cardiac myoblasts, and ventricular adult myocytes from WKY rats, the total cell lysates were immunoprecipitated using anti-CaMKII antibody or anti-CaMKII α, β, γ or δ specific antibodies and together to total samples were analyzed by western blot with anti-CaMKII α, β, γ or δ antibodies as indicated. Total extracts from rat brain, mouse brain and mouse heart were used as controls.

B. Total RNA from H9C2 cell line and isolated ventricular cardiomyocytes was extracted with standard methods. RT-PCR for CaMKII α, β, γ and δ was performed as indicated above. The representative graph indicates the relative amounts of transcripts for CaMKII

isoforms (α, β, γ and δ) in H9C2s and ventricular adult myocytes WKY rats. Cycle threshold (Ct) values from 3 independent experiments were normalized to the internal β-actin control. The ratio of fold change was calculated using the Pfaffl method. * = $p<0.05$ vs CaMKII; # = $p<0.05$ vs CaMKII.

4.4 The inhibition of CaMKII significantly reduces the activation of ERK induced by phenylephrine.

To evaluate the effect of CaMKII inhibition on Erk activation induced by PE, H9C2 cells were treated with CaMKs inhibitor (KN93), specific inhibitors of CaMKII (AntCaNtide and AntCaNtide minimal inhibitory sequence tat-CN17β). The activation of CaMKII induced by PE is significantly reduced following treatment with inhibitors (Figure 4A). The results also show that both the specific inhibitor of CaMKs, KN93, the specific inhibitors of CaMKII, inhibit ERK phosphorylation induced by PE (Figure 4B). Furthermore, inhibition of ERK obtained by treatment with the inhibitor of Mek/Erk pathway (UO126) reduced the CaMKII phosphorylation (Figure 5A and 5B). These results suggest that a specific role of CaMKII in cardiomyocytes, and in particular that stimulation with PE in H9C2 active Erk in a $Ca2^{+}$/CaMKII dependent mode.

4.5 The activation of CaMKII regulates its association with Erk.

The mutual interaction between CaMKII and Erk was evaluated by co-immunoprecipitation: the two kinases were associated in basal condition, and this interaction increased after stimulation with PE. The association was significantly inhibited after treatment with inhibitors of CaMKs (KN93), CaMKII (AntCaNtide and tat-CN17β). Furthermore, inhibition of ERK obtained by treatment with the inhibitor of Mek/Erk pathway (UO126) and the inhibition of CaMKII obtained by ANT 1-17 inhibits the interaction of both kinases (Figure 5C and 5D). These results confirm that the activation of CaMKII promotes its association with active Erk (Figure 4C and 4D).

4.6 Inhibition of CaMKII regulates the subcellular compartmentalization of CaMKII and Erk.

To evaluate the effect of the activation of CaMKII on the subcellular localization of the kinase and ERK, H9C2 cells were treated with PE, after stavation, in the presence and in the absence of inhibitors. The PE induced the accumulation/localization of CaMKII in the nucleus. Treatment with inhibitors (KN93, AntCaNtide and tat-CN17β), significantly reduced the nuclear localization of both kinases (Figure 4E and 4F). Furthermore, inhibition of ERK obtained by treatment with the inhibitor of Mek/Erk pathway (UO126) significantly reduced the nuclear localization of ERK and CaMKII (Figure 5E and 5F). These results support the hypothesis that the activation of CaMKII promotes nuclear localization of both kinases.

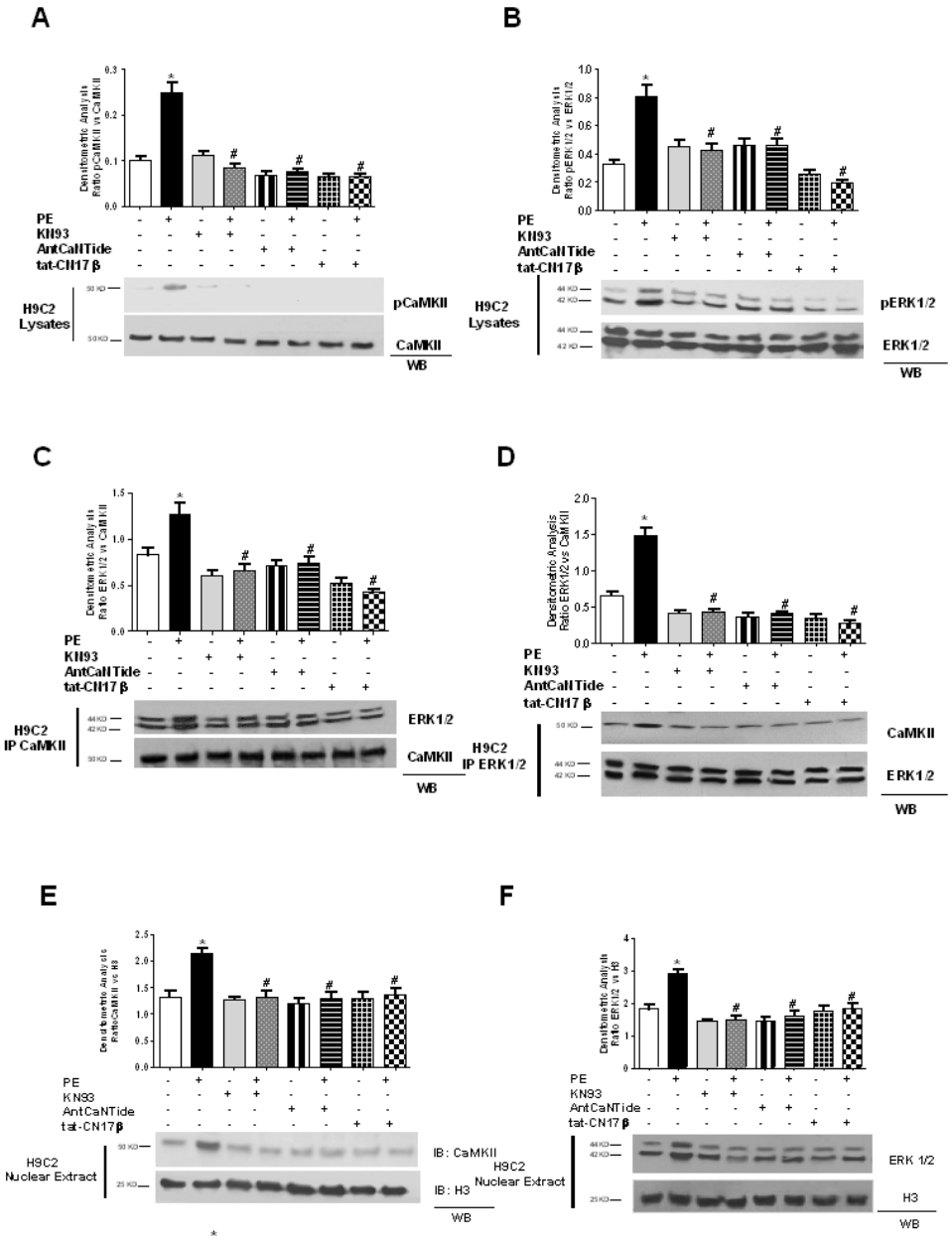

Figure 4. Activation of CaMKII pathway in H9C2 cardiomyoblasts.

To investigate the role the cross talk between CaMKII and ERK on cardiac hypertrophy, we assessed the effect of CaMKII inhibition on PE-induced ERK activation. H9C2s were pretreated with KN93 (5 µmol/L) or AntCaNtide (10 µmol/L) and tat-CN17β (10 µmol/L) for 30 minutes and then stimulated with PE (100 nmol/L for 15 minutes).

A. H9C2 total cell lysates were analyzed by Western blotting (WB) for phosphothreonine 286 CaMKII (pCaMKII) and total CaMKII (CaMKII), with specific antibodies.

Data from immunoblots were quantified by densitometric analysis (DA). pCaMKII levels were corrected by total CaMKII densitometry. * = p<0.05 vs Ctrl; # = p<0.05 vs PE.

B. Total H9C2 cell extracts were be subjected to WB analysis to visualize phosphotyrosine (pERK1/2) and total ERK (ERK1/2) cell content using anti-pERK1/2 or anti-total ERK1/2 antibodies. pERK1/2 levels and were corrected by total ERK1/2 densitometry. * = p<0.05 vs Ctrl; # = p<0.05 vs PE.

C. The H9C2 total lysates were immunoprecipitated using anti-CaMKII antibody. The protein samples underwent WB procedure to visualize ERK and evaluate the association with CaMKII. The experiments were normalized by WB for total CaMKII. ERK1/2 levels were corrected by CaMKII densitometry. * = p<0.05 vs Ctrl; # = p<0.05 vs PE.

D. To confirm the interaction between CaMKII and ERK in H9C2, total cell lysates were immunoprecipitated using anti-ERK1/2 antibody, and subjected to WB using anti-CAMKII antibody. CaMKII levels were corrected by ERK1/2 densitometry. * = p<0.05 vs Ctrl; # = p<0.05 vs PE.

E. Nuclear extract from H9C2s were prepared as indicated in the Methods. Nuclear extracts were analyzed by WB for total CaMKII with selective antibody. CaMKII levels were averaged and normalized to histone 3 densitometry. * = p<0.05 vs Ctrl; # = p<0.05 vs PE.

F. To evaluate nuclear ERK localization, the nuclear extracts were analyzed by WB for total ERK with specific antibody.. ERK1/2 levels were normalized to histone 3 densitometry. *, $P < 0.05$ *vs.* Ctrl; #, $P < 0.05$ *vs.* PE. Data from all immunoblots were quantified by densitometric analysis Each data point in all graphs represent the mean±SEM of 3 independent experiments.

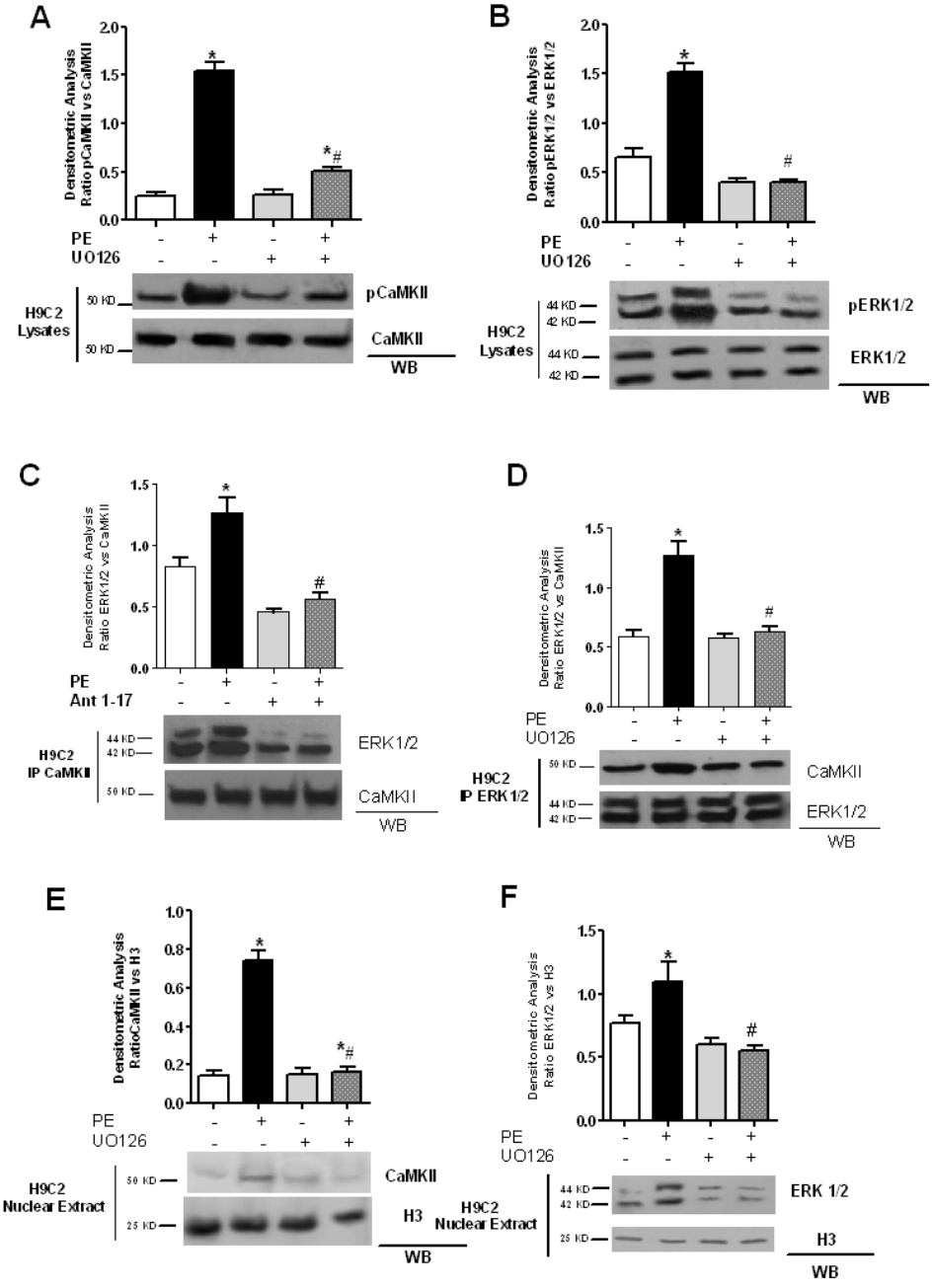

Figure 5. Inhibition of ERK pathway downregulates PE-induced CaMKII activation in H9C2 cardiomyoblasts

A. H9C2 cardiomyoblasts were exposed to UO126 (10 µmol/L) for 30 minutes, and then stimulated with 100 nm phenylephrine (PE) for 15 minutes. Total cell extracts were analyzed by western blot for phosphothreonine 286 CaMKII (pCaMKII) and CaMKII with specific antibodies. pCaMKII levels were corrected by CaMKII densitometry. *, $P < 0.05$ *vs.* Ctrl; #, $P < 0.05$ *vs.* PE.

B. H9C2s were stimulated with PE after pretreatment with UO126 (10 µmol/L for 30 minutes). Total lisates were analyzed by WB for pERK with specific antibody. pERK1/2 levels were corrected by ERK1/2 densitometry. * = p<0.05 vs Ctrl.

C. H9C2s were stimulated with 100 nmol/L PE for 15 minutes following 30 min pretreatment with UO 126 (10 µmol/L). CaMKII was immunoprecipitated from cell lysates using a specific anti-CaMKII antibody, and ERK (ERK1/2) was visualized by WB to evaluate its association with CaMKII. ERK1/2 levels were corrected by CaMKII densitometry. * = p<0.05 vs Ctrl.

D. To confirm the interaction between CaMKII and ERK in H9C2s, total cell lysates were immunoprecipitated using anti-ERK1/2 antibody, and subjected to western blot using anti-CAMKII antibody. CaMKII levels were corrected by ERK1/2 densitometry.* = p<0.05 vs Ctrl.

E. After pharmacological inhibition of ERK and stimulation with PE, nuclear extract from H9C2s were prepared as indicated in the methods section. Nuclear extracts were analyzed by WB for total CaMKII with specific antibody. CaMKII levels were averaged and normalized to histone 3 densitometry. *, $P < 0.05$ *vs.* Ctrl.

F. To confirm the effects PE induced ERK nuclear localization after pretreatment with UO126, nuclear extracts were analyzed by WB for total ERK1/2 with specific antibody. ERK1/2 levels were normalized to histone 3 densitometry. *, $P < 0.05$ *vs.* Ctrl. Data from all immunoblots were quantified by densitometric analysis Each data point in all graphs represent the mean±SEM of 3 independent experiments.

4.7 Activation of CaMKII and Erk regulates the expression of ANF induced by phenylephrine.

The expression of ANF is commonly considered an indicator of cardiac hypertrophy. To evaluate the effect of CaMKII on the expression of ANF, H9C2 cells were stimulated with PE, after stavation, in the presence and in the absence of CaMKs inhibitors (KN93) and the selective inhibitors oc CaMKII (AntCaNtide and tat-CN17β), as well as the inhibitor of Mek/Erk pathway (UO126). Stimulation with PE induced an increased expression of ANF from baseline, that was significantly reduced by treatment with the inhibitors (Figure 6). On the other hand, CaMKIIDN infection significantly reduces ANF levels (Figure 6A). To confirm the effectiveness of CaMKIIWT and CaMKII-DN infection in H9C2s, the total cell lysates were subjected to western blot analysis for CaMKII (Figure 6B). Finally, the efficacy of CaMKII inhibitors to prevent PE induced hypertrophic responses in H9C2s, is confirmed by KN93 such as AntCaNtide and tat-CN17β significantly reduction of the levels of expression of ANF induced by PE (Figure 6C). A similar result is obtained by the inhibition of ERK pathway with UO126 (Figure 6C). These data are consistent with the crosstalk between CaMKII and ERK pathways, and suggest the existence of a correlation between the activation of CaMKII-ERK pathway and ANF activation.

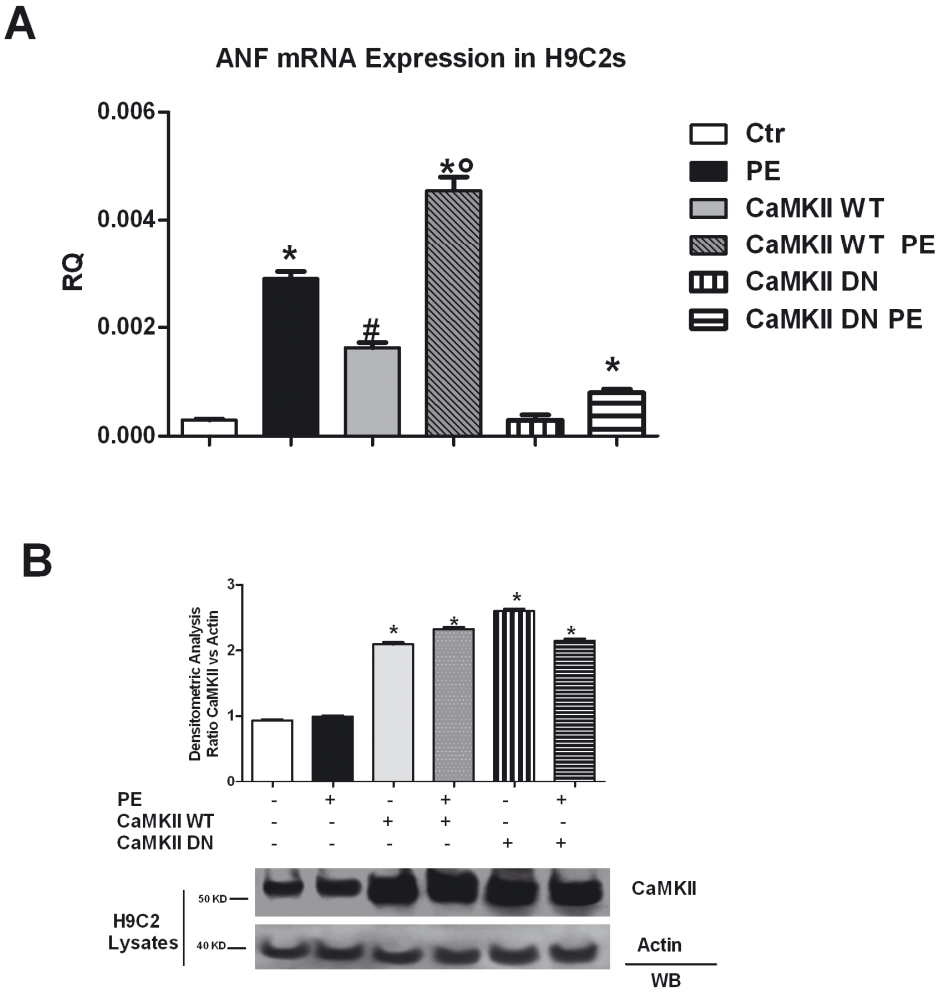

Figure 6. CaMKII/ERK-dependent regulation of the hypertrophy marker ANF.

A. H9C2 cells at 70% confluence were incubated 1 h at 37°C with 5 mL DMEM containing purified adenovirus at a multiplicity of infection (moi) of 100:1, encoding either the kinase-dead (CaMKII-DN,

rCaMKIIdelta, K42M, impaired ATP binding pocket), or the wild type (CaMKII-WT, rCaMKIIdelta) variant of CaMKII or the empty virus as a negative control (Ctr). 48 h after the infection, the cells were stimulated with PE 100 nM for 24 h. Total RNA was isolated from H9C2s using TRIzol reagent, and cDNA was synthesized by means of a Thermo-Script RT-PCR System, following the manufacturer's instruction. Then ANF gene expression was evaluated by real-time PCR. Results are expressed as mean±SEM from 3 independent experiments. The ratio of fold change was calculated using the Pfaffl method[34]. * = $p<0.05$ vs Ctrl; # = $p<0.05$ vs PE.

B. The H9C2s infected with adenoviruses encoding wilde type CaMKII (CaMKII-WT) and kinase dead CaMKII (CaMKII-DN) were stimulated with PE 100 nM for 24 h. Total cell lisates were analyzed by WB for total CaMKII with specific antibody. CaMKII levels were corrected by Actin densitometry. Data from the immunoblots were quantified by densitometric analysis .* = $p<0.05$ vs Ctrl # = $p<0.05$ vs PE. Each data point in all graphs represent the mean±SEM of 3 independent experiments.

C. H9C2 cells were pretreated with CaMK inhibitor KN93 (5 µmol/L), the selective inhibitors AntCaNtide (10 µmol/L) and tat-CN17β (10 µmol/L) and ERK specific inhibitor pathway UO126 (10 µmol/L) for 30 minutes and then stimulated with PE (100 nmol/L) for 24 hours. cDNA was synthesized from RNA obtained from H9C2s as indicated above. The ANF gene expression was evaluated by real-time PCR. Results are expressed as mean±SEM from 3 independent experiments. The ratio of fold change was calculated using the Pfaffl method. * = $p<0.05$ vs Ctrl; # = $p<0.05$ vs PE.

4.8 Inhibition of CaMKII reduces left ventricular hypertrophy in hypertensive rats (SHR)

To confirm that CaMKII inhibition prevents the left ventricular hypertrophy (LVH), we used Spontaneous Hypertensive Rat (SHR), an animal model of hypertension-induced LVH and in normotensive Wistar Kyoto rats (WKY). AntCantide, tat-CN17β or a saline solution were injected in the cardiac wall of the SHR and WKY. LVH was evaluated weekly for 4 weeks by echocardiography.

Both AntCantide and tat-CN17β treatments reduced interventricular septum (IVS) thickness (Figure 7A) and Left Ventricular Mass (LVM)/body weight (BW) (Figure 7C). At the end of the observation period, heart weight (HW)/BW ratio saccordingly showed a significant reduction (Figure 7B). A similar beneficial effect could be observed on left ventricle function, assessed by ultrasound. Indeed, Lelf Ventricular Ejection Fraction (LVEF) and Lelf Ventricular fractional shortening (LVES), are depressed in the SHR control group with respect to the WKY rat and normalized after treatment with either AntCantide or tat-CN17β (Figure 7D). To rule out changes in hemodynamics as a possible mechanism of regulation of LVH, we measured blood pressure in rats after 3 injections and verified that there were no effects of treatments in the hypertrensive states of the 3 SHR groups. (Figure 7E). Biochemical analysis confirmed the reduction of LVH induced by AntCantide and tat-CN17β, as assessed by the expression of the hypertrophy marker gene ANF. (Figure 7F). Histological analysis of *SHR* cardiomyocytes showed common elements of hypertension-induced LVH, including increased cell size and augmented interstitial fibrosis compared to WKY. Both parameters were significantly reverted after treatment with either AntCantide or tat-CN17β (Figure 7G, 7H, 7I). Finally, CaMKII expression levels were increased after the treatment with AntCantide and tat-CN17β in SHR compared to WKY rat (Figure 8A), on the other hand, CaMKII and ERK phosphorylation were reduced by AntCantide and tat-CN17β (Figure 8B and 8C). In the hypertrophic hearts, the nuclear accumulation of CaMKII (Figure 8D) and ERK (Figure 8E) was also enhanced, as well as the interaction between both kinases (Figure 8F and 8G). We can confirm that CaMKII inhibition by AntCantide or tat-CN17β blocked both Erk phosphorylation, CaMKII/Erk interaction and the nuclear localization of both kinases.

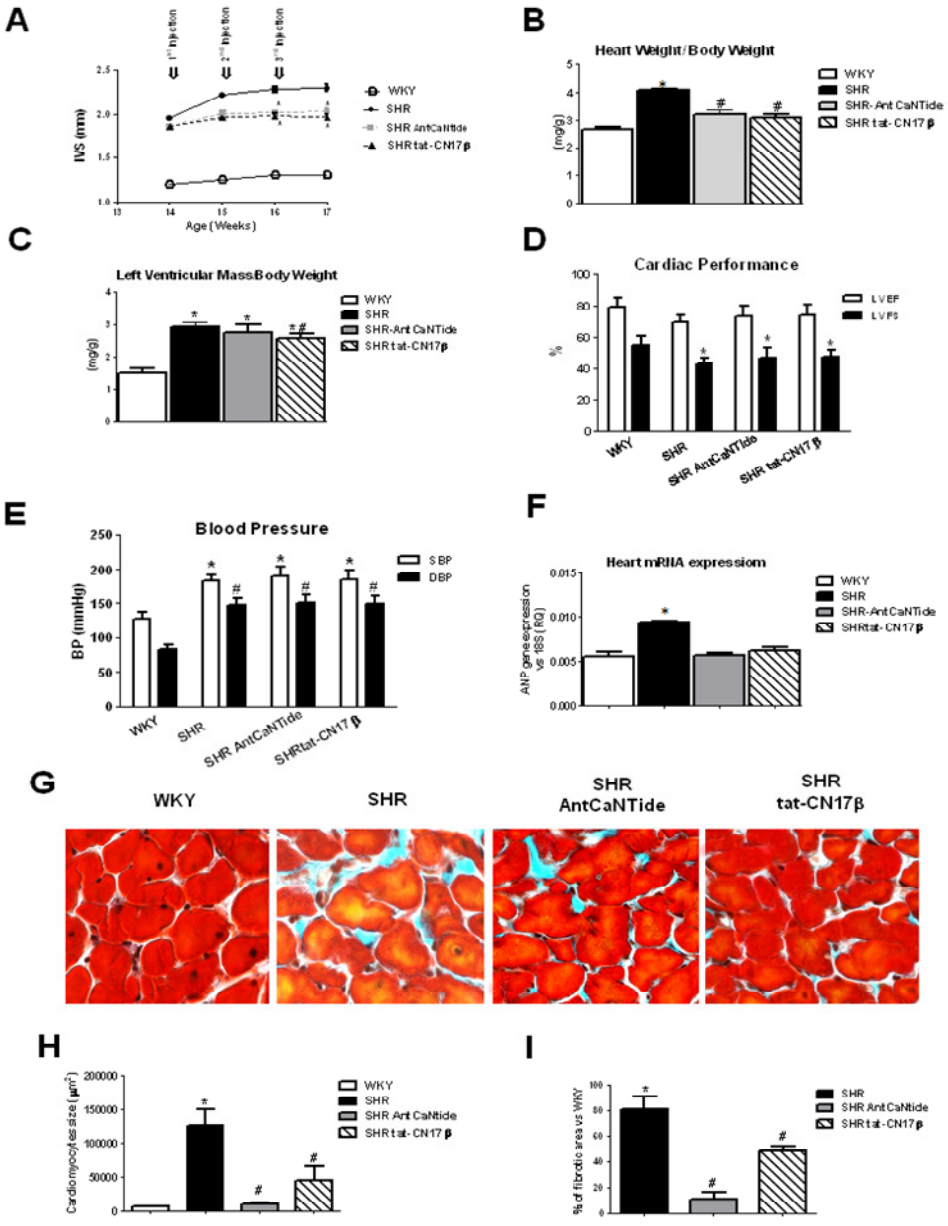

Figure 7. The effect of CaMKII selective peptide inhibitors in vivo in SHRs.

A. SHR rats were subjected to intracardiac injection at the age of 14 week and repeated after 7 and 14 days. Transthoracic echocardiography was performed at each drug administration and once more at the day of euthanasia in 7 untreated WKY rats, 8 AntCaNtide SHRs and 7 tat-CN17β SHRs, and 5 control SHRs (SHR) using a dedicated small-animal high-resolution-ultrasound system (Vevo 770, VisualSonics). ANTCaNtide-SHRs and tat-CN17¤-SHRs the reduction of interventricularseptal (IVS) thickness compared to SHR (*P<0.05 vs WKY; #P<0.05 vs SHR).

B, C. At the end of treatment, rats were weighed and then euthanized. Hearts were immediately removed, rinsed 3 times in cold PBS and blotted dry, weighed and then rapidly frozen. The HW/BW ratio (B) and LVM/BW ratio (C) were measured in ANTCaNtide-SHRs and tat-CN17β- SHRs and compared to WKY and SHR hearts (**P*<0.05 vs WKY; #*P*<0.05 vs SHR).

D. Left ventricular ejection fraction (LVEF) at the end of the treatment was calculated using VeVo770 to evaluate cardiac performance. All measurements were averaged on 5 consecutive cardiac cycles and analyzed by two experienced investigators blinded to treatment. No differences were observed in LVFS and LVEF in ANTCaNtide and tat-CN17β SHRs when compared with SHR (*P<0.05 vs WKY).

E. To evaluate the involvement of BP values in CaMKII–dependent regulation of cardiac hypertrophy, SBP and DBP values were assessed weekly in AntCaNtide- and tat-CN17β- treated and control. The pressure values in the graph represent the average of the measurements made in the course of 4 weeks of treatment (**P*<0.05 vs WKY).

F. At the end of the treatment the total RNA was isolated from myocardial sample using TRIzol reagent, and cDNA was synthesized by means of a Thermo-Script RT-PCR System, following the manufacturer's instruction. ANF gene expression was evaluated by real-time PCR in AntCaNtideand tat-CN17β-SHR rats compared with WKY and SHR (**P*<0.05 vs WKY). Results are expressed as mean±SEM from 3 independent experiments. The ratio of fold change was calculated using the Pfaffl method.

G. Paraffin-embedded sections of control and treated hearts were stained by Masson's trichrome staining to evaluate collagen fibers. The treatment with AntCaNtide and tat-CN17β reduced fibrosis compared with SHR.

H. Cardiomyocytes size was measured by Image J software, and means of areas are showed in the histogram ((*P<0.05 vs WKY; # P<0.05 vs SHR). Images are representative of 3 independent experiments (magnification ×60; black bar = 100 μm)

I. Fibrotic areas were measured by Image J software, and percent of fibrotic areas compared to WKU are shown in the histogram (*P<0.05 vs WKY). Images are representative of 3 independent experiments.

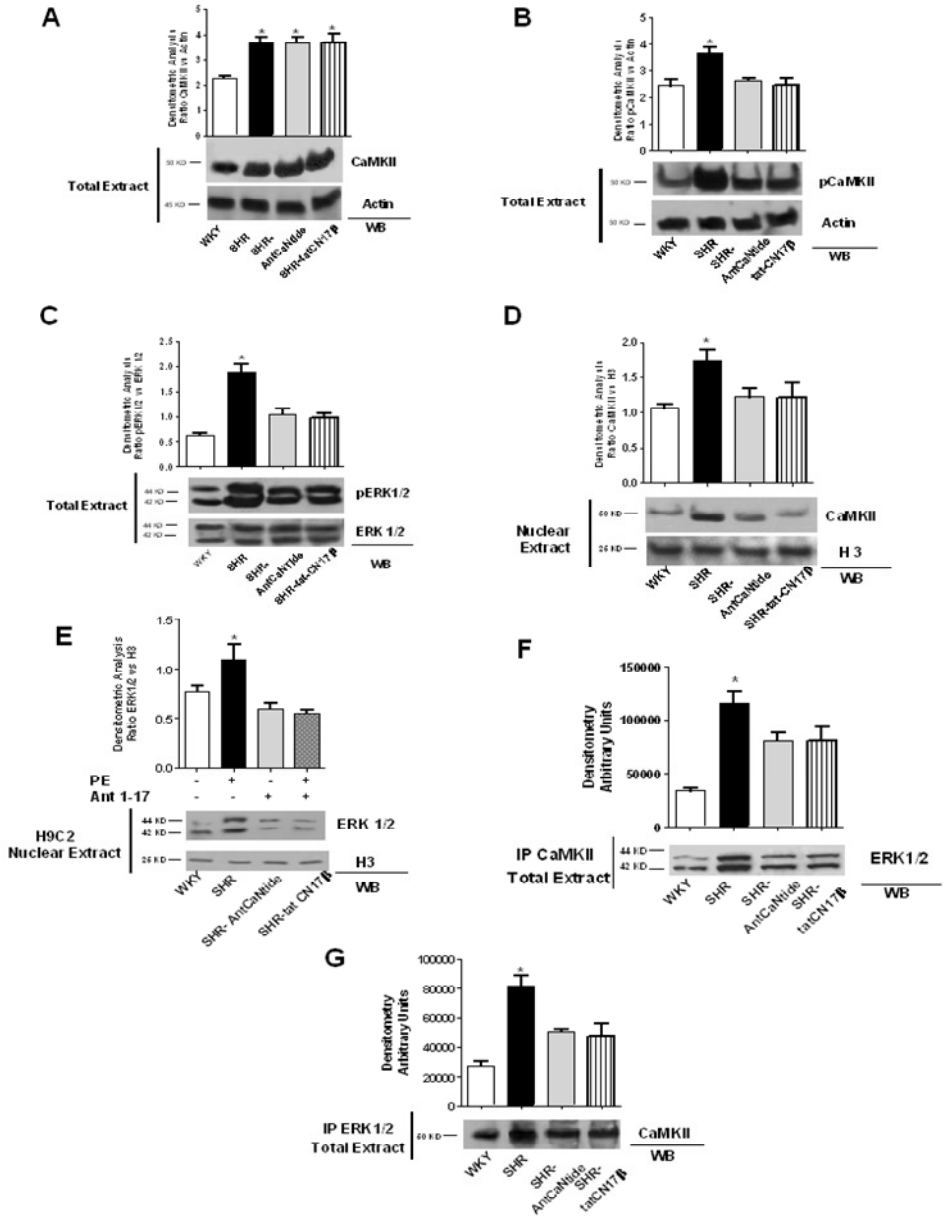

Figure 8. Activation of CaMKII/ERK pathway in vivo in SHR

A. Three weeks after the injections, samples from WKY, SHR- AntCaNtide, SHR- tat-CN17β, and SHR-Control total lysates were prepared from the left ventricular samples. Whole lysates were subjected western blotting analysis with anti□CAMKII antibody. CAMKII levels were corrected by Actin densitometry.* = p<0.05 vs WKY.

B. To assess CAMKII phosphorylation levels in LV after CaMKII inhibitors pretreatment, total lysate samples from WKY, SHR-AntCaNtide, SHR- tat-CN17β, and SHR-Control were analyzed by WB for anti-phosphothreonine 286 CaMKII antibody (pCaMKII). pCaMKII levels were corrected by Actin densitometry.* = p<0.01 vs 0'.

C. Total cell extracts of LV from WKY, SHR-AntCaNtide, SHR- tat-CN17β, and SHR-Control were analyzed by WB with anti-pERK (pERK1/2) or anti- total ERK ½ (ERK ½). pERK½ levels were corrected by total ERK½ densitometry. * = p<0.01 vs Ctr; # = p<0.01 vs WKY.

D. To evaluate the effects of CaMKII inhibition on CaMKII subcellular compartmentalization, nuclear extract from WKY, SHR, SHR-AntCaNtide and SHR-tat-CN17β were prepared as indicated above. Nuclear extracts were analyzed by WB for total CaMKII with specific antibody. CaMKII levels were averaged and normalized to histone 3 densitometry.*, P < 0.05 vs. WKY.

E. The nuclear extract from WKY, SHR, SHR-AntCaNtide and SHR-tat-CN17β were analyzed by WB for total ERK with specific antibody to test the effects of CaMKII inhibitors on ERK subcellular compartmentalization. CaMKII levels were averaged and normalized to histone 3 densitometry.*, P < 0.05 vs. WKY.

F. To examine the association between CaMKII and ERK in the left ventricule from SHR following intracardiac injections, total cell lysate from WKY, SHR, SHR-AntCaNtide and SHR-tat- CN17β, total lysates were immunoprecipitated using anti-CaMKII antibody and subjected to WB with anti-ERK antibody. ERK levels were averaged and normalized to IgG densitometry. *P < 0.05 vs. WKY. Data from all immunoblots presented here were quantified by densitometric analysis Each data point in all graphs represent the mean±SEM of 3 independent experiments.

5. DISCUSSION

Cardiac remodeling is generally accepted as a determinant of the clinical course of heart failure (HF). Defined as genome expression resulting in molecular, cellular and interstitial changes and manifested clinically as changes in size, shape and function of the heart resulting from cardiac load or injury, cardiac remodeling is influenced by hemodynamic load, neurohormonal activation and other factors still under investigation (Cohn JN et al. 2000). The calcium calmodulin-dependent protein kinase II (CaMKII) is a multifunctional serine/threonine kinase enriched incardiomyocytes. CaMKII regulates the physiology of excitation-contraction coupling (Maier and Bers, 2007) and plays a role in the pathophysiology of structural heartdisease, hypertrophy, and arrhythmias (Anderson, 2005; Hund and Rudy, 2006). CaMKII directly contributes to protein phosphorylation, a dynamic post-translational modification important for multiple cellular functions such as membrane excitability, cellular Ca^{2+} homeostasis, metabolism, vesicle secretion, gene transcription, protein trafficking and cell survival and differentiation. Neurohormonal signals elevated in heart disease can stimulate CaMKII activity. Of interest, β-adrenergic (W. Z. Zhu, et al. 2003) and Ang II (Erickson, et al. 2008) receptor stimulation both independently result in CaMKII activation. Likewise, CaMKII inhibition reverses the hypertrophic and anti-apoptotic effects of these agonists, respectively (Erickson, et al., 2008; Sucharov, et al., 2006; R. Zhang, et al., 2005). Angiotensin II,endothelin-1 and catecholamines (α-adrenergic) bind to specific seven-transmembrane-spanning receptors that are coupled to heterotrimeric G proteins of the Gαq/α11 subclass. These G proteins are coupled to phospholipase Cβ (PLCβ). This coupling induces the generation of diacylglycerol (DAG), which functions as an intracellular ligand for protein kinase C (PKC), leading to PKC activation, and production of inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$. Accumulation of $Ins(1,4,5)P_3$ leads to the mobilization of internal Ca^{2+} by directly binding to the $Ins(1,4,5)P_3$ receptor located in the endoplasmic reticulum or the nuclear envelope. Recently, the generation of $Ins(1,4,5)P_3$ and the associated liberation of internal Ca^{2+} stores was shown to mediate hypertrophic signaling through calcineurin–NFAT activation or calmodulin dependent kinase (CaMK)–HDAC inactivation (Wilkins, B. J. &Molkentin, 2004; J. Wu, X. et al. 2006). Activation of Gαq/α11 is also a potent inducer of MAPK signalling in cardiacmyocytes, although the exact mechanism of MAPK coupling has not been described (Clerk, et al 1999). In response to agonist stimulation or cell stretching, ERK1 and 2 become activated both in cultured cardiac myocytes and in isolated perfused hearts (Yamazaki T, et al 1993). Moreover ANG II, via AT1 receptors, carries out its functions via MAP kinases (ERK 1/2, JNK, p38MAPK), receptor tyrosine kinases [PDGF, EGFR, insulin receptor], and nonreceptor tyrosine kinases [Src, JAK/STAT, focal adhesion kinase (FAK)]. AT1R-mediated NAD(P)H oxidase activation leads to generation of

reactive oxygen species, widely implicated in vascular inflammation and fibrosis. ANG II also promotes the association of scaffolding proteins, such as paxillin, talin, and p130Cas, leading to focal adhesion and extracellular matrix formation. These signaling cascades lead to contraction, smooth muscle cell growth, hypertrophy, and cell migration, events that contribute to normal vascular function, and to disease progression. (Puja K. et al 2007).

In the present study, we provide for the first time the compelling evidence that pharmacological selective inhibition of CaMKII results in the reduction of cardiac hypertrophy both *in vitro* and *in vivo* models. Indeed, so far the role of CaMKII in LVH was essentially demonstrated by gene deletion strategies (Anderson et al. 2011). Here we demonstrate that peptides designed on the CaMtide sequence can inhibit CaMKII and block hypertrophy responses. Furthermore, our data indicate that a crosstalk between CaMKII and ERK occurs also in cardiac myocytes and promoted cardiac hypertrophy. Our data exploit the pathogenetic role of CaMKII in the settings of cardiac myocyte hypertrophy, showing that a targeting strategy based on selective peptides can prevent this maladaptive response of the heart. Since CaMKII is present in humans in 4 isoforms, namely CaMKIIα, CaMKIIβ,CaMKIIγ and CaMKIIδ and the adult heart is composed of ~56% myocytes, 27% fibroblasts, 7% endothelial cells, and 10% vascular smooth muscle cells (Banerjee et al. 2007), we felt the urge to characterize the expression of CaMKII isoforms in our cardiac myocyte models, namely the H9C2 myoblasts and adult rat ventricular cardiomyocytes. Both H9C2 and adult rat cardiomyocytes express CaMKII α, β, γ and δ isoforms, and this finding is in accordance with previous literature (Tobimatsu and Fujisawa 1989; Singer et al. 1997; Edman and Schulman 1994; Baltas et al. 1995; Mayer et al. 1995; Tombes et al. 2003), Albeit other reports suggest that the heart does not express CaMKII α and □β□ isoforms (Tobimatsu and Fujisawa 1989). Of note, though, our results indicate that the most abundant CaMKII isoform in adult rat cardiomyocytes is CaMKIIβ, since CaMKII α, δ and γ are only detectable when concentrated by immunoprecipitation. These data contradict the notion that CaMKIIδ is the most important CaMKII isoform in the heart. By a critical perusal of the literature, it appears that this assumption is based on early reports that analyzed CaMKII expression by mRNA expression from mRNA extracted by the whole heart (Colomer et al. 2003; Lu et al. 2010; Little et al 2009; Hagemann et al. 2001). On the contrary, we evaluated expression of the isoforms using commercial, validated antibodies which are of wide use by researcher in the field and that recognizes in rat the respective isoforms of CaMKII. Furthermore, our results are obtained in cultured cardiac myoblasts or freshly isolated ventricular myocytes; we also compared the relative expression in these cells with the most abundant source of CaMKII isoforms in the body of the rat, the brain. Therefore, we are convinced that differences in the model used and dissimilarities 17 between mRNA and proteins may account for the divergences observed between our and previous reports. Our data do not exclude that less expressed isoforms of CaMKII might play a role in LVH; on

the contrary, we believe that our results point in particular to the nuclear isoforms of CaMKII. On this regard, we confirm that CaMKIIδ is the most important nuclear isoform in cardiac myocytes. Our data, furthermore, indicate that the recently described mechanism of stimulation of nuclear transcription by CaMKII activation of ERK is possibly relevant also in the setup of LVH. In particular, we demonstrate that inhibition of CaMKII results in the loss of ERK accumulation within the nucleus. These data are in agreement and help to reconcile previous literature. In particular, it has been observed that nuclear inhibition of CaMKII does not prevent hypertrophy in response to physiological stimulation (Li et al. 2006). On the contrary, another report shows that CaMKII isoforms deletion results in attenuation of hypertrophy (Anderson et al. 2011; Mani et al. 2010). According to our results both findings can co-exists, since the crosstalk between ERK and CaMKII takes play in the cytosol and favors accumulation of ERK in the nucleus. Selective inhibition of nuclear CaMKII isoform will not prevent ERK to enter the nucleus and determine activation of ERK dependent transcription factors related to hypertrophy (Proud 2004). The mechanism of inhibition of CaMKII with CaMKII-INB based peptide represents an alternative strategy based on the sterical inhibition of a conformational change of the kinase which is needed for its activation, rather than with the occupation of the ATP pocket (Sumi et al. 1991). Although previously used for proof of concept studies, these peptides, and in particular AntCaNtide, were never used before *in vivo*. CaMKII selective inhibition with both AntCaNtide and tat-CN17β efficiently reduced the hypertrophy of cardiac myocytes, as well as the remodeling of the heart. We therefore considered antCaNtide as a leading compound, and identified its minimal inhibitory sequence, that resides in residues 1-17 (Gomez-Monterrey et al. 2013). The novel tat-CN17β peptide recapitulates the inhibitory properties of the parental AntCaNtide peptide, both *in vitro* and *in vivo*, and the shorter sequence poses the basis for the identification of the minimal inhibitory sequence and the future molecular design of pharmacological inhibitors.

6. CONCLUSIONS

This study confirms that the crosstalk between CaMKII and ERK sustains the activation of both kinases *in vitro* and *in vivo* in cardiac myoblasts and hypertrophic hearts.

CaMKII-ERK interaction offers a novel therapeutic approach to limit cardiac remodeling.

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