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Calcium/calmodulin-dependent protein phosphatase, calcineurin, has recently been found to play a principal role in activating hypertrophy-sensitive gene expression upon the initiation of cardiac hypertrophy. In order to further quantitatively characterize the roles of calcineurin, we have established an inducible calcineurin expression system in which the expression of constitutively active form of calcineurin is under the control of a tetracycline-dependent transactivator (tTA). By using a reporter gene as a tTA target, we confirm that expression of the target gene can be dramatically induced by tTA in primary cardiomyocytes. In consistent with the results from calcineurin transgenic study, our transfection studies have indicated that the transcription of hypertrophy-sensitive genes, including cardiac α-actin, ANF, and skeletal α-actin gene, was obviously up-regulated by induced expression of active calcineurin in cardiomyocytes. The transcription activation of three hypertrophy response genes mediated by active calcineurin is both active calcineurin plasmid DNA dose and time-dependent. Furthermore, the calcineurin induction of hypertrophy-sensitive genes can be significantly blocked by a specific calcineurin inhibitor, cyclosporin A (CsA), and the inhibition of hypertrophy response gene expression is CsA concentration-dependent. More importantly, the transcription of hypertrophy-sensitive genes mediated by active

calcineurin can be regulated reversibly by CsA manipulation. In addition, the expression of tTA target gene can also be inhibited by effector doxcycline, but doxcycline inhibition is not complete. Based on these results, we conclude that transcription of hypertrophy-sensitive genes can be regulated conveniently by this inducible system. Therefore, the experimental manipulation of temporal and quantitative expression of calcineurin would be useful for further elucidating precise molecular mechanisms for calcineurin-dependent signaling transduction during the onset of cardiac hypertrophy.

TRANSCRIPTIONAL REGULATION OF HYPERTROPHY-SENSITIVE GENES BY AN INDUCIBLE CALCINEURIN EXPRESSION SYSTEM

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TRANSCRIPTIONAL REGULATION OF HYPERTROPHY-SENSITIVE GENES BY AN INDUCIBLE CALCINEURIN EXPRESSION SYSTEM

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TABLE OF CONTENTS

LIST OF TABLE AND ILLUSTRATIONS

ABBREVIATIONS

ANF	atrial natriuretic factor
AT-II	angiotensin-II
ATP	adenosine 5'-triphosphate
bp	base pair
CaM Kinase II	calcium/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CaM	calmodulin
CaN	calcineurin
CardA	cardiac α-actin
C/EBP	CCAAT/enhancer binding protein
CMV	cytomegalovirus
CoA	coenzyme A
CREB	cAMP response element binding protein
CsA	cyclosporin A
DAG	diacylglycerol
Dox	doxycycline
EDTA	ethylenediaminetetracetic acid
ET-I	endothelin-I
FGF	fibroblast growth factor
GATA-4	5'-GATA-3' motif binding protein
G-protein	heterotrimeric GTP-binding protein
IL-2, 2,4	interleukin-2,3,4
IP3	inositol-1,4,5,-trisphosphate
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LUC	luciferase
MAP	mitogen-activated protein

MEF	myocyte-specific enhancer binding factor
MEK	MAP kinase kinase
MEKK	MAP kinase kinase kinase
MHC	myosin heavy chain
MLC	myosin light chain
PBS	phosphate-buffered saline
PE	phenylephrine
PERE	PE response element
PKA	protein kinase A
PKC	protein kinase C
PIP_2	phosphatidylinositol-4,5-bisphophate
PLC	protein phospholipase C
Raf	a MAP kinase kinase kinase
Ras	a monomeric GTPase (p21)
SkA	skeletal α-actin
SR	sarcoplasmic reticulum
SRE	serum response element
STAT	signal transducer and activator of transcription
SV40	simian virus 40
TEF-1	transcriptional exhancer factor-1
tet	tetracycline
tTA	tetracycline-regulated transactivator
VDCC	voltage-dependent L-type Ca^{2+} channel

CHAPTER I

INTRODUCTION

Heart Development

Heart formation during embryogenesis is a complex process that requires a commitment of mesodermal cells to the cardiac muscle cell lineage in response to a specific inducing signal (Litvin et al., 1992; Schultheiss et al., 1995). Subsequently, these precursors differentiate into cardiomyocytes and develop to form the primitive cardiac tube. After cardiac looping, the ventricular and atrial chambers of the heart become distinguished. Atrial and ventricular myocytes arise from separate lineages that are specified at the early stage of cardiogenesis, and they will finally exhibit the divergent morphological, electrophysiological, biochemical and contractile properties because of the activation of distinct subsets of cardiac muscle gene expression (Review by van Bilsen and Chien, 1993; Olson and Srivastava, 1996). The enlargement of the embryonic heart is largely dependent on proliferation of the differentiated myocytes. However, after birth, cardiac myocytes rapidly lose their ability to proliferate and show the terminally differentiated phenotype of adult cardiomyocytes (Chien et al, 1993). Thus, postnatal growth of heart is dependent on the enlargement of preexisting adult cardiomycytes.

Although little is known about the mechanisms that control the differentiation and diversification of cardiogenic precursors to distinct adult phenotypes, recent studies have shown that embryonic and adult heart have obvious differences in some cardiac gene expression. For example, during the early stage of cardiac development, atrial natriuretic factor (ANF) is expressed in both atrial and ventricular regions, whereas just after birth, ANF becomes restricted to the atrium (Zeller et al., 1987) (Table I). In rodents, β myosin heavy chain (βMHC) is the predominant MHC isoform in fetal heart. However, αMHC becomes the most abundant isoform in adult rat cardiomyocytes (Lompre et al., 1984) (Table I). In addition, ventricular myosin light chain 1 (MLC-1) gene is expressed in all embryonic heart chambers but is selectively down-regulated in atrium after birth (Lyons et al.,1990). Therefore, the temporal and spatial expression of cardiac muscle genes becomes a remarkable feature of the developing heart.

Cardiac Hypertrophy

In response to mechanical load, hypertension or a variety of pathological stimuli, including endocrine disorder, myocardial injury and myocardial infarction, adult cardiomycytes can initially adapt to increasing cardiac muscle mass through the activation of a hypertrophied process in order to augment cardiac output. A cardiac hypertrophic response is not accompanied by muscle cell proliferation, but characterized by an obvious increase in myocyte mass and size, increased synthesis of cellular contractile proteins, and activation of cardiac embryonic marker gene

expression (van Bilsen and Chien, 1993). Actually, cardiac hypertrophy is an important compensatory response of the heart to various forms of cardiac diseases. Although it first may have a beneficial function by improving cardiac output, prolonged hypertrophy can eventually lead to dilated cardiomyopathy and heart failure. It has been known that some patients with cardiac hypertrophy develop heart failure and this is associated with a near 50% mortality rate (Levy et al., 1990).

Cardiac hypertrophy is quite different from normal postnatal growth. One of the important biochemical features of hypertrophied myocardium is the induction of an embryonic gene program (Chien et al., 1993) (Table I). There is abundant evidence not only from rat model systems, but also from other mammalian model systems, such as pig, canine, and humans. A common characteristic of hypertrophy is the reexpression of both atrial natriuretic factor (ANF) and B-type natriuretic peptide (BNP) genes in the adult ventricle (Takahashi et al., 1992). These two different genes of cardiac origin encode the two peptide hormones known for potent natriuretic, vasodilatory and diuretic effects (Steinhelper, 1993). As mentioned above, the expression of ANF is down-regulated in the ventricle shortly after birth. It is primarily synthesized within the mature heart atria and is hardly detectable in the normal adult ventricle. Upon the onset of pathologic hypertrophy, it may play a compensatoryly regulatory role in maintaining blood pressure and inhibiting activation of the Renin-Angiotensin-Aldosterone system (Brandt et al., 1993). Induction of ANF gene has been found in hypertrophied ventricular muscle cells of all vertebrate species currently examined, thus, it serves as one of the conserved genetic markers for cardiac hypertrophy (Chien et al., 1991).

Table I. Expression levels of cardiac hypertrophic response genes in ventricular myocytes during different normal developmental or hypertrophic periods.

+++: very high level, ++: high level, +: low level, -: very low level.

Cardiac Hypertrophic Gene Responses

	EXPRESSION		
	FETAL	ADULT	
		Normal	Hypertrophy
Embryonic Genetic Program			
Atrial Naturiatic Factor (ANF)	+++	+/-	++
Skeletal α-Actin	+++	+/-	++
βMyosin Heavy Chain (βMHC)	+++	+/-	++
Post-Diff. Genetic Program			
α Myosin Heavy Chain (αMHC)	+/-	+++	++
Ca++-ATPase	+/-	++	+/-
Constitutive Genetic Program			
Cardiac α-Actin	++	++	+++
Myosin Light Chain-2	++	++	+++
Early/Immediate Genetic Program			
c-fos	+++	+/-	++
c-jun	+++	+/-	++
Egr-1	+++	+/-	++
nfat-3	+++	+/-	++

Similarly, BNP expression level in the embryonic ventricle is much higher than that in adult ventricle (Dagnino et al., 1991). Under most circumstances, the expression of BNP and ANF genes is consistent. It has been found that BNP expression in adult heart was dramatically increased during hypertrophy (Mukoyama et al., 1991). Recently, many studies have indicated that BNP gene in primary neonatal cardiomyocytes was induced by various hypertrophic stimuli (Thuerauf et al., 1994; Molkentin et al, 1998). Accordingly, the typical alteration of ANF and BNP gene expression has been used as the important molecular markers for hypertrophy.

During hypertrophic growth, cardiac fetal genes, including not only ANF and BNP but also many cardiac contractile protein genes, are induced for expression. In heart, the major contractile proteins are MHC, MLC, actin, tropomyosin (TM), and troponin complex (TnT,TnI,TnC), which have multiple isoforms. Expression of a distinct isoform of these multigene families is developmentally regulated at the transcriptional or posttranscriptional level (Komuro et al., 1993). In hypertrophied heart, some of the fetal isoforms are selectively activated. Previous studies have documented that ventricular hypertrophy in rodent species was associated with the reactivation of βMHC and skeletal α-actin (SkA) genes. Normally, these genes are highly expressed in rat embryonic ventricular myocytes, but not in the adult cardiomyocytes (Boeheler et al., 1991; Izumo et al., 1987; Bishopric et al., 1991; Lompre et al., 1991) (Table I). In addition, it has also been reported that the hypertrophic stimuli lead to the up-regulation of another embryonic gene marker, the smooth muscle actin gene (Black et al., 1991).

Another biochemical feature of myocardial hypertrophy is a quantitative increase in the transcription of several constitutively expressed contractile protein genes (Table I). This results in the elevated accumulation of these contractile proteins and enhanced sarcomeric organization (Chien et al., 1993). The α-adrenergic-mediated myocardial cell hypertrophy displays an increase in total transcriptional activity and a concomitant acceleration in the transcription of individual contractile protein genes such as cardiac α-actin (Long et al., 1989) and myosin light chain-2 (MLC-2) (Lee et al., 1988; Knowlton et al., 1991; Zhu et al., 1991). Cardiac α-actin and MLC-2, the sarcomeric post-differentiated isoforms, are predominantly expressed in adult cardiomyocytes, but their levels are usually constant. The inducibility of these constitutively expressed contractile protein genes enhances total cell volume and cellular sarcomeric organization.

A wealth of information has recently been obtained concerning the transient expression of immediate-early genes such as *c-fos*, *c-jun*, and *c-myc* during cardiac hypertrophy (van Bilson and Chien, 1993). In other cell types, the expression of a subset of immediate-early genes usually indicates an activation of cell proliferation. Indeed, the induction of a subset of immediate-early genes in the heart is rapid and transient, and associated with subsequent hypertrophic phenotypes. Obviously, the induction of immediate-early genes is an important feature of cardiac myocyte hypertrophy.

Although it is unclear what are the molecular mechanisms by which these cardiac genes are reactivated or up-regulated in response to a variety of hypertrophic

stimuli, some observations have implicated that different hypertrophic stimuli lead to the expression of different panels of immediate-early genes, and are associated with distinct hypertrophic phenotypes of cardiomyocytes. For example, transient expression of Egr-1 (early growth response gene-1) is stimulated by α adrenergic, but not by β adrenergic agonists; *c-fos/c-jun* expression is associated with sarcomere assembly phenotype in rat neonatal cardiac myocytes (Iwaki et al., 1990). It is known that the products of immediate early genes serve as distinct transcription factors which function in regulating multiple gene expression. Some studies have also showed that primary cultures of rat neonatal cardiomyocytes stimulated with either α or β adrenergic agonists have differences in the expression of various contractile proteins, such as cardiac and skeletal α-actin (Long et al., 1989), and MLC-2 (Lee et al., 1990). Taken together, it is likely that the induction of the immediate-early genes is required for the expression of cardiac embryonic or contractile protein genes. In addition, there is evidence showing that the overexpression of *c-jun* may up-regulate an ANF promoter-reporter expression; *c-fos/c-jun* complex interacts with ANF gene 5' flanking region; protein kinase C (PKC) stimulates the expression of both the *c-fos* and *c-jun* genes, and also induces the ANF gene expression and secretion (Kovacic-Milivojevic et al., 1992; La Pointe et al., 1990). Numerous candidate immediate-early gene products, including Egr-1, SRF, CREB etc, have been proposed to potentially regulate the ANF gene expression. However, so far, it is unclear whether the reactivation of those fetal protein isoforms during hypertrophy is only controlled by immediate-early gene

products or whether there are other mechanisms involved in coordinating the expression of cardiac hypertrophy-related genes.

Signaling Mechanisms of Cardiac Hypertrophy

Over the past decade, a large body of work has shown that the hypertrophy phenotypes are largely dependent on the regulation of a cardiac hypertrophic gene program (Table I). Based on *in vitro* cardiomyocyte models, the earliest alteration on gene expression is the rapid and transient induction of a subset of immediate-early genes within 30 min of exposure to a hypertrophic stimulus. After 6-12 hour, some specific embryonic genes, like ANF, skeletal α-actin, and β-MHC, are reactivated. Following 12-24 hour, the upregulation of constitutively expressed contractile protein genes such as cardiac α-actin and MLC-2 is observed. (Iwaki et al., 1990; Long et al., 1989; Lee et al., 1988). In contrast to these upregulated genes, the gene encoding Ca^{2+}-ATPase on the sarcoplasmic reticulum is gradually down-regulated by hypertrophic signals including mechanical stress or some growth factors (Nagai et al., 1989; Parker et al., 1991). A low level expression of Ca^{2+}-ATPase gene has been observed during the fetal stage of cardiac development (Komuro et al., 1989). Thus, the down-regulation of Ca^{2+} -ATPase is consistent with fetal gene program. Possibly, there is a similar mechanism of transcriptional regulation between embryonic heart and hypertrophic heart. However, the precise hypertrophic signaling pathways that lead to the regulation of these cardiac gene expression are currently unclear. Therefore, the

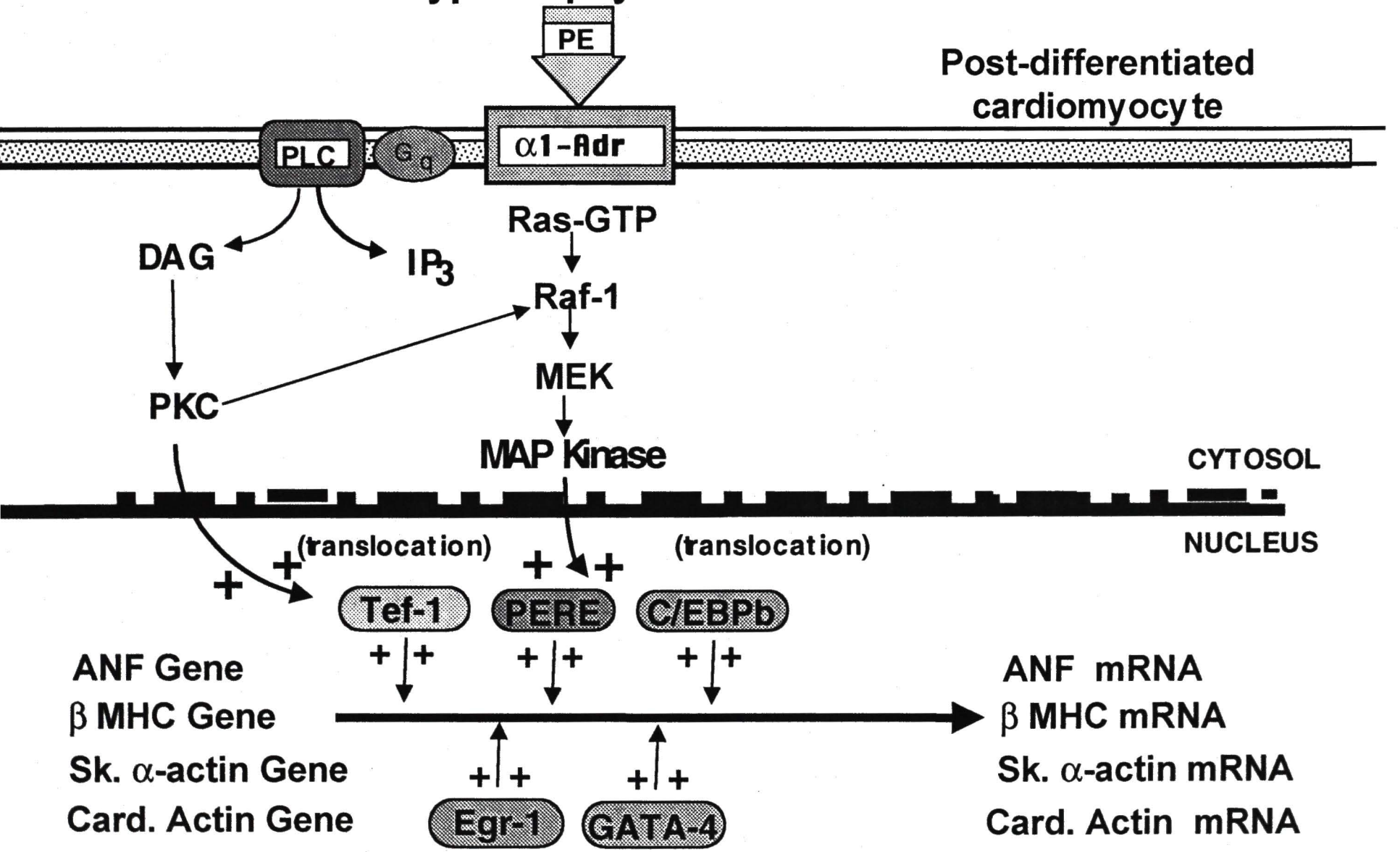
identification of signal transduction and gene regulation mechanisms for hypertrophy and heart failure become the central issue of molecular cardiology.

Great progress in understanding the molecular events involved in cardiac hypertrophy have been made through the recent applications of molecular biology to cardiac myocyte research. New techniques and strategies, including transient transfection, microinjection, transgene technology, and *in vivo* transfection, have allowed us to explore the intracellular molecular events and signaling mechanisms of cardiac myocytes. Previous studies have indicated that the growth response of primary myocardial cells to many hypertrophic stimuli virtually identical to that observed in the pathologic adult myocardium (van Bilsen and Chien, 1993), the development of experimental procedures for primary cardiomyocyte culture provides us a suitable *in vitro* model for detailed analysis of the hypertrophic responses at the cellular level with regard to individual stimuli and their signaling pathways.

Using the cultured myocardial cell model and the genetic markers for hypertrophy, some important intracellular effectors and a number of extracellular neurohumoral factors in hypertrophic signaling pathways have been identified (Reviewed by Hefti et al., 1997). A whole range of substances including adrenergic agonists, angiotensin-II, endothelin-1, various polypeptide growth factors, cytokines and Thyroid 3 have been recognized as hypertrophic stimuli. Moreover, in response to these extracellular stimuli, two intracellular signaling pathways are thought to be involved in mediating hypertrophic signals to the nucleus and ultimately leading to activation of the hypertrophy-related genetic programs (Hefti et al., 1997) (Figure 1).

Figure 1. Schematic outline of putative hypertrophy signaling pathways and transcriptional induction of hypertrophy-sensitive genes. Hypertrophic stimuli such as PE lead to the activation of small G protein-coupled Ras and linked-Mitogen-activated protein (MAP) kinase pathway. In parallel, G protein-coupled receptor results the activation of phospholipase C (PLC), which hydrolyzes PIP2 to produce diacylglycerol (DAG) and inositol triphosphate (IP3), in turn leading to the activation of protein kinase C (PKC). Subsequently, MAP kinase and PKC directly or indirectly activate specific transcription factors, which translocate into nucleus and bind to their specific binding sequences such as tef-1, CRE, GATA in the promoter regions of hypertrophic response genes and finally result in the activation of these hypertrophy-sensitive genes.

Nuclear Signaling and Transcriptional Regulation of Hypertrophy-Sensitive Genes



For example, accumulating evidence suggests that the Ras and distinct motigen-activated protein kinase (MAPK) cascades may play a central role in transmitting extracellular growth and stress stimuli to the nucleus and resulting in genetic (expression of embryonic marker genes) and morphological (changes in the sarcomeric organization) effects associated with hypertrophy (Bogoyevitch et al., 1993a, 1993b; Thorburn et al., 1993; Thorburn and Thorburn, 1994; Zechner et al., 1997).

Several other second messenger systems for hypertrophic signal transduction have been reported in the myocardium. Adrenergic agonists, angiotensin II and endothelin-1 are capable of activating their specific receptors which link to G-proteins (Gq, Gs, Gi). G proteins may directly associate with the Ras-MAPK pathways. More importantly, numerous studies have shown that activated Gq, in turn, stimulates the PIP2 signaling pathway producing two messengers 1,2-diacylglycerol (DAG) and inositol triphosphate (IP3) (Sugden and Boboyevitch, 1996). DAG is known to activate protein kinase C (PKC), which may mediate different downstream cascades through the phosphorylation of a number of nuclear and cytosolic signaling molecules (Figure 1). For example, PKC has been shown to phosphorylate a nuclear transcription factor CREB and cytosolic regulator IκB *in vitro* (Yamamoto et al., 1988; Ghosh et al., 1990), suggesting PKC may directly and indirectly regulate the activity of nuclear transcription factors. Recently, it has been reported to activate Raf-1 (a MAPK kinase kinase) (Kolch et al., 1993). As a consequence, PKC may crosslink the G protein-mediated hypertrophic signal pathway to MAPK cascade (Denhardt, 1996). In parallel, the messenger IP3 induces the release of Ca^{2+} from intracellular Ca-stores. The elevated

intracellular calcium level is an obvious response of cardiomyocytes to those hypertrophic stimuli. In addition, it is known that β-adrenergic agonists are able to modulate protein kinase A (PKA) activity through receptor-coupled G protein (Gs,Gi), adenylate cyclase, and the cAMP cascade. An important effect of PKA is the elevation of intracellular Ca^{2+} concentration (Hefti et al., 1997). It is also known that the L-type Ca channel operated by coupled Gs protein can be opened in response to β adrenergic agonist stimulation, thereby leading to an enhancement of the Ca^{2+} influx (Yatani et al., 1989; Matsuda et al., 1996). It is noted that all of these intracellular pathways are associated with an increase of intracellular Ca^{2+} level (Sadoshima and Izumo, 1997).

In fact, it has been observed that the expression of ANF was increased up to 10 fold in myocardial cells in the presence of a Ca^{2+} channel agonist, BAY K8644; ANF expression induced by both α-adrenergic agonist--Phenylephrine (PE) and the Ca^{2+} channel agonist was completely blocked by a calcium/calmodulin inhibitor,W7; the PKC inhibitor, H7, only partially decreased the ANF expression stimulated by PE, but had no effect on the Ca^{2+} channel agonist-stimulated ANF expression. These results indicate that the changes in intracellular levels of Ca^{2+} appear an important signal for cardiac hypertrophy (Sei et al., 1991).

More importantly, overexpression of calmodulin in the heart of transgenic mice is known to cause cardiac hypertrophy (Gruver et al., 1993). Accordingly, there may be a calcium/calmodulin-dependent signaling pathway involved in hypertrophy. Furthermore, it has been known that those stimuli that increase intracellular calcium can activate calcium/calmodulin-dependent kinase II, which in turn can directly activate the

C/EBP transcription factor family (Wegner et al., 1992). The regulation of ANF secretion in primary cardiomyocytes was shown to depend on the activity of calcium/calmodulin-dependent protein kinase II (McDonough et al., 1994). Recent, other studied have showed that the elevation of intracellular Ca^{2+} induced by angiotensin II results in the activation of c-Jun N-terminal kinases (JNKs, one of three MAP kinases) (Zohn et al., 1995). These data implicate the significant roles of calcium/calmodulin signaling in multiple hypertrophic signal pathways. However, other components of the intracellular calcium-dependent signaling pathway have not yet been identified, also how alterations in intracellular calcium level synergistically regulate other signaling molecules and eventually affect hypertrophy-related gene expression in cardiomyocytes remains elusive.

Calcium/calmodulin-dependent Protein Phosphatase--Calcineurin

It has been known that the activities of several intracellular proteins, including calmodulin, calcium/calmodulin-dependent protein kinase (CaM kinase) family, and calcineurin, can be modulated by intracellular calcium concentrations. Calcineurin(protein phosphatase2B) is a calcium/calmodulin-regulated serine/threonine phosphatase that is ubiquitously expressed in most tissues. In most rat tissues, calcineurin concentrations range from 0.2-0.6 ug/mg protein (Su et al., 1995). The amino acid sequence of calcineurin is highly conserved from yeast to humans with over 50% sequence identity (Yakel, 1997). Calcineurin comprises two subunits, a calmodulin-binding catalytic subunit (CaNA) and an intrinsic Ca^{2+} -binding regulatory

subunit (CaNB), which regulates enzymatic activity through the binding to subunit A. In vitro studies of activation of calcineurin have shown that calmodulin can bind in an inactive enzymatic complex with calcineurin. Under these conditions, only two of calmodulin's four calcium-binding sites are occupied. Thus only an additional small increase in calcium concentration can result in the binding of calcium to the two remaining binding sites on calmodulin. In this state, the phosphatase activity of calcineurin will be dramatically enhanced (Crabtree and Clipstone, 1994).

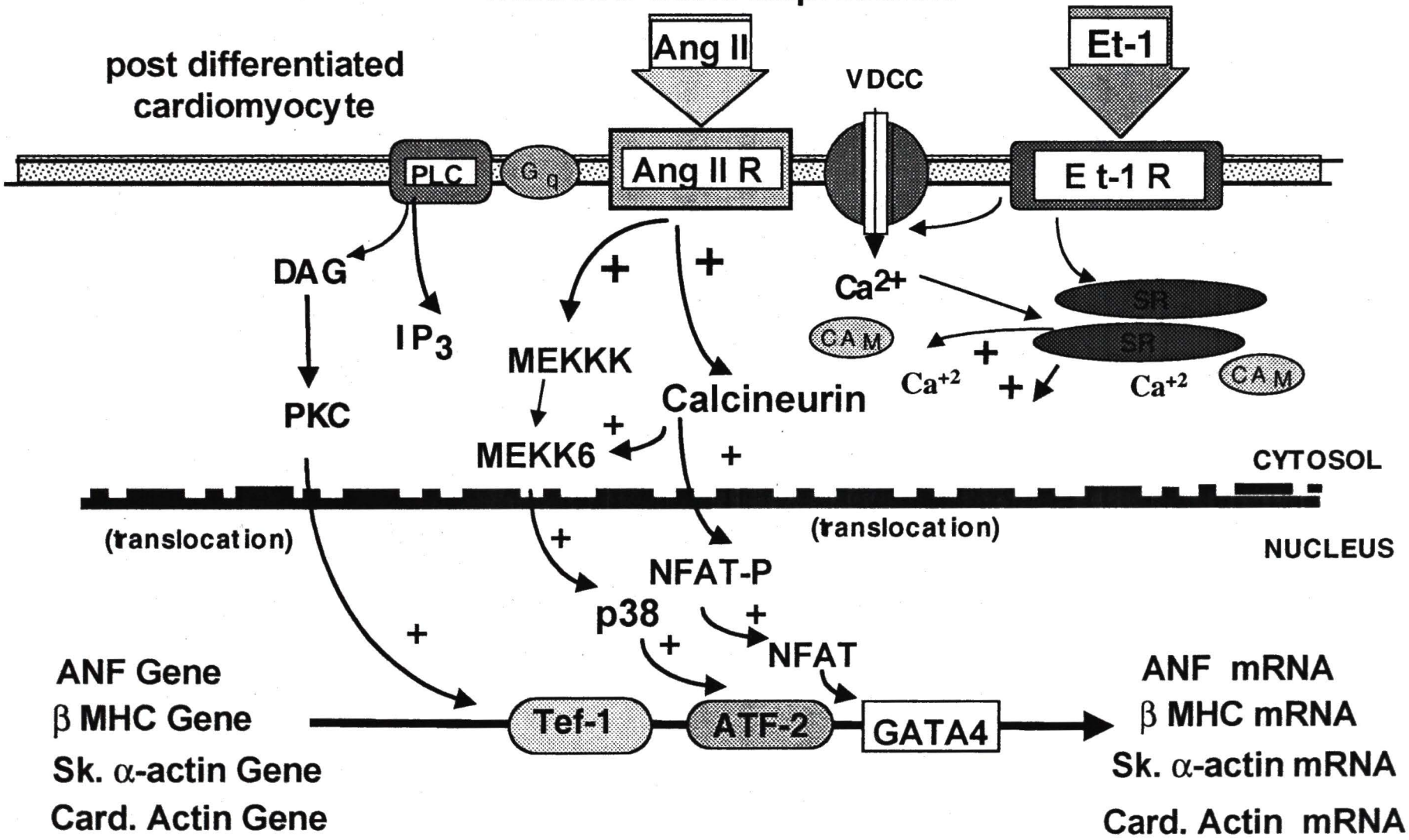
Calcineurin has been implicated in control of yeast growth (Crabtree and Clipstone, 1994). In mammalian cells, it has been shown to regulate the activity of ion channels as well as neurotransmitter and hormone release in neurons (Yakel, 1997). In addition, calcineurin is found to play a role in induction of gene transcription in pancreatic islet cells as well as in sodium/potassium transport in the nephron (Schwaninger et al., 1995; Tumlin, 1997). Recently, significant findings involved in T cell activation and the regulation of immune response gene expression have demonstrated that calcineurin is a major downstream effector of the calcium signal in the T and B cell signal transduction cascade, and activated calcineurin mediates the expression of lymphokine genes and other immune response genes through dephosphorylation of a family of transcription factors known as NFATs (nuclear factors of activated T cells). Dephosphorylated NFAT by calcineurin can translocate into the nucleus and directly activates immune response genes such as interleukin 2 and interleukin 4 (Crabtree and Clipstone, 1994; Rao et al., 1997). In particular, it is identified that the immunosuppressant drug cyclosporin A (CsA) dominantly interferes

with the immune response by its inhibitory effects on the activity of calcineurin (Shaw et al.,1995; Loh et al., 1996). Clearly, calcineurin as an essential signaling intermediate plays a pivotal role in the transduction of signals from T cell membrane to gene regulatory events in the nucleus.

Although calcineurin exists in the cardiomyocytes, little was known about its biological function(s) in myocardium only a couple of years ago. Furthermore, it was unknown whether calcineurin plays a role in transducing calcium signals to alterations in specific cardiac gene expression during cardiac hypertrophy. More recently, preliminary transfection studies from our laboratory suggest that calcineurin in primary rat cardiomyocytes plays an important role in the activation of cardiac embryonic gene expression (Zeng et al, unpublished data). Strikingly, transgenic mice overexpressing the constitutively active form of calcineurin in the heart develop dramatic cardiac hypertrophy and heart failure; moreover, calcineurin has been shown to dephosphorylate transcription factor NFAT3 (NFAT isoform) in vitro; and the overexpression of active form of NFAT3 in the heart of transgenic mice leads to similar phenotypes as those of transgenic mice overexpressing the active form of calcineurin (Molkentin et al., 1998). These findings demonstrate that the activation of calcineurin is critical for the induction of cardiac hypertrophy (Figure 2), and the finding suggests that calcineurin may be the nodal point through which the elevated intracellular calcium signals are linked to the regulation of cardiac gene expression in the nucleus during hypertrophy.

Figure 2. Schematic outline of major molecular signaling pathways involved in the initiation of cardiac hypertrophy. Adult cardiomyocytes transduce hypertrophic signals into nucleus not only through Ras-MAP kinase and PKC pathways, but also through a calcineurin-dependent pathway. In response to multiple hypertrophic stimuli such as endothelin-I, their receptors lead to the activation of G protein and the production of second messengers. G protein can trigger the opening of membrane bound calcium channels. Moreover, calcium can also be released from intracellular stores sarcomere reticulum in response to those messengers. Elevation of intracellular calcium by the opening of L-type calcium channels or/and release of intracellular calcium stores results in activation of calcium binding protein, calmodulin. Subsequently, calcium/calmodulin-dependent protein phosphatase, calcineurin is activated. In turn, activated calcineurin dephosphorylates transcription factor NFAT, which finally induces the expression of hypertrophic response genes.

Nuclear Signaling and Transcriptional Control of Hypertrophy Induced Gene Expression



In addition, there is evidence indicating that targets of calcineurin may be JNK family kinases and IκBα (an inhibitor of NFκB) other than NFAT in T lymphocytes (Su et al., 1994; Frantz et al., 1994). Thus, it is possible that calcineurin, acting in concert with other signal pathways, may modulate the synthesis of transcription factors that are required for the subsequent activation of immune response genes. Alternatively, it is also likely that calcineurin may increase activities of some specific transcription factors like NFκB by inactivating their inhibitors (Jain et al., 1995). It is conceivable that calcineurin in cardiac myocytes might utilize similar mechanisms as those in lymphocytes to modulate complicated hypertrophy response genes. Indeed, the functions of calcineurin in cardiomyocytes and its cooperative regulation to hypertrophy response genes are still poorly understood. The molecular mechanism(s) by which calcineurin precisely controls the transcription of hypertrophy-related genes remains to be elucidated.

Tetracycline Regulatory Expression Systems

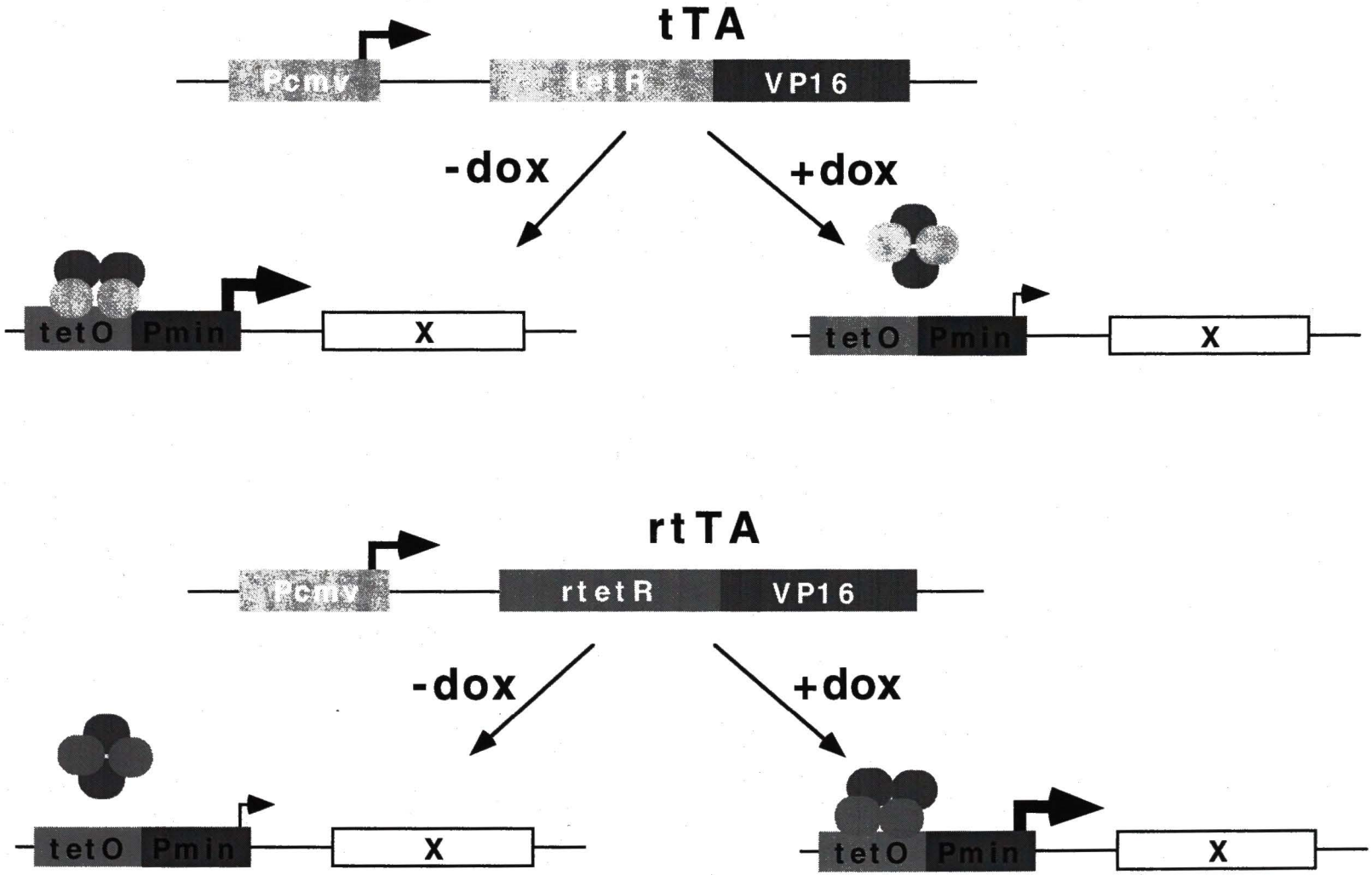
To better understand the roles of calcineurin in complex genetic environments and further facilitate the analysis of hypertrophic signal transduction mechanisms, it is required not only to be able to reversibly turn on or off molecular switches for activating the gene, but also to quantitatively adjust its expression level in a temporal manner. Application of the tetracycline-controlled expression systems will allow us to finely regulate activity of a given gene in mammalian cells, even in whole animals.

One of the tetracycline-regulated gene expression systems is based on two basic elements: a chimeric transcription factor termed tetracycline-controlled transactivator (tTA) and the tTA-controlled human cytomegalovirus minimal promotor (P_{hCMV-1}), which contains up to seven repetitive tetracycline operator (tetO) sequences upstream of minimal promoter. The chimeric transactivator, tTA, is a fusion protein between the DNA binding domain of tetracycline repressor (TetR) and the transcriptional transactivation domain of virus protein 16 (VP16), which is essential for the transcription of the immediate early viral genes. tTA can specifically bind to upstream tetO sequences in the responsive promoter--P_{hCMV-1} and markedly stimulate certain gene transcription by interaction between VP16 transactivation domain and basal transcription machinery. In the presence of an effector substance such as tetracycline or doxycycline, conformational changes in the Tet repressor domain of tTA prevent tTA from binding to tetO sequences, thus the tTA-dependent gene expression will be competitively inhibited (Bujard, 1996) (Figure 3).

Another similar regulatory system called "reverse tetracycline-controlled transactivator" (rtTA) system has recently been developed to use for regulation of mammalian genes as well (Gossen et al., 1995; Kistner et al., 1996). This rtTA system is identical to the tTA system only with exception of four animo acid exchanges in the tetR moiety. These alterations result in a complete reverse phenotype for DNA binding of rtTA. The rtTA requires doxycycline for binding to tetO sequences and thus for activating transcription of downstream gene (Figure 3).

Figure 3. Diagram of the tetracycline regulatory gene expression systems. The tTA and rtTA synthesis is controlled by human cytomegalovirus promoter (P_{CMV}). The fusion protein tTA is composed of the repressor (tetR) and a C-terminal portion of protein VP16. tTA binds in absence of doxycycline but not in its presence to an array of seven cognate operator sequences (tetO) and activates downstream minimal human cytomegalovirus (hCMV) promoter (P_{min}), which itself is inactive. Subsequently, transcription of downstream gene X is induced. rtTA system is identical to the tTA system with the exception of 4 amino acid exchanges in the tetR moiety. These changes convey a reverse phenotype to the repressor (rtetR). The rtTA requires doxycycline for binding to tetO and for transcription activation of gene X.

Schematic Outline of the Tet Regulatory Systems



Due to the specific recognition of the tetR/rtetR domain to tetO sequences as well as the high affinity of tetracycline for tetR or rtetR , two inducible expression systems have shown their abilities to tightly regulate gene expression in cultured mammalian cells (Gossen and Bujard, 1992); moreover, because of the strong transactivation ability from VP16 transactivator, the tTA as well as rtTA systems are able to efficiently induce target gene transcription. A very low concentration of tetracycline results in a rapid repression of tTA-induced gene expression (Gossen et al., 1993). Furthermore, it has been shown that doxycycline, a water soluble analog of tetracycline, was higher effective than tetracycline (Gossen et al., 1995). Many investigators have successfully utilized the systems to control the activity of their gene of interest in cell cultures as well as in transgenic organisms. For example, tetracycline-regulated expression of the STAT5-JAK2 fusion gene in cultured cells allows the study of the function of STAT5 independent on upstream activation events (Berchtold et al., 1997). In particular, the tTA system has been applied to quantitatively controlling cardiac gene activities *in vivo* in a highly tissue-specific manner (Fishman et al., 1994). Furthermore, transgenic mice overexpressing tTA in heart have been generated. They have been shown to facilitate the studies of cardiac gene functions in mammalian animals (Passman and Fishman, 1994). These results indicate that the tetracycline-regulated expression systems will be high useful for exploring cardiac gene functions.

Overview of the present study

Based on our preliminary data and others' studies, we proposed that the activation of calcineurin up-regulates expression of several hypertrophy-sensitive genes in cardiomyocytes. In the present study, a tetracycline-regulated calcineurin expression system has been established to control calcineurin expression. The activation of hypertrophy-sensitive gene transcription mediated by induced active calcineurin depends on active calcineurin plasmid DNA dose as well as time. Furthermore, calcineurin specific inhibitor-- cyclosporin A can significantly inhibit transcription of the hypertrophic response genes induced by active calcineurin. The calcineurin induction of hypertrophy-sensitive gene expression could be reversibly regulated by cyclosporin A manipulation. The goals of this project are to establish a finely controlled expression system for the studies of cardiac gene functions, *in vivo* gene therapies, as well as drug discovery.

CHAPTER II

MATERIALS AND METHODS

Plasmid Constructs.

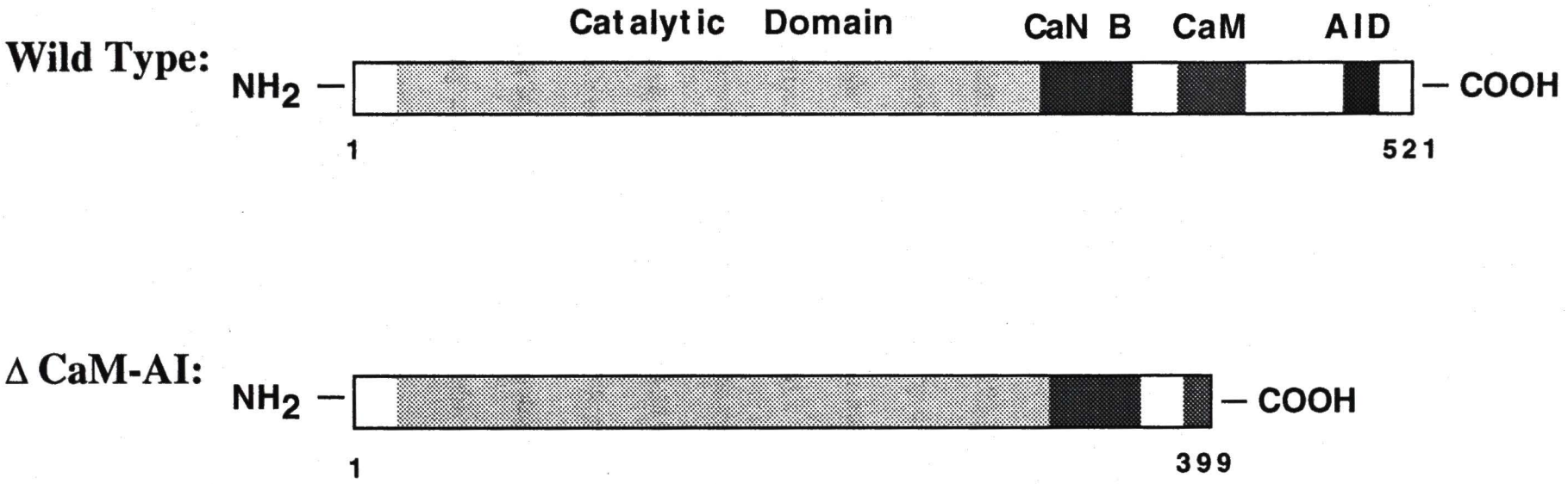
Tetracycline-controlled transactivator (tTA) expression plasmid pUHD15-1 is a generous gift of Dr. Glenn Fishman (Albert Einstein College of Medicine, NY). Plasmid pUHD15-1 encodes chimeric protein tTA under the control of the human cytomegalovirus (CMV) promoter and enhancer. tTA gene consists of the sequences encoding amino acid residues 1-207 from the tetR protein, which are able to specifically bind to the tet operator DNA sequences, and the sequences encoding residues 363-490 from VP16, which have strong transactivation function (Gossen and Bujard, 1992).

Plasmids pUHD10-3 and pUHC13-3 are also provided by Dr. Glenn Fishman. Plasmid pUHD10-3 contains a chimeric tet operon/CMV minimal promoter upstream of multiple cloning sites (Bujard, 1996). pUHC13-3 is a firefly luciferase reporter gene expression plasmid driven by the chimeric tet operon/ CMV minimal promoter (Gossen and Bujard, 1992).

Constitutively active gene form of calcineurin (ΔCaM-AI) (Figure 4) was originally recovered from plasmid pCN(α)ΔCaM-AI which was generated by subcloning the auto-inhibitory domain-deleted calcineurin gene mutant into pSRα

Figure 4. Schematic structures of the wild type and the constitutively active form of the calcineurin A subunit. The four variable domains are shown by unfilled frames. CaN B represents the calcineurin B subunit binding domain. CaM is calmodulin binding domain. AID is the autoinhibition domain. In ΔCaM-AI, amino acid residues 400-521 are deleted.

Schematic Structures of Wild Type and Deletion Mutant of the CaN A Subunit



expression vector (Parson et al., 1994). This construct was kindly sent as a generous gift by Dr. Stephen O'Keefe (Merck &Co. Inc., Rahway, NJ). To generate tTA inducible calcineurin construct p10-3CaN, a 1.2 Kbp EcoR I fragment of pCN(α)ΔCaM-AI coding the constitutively active calcineurin was subcloned into the unique EcoR I cleavage site of the plasmid pUHD10-3, which contains a tTA-dependent cytomegalovirus (CMV) minimal promoter in front of the EcoR I cloning site (Bujard, 1996) (Figure 5).

ANF-luciferase promoter-reporter construct, NP337 (a gift from Dr. Mona Nemer, Univ. of Montreal, Quebec, Canada) was made by cloning about 700-bp nucleotides of ANF proximal promoter fragment into luciferase-based pXP-2 vector as previously described elsewhere (McBride et al., 1993) (Figure 6).

The skeletal α-actin reporter, SkA-Luc, was constructed by subcloning approximately 420bp proximal upstream promoter (-394 bp to +24 bp) of avian skeletal α-actin gene into the firefly luciferase expression vector pXP1 (MacLellan et al., 1994) (Figure 6). Another reporter construct, cardiac α-actin promoter-luciferase (CardA-Luc), was generated by subcloning a 330-bp fragment of chicken cardiac α-actin promoter and immediate upstream region (from -315 to +15, relative to the transcription start site) into the HindIII cloning site of the pGL2-basic luciferase vector (Chen and Schwartz, 1996) (Figure 6). These two reporter constructs were generous gifts from Dr. Robert J. Schwartz and Dr. Micheal D. Schneider (Balyor College of Medicine, Houston, TX).

Figure 5. Strategy of subcloning the gene encoding the constitutively active calcineurin into tTA-dependent plasmid vector pUHD10-3.

Subcloning Constitutively Active Calcineurin Gene into tTA-dependent vector pUHD10-3

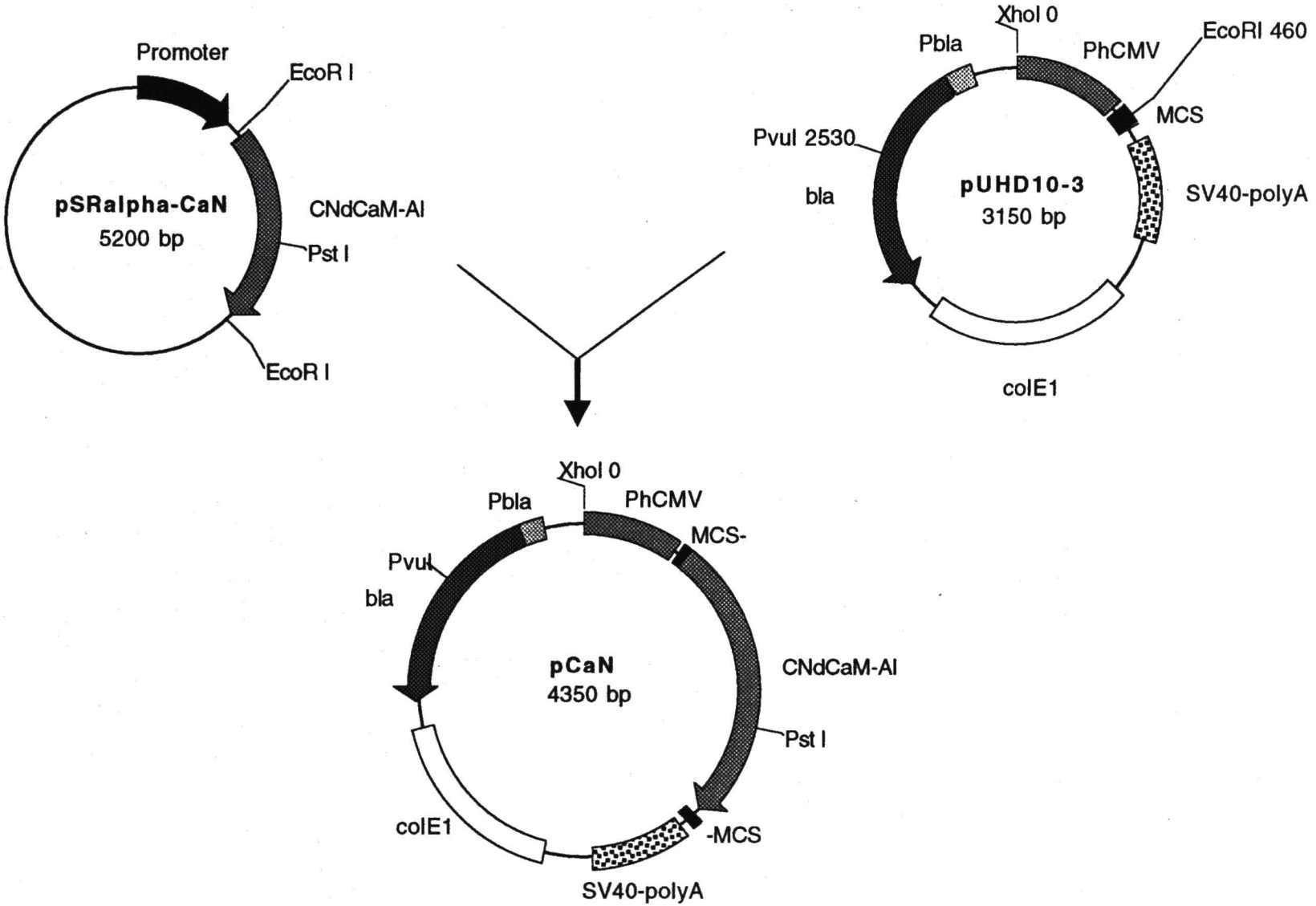
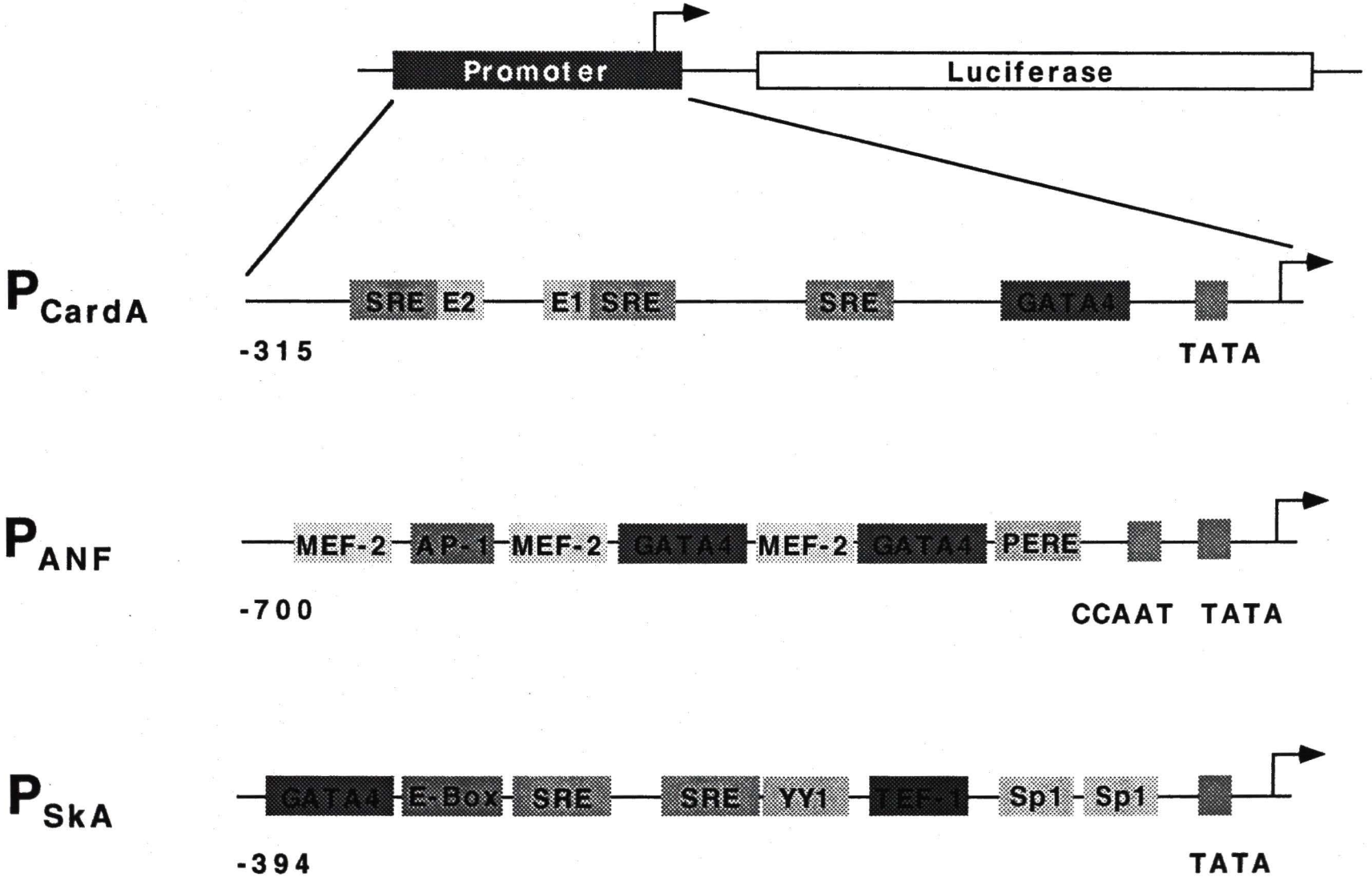


Figure 6. Diagram of important cis-elements in the promoter regions of three cardiac reporter genes.

SRE: serum response element; E1, E2: E-box; PERE: PE response element.

Promoter Regions of Reporter Genes



Cell Cultures

Primary myocardial cell cultures. Primary ventricular myocytes were prepared from 1-4 day old Sprague-Dawley rats. Rat pups were decapitated and hearts were removed sterilely from thoracic cavity and washed with Medium 199 (Life Technologies, Gaithersburg, MD) to get rid of residual blood. The atria were removed and ventricles were finely minced and subjected to three sequential Viokase (A. H. Robbins Company, Richmond, VA) digestion of 18 min each (Harary et al., 1975). Viokase containing lipase, protease and amylase as well as other pancreatic enzymes was made at 1mg/ml in PBS buffer and used at a rate of 8 ml per heart. Supernatants transferring from the three digestions were put on ice in order to quench the digestion reaction. Then the supernatants were centrifuged at 2000 g for 8 min at room temperature. Cell pellets were resuspended in Medium 199 supplemented with 10% fetal bovine serum and 50 ug/ml gentamicin. Cells were then plated in 100-mm tissue culture dishes and incubated in 37°C cell culture incubator for 45-50 mins. This "preplating process" can selectively removed rapidly attaching, non-cardial myocytes such as fibroblasts from the cell population, thus enriching the cell population for cardiomyocytes. Subsequently, the non-attached cells were transferred to fresh 35-mm six-well tissue culture dishes for the initiation of long-term culture. After 20-24 hours, myocardial cell cultures were washed and maintained in fresh medium 199 supplemented with 10% fetal bovine serum and 50 ug/ml gentamicin. The primary cultures of cardiomyocyte would be ready for use on day 2, and could be maintained for up to 7 days.

Pac-1 cell cultures. Frozen Pac-1 cultures were first propagated in medium 199 containing 15% fetal bovine serum for two generations. Then Pac-1 smooth muscle cells were plated in 35-mm cell culture dishes and maintained in medium 199 containing 10% fetal bovine serum and 50 ug/ml gentamicin.

Transient Transfection

Primary myocardial cultures were first washed with serum-free medium to get rid of serum. Subsequently, by using the commercial cationic liposome-based transfecting agent, Lipofectamine^{TM} (Life Technologies, Grand Island, NY), the transfection procedures were performed as the manufacturer's instructions. The amounts of plasmid DNA were determined in each experiment described in Results. Briefly, for each transfection, DNA and Lipofectamine were respectively diluted into separate vials containing 100 ul serum-free medium supplemented with Nutridoma HU (Boehringer Mannheim Corporation, Indianapolis, IN), which contains human serum albumin, bovine insulin and human transferrin. Then the two solutions were combined and incubated at room temperature for 30 min to allow to form DNA-liposome complexes. Following this step, 800 ul serum-free Nutridoma supplemented medium were added into the tube containing the DNA-liposome complexes, thus the diluted mixture can cover well on the adherent cardiomyocytes in 35-mm culture dish. The cell cultures were incubated with the diluted mixture at 37^{0}C for about 16 hours. After transfection, the transfection solution was removed and cells were maintained in serum-free Nutridoma supplemented medium 199 .

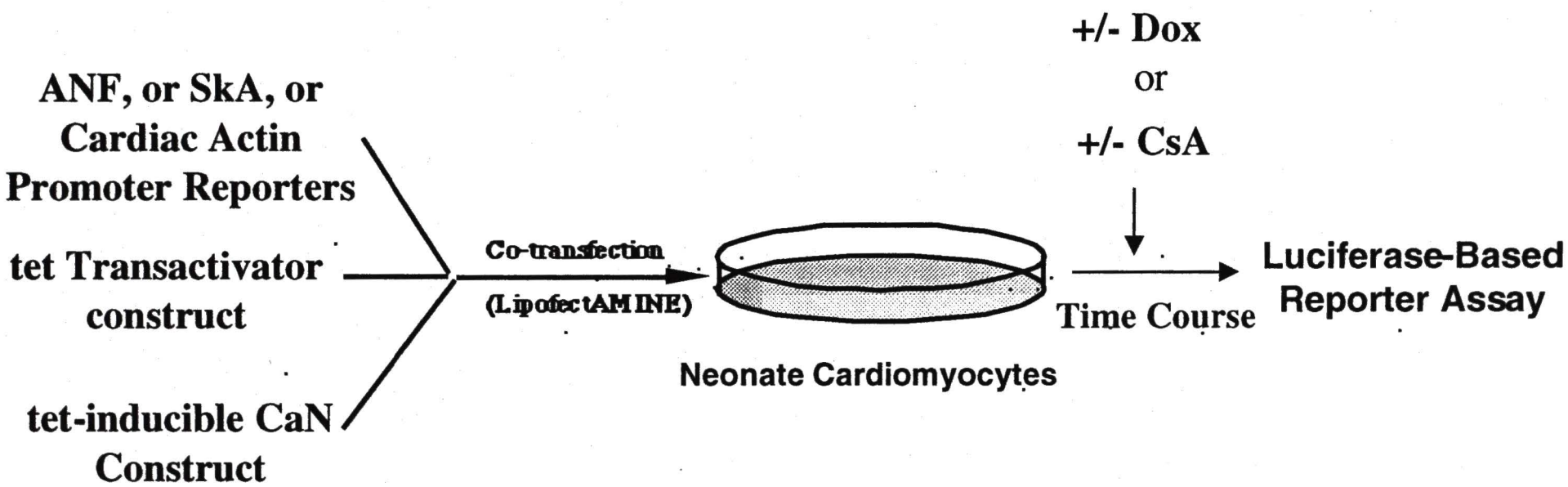
In the present study, tTA expression construct, tTA-dependent calcineurin expression plasmid, and a cardiac promoter-reporter construct will be co-transfected into cardiomyocyte cultures (Figure 7). Usually, the constitutive expressed β-gal gene plasmid may be simultaneously co-transfected and used to normalize the data. However, due to the specificity of the inducible systems, in each experiment, same quantitative cells will be transfected with the same amounts of total DNA. Specifically, in some experiments, empty vector will be used to adjust total amount of DNA in order to keep equal transfection efficiency.

Firefly Luciferase Assay

The cells to be assayed for luciferase activity were washed twice with PBS buffer and lyzed with 100ul of Reporter Lysis Buffer (Promega, Madison, WI). The lysates were scraped from dishes and briefly centrifuged to get rid of large debris. The supernatants were tested for luciferase activity by using a Turner Designs Luminometer Model 20 (Promega). For each luciferase assay, 20 ul of cell extract was mixed with 100 µl Luciferase Assay Reagent (Promega, Madison, WI) which contains 20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_25H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 uM coenzyme A, 470 uM luciferin, and 530 uM ATP. Integrated luminescence emission was measured over 10 seconds to 5 minutes. All assays were performed at room temperature. The reaction catalyzed by luciferase has been described previously (Deluca and McElroy, 1978). In the presence of luciferin, ATP-Mg^{2+}, coenzyme A, and

Figure 7. Protocol for the studies of the inducible calcineurin expression system in primary neonate rat cardiomyocytes.

Protocol for Studying Hypertrophy-Sensitive Gene Responses to Manipulated CaN Expression in Neonate Cardiomyocytes



molecular oxygen, luciferase can catalyze these substances to react each other and finally lead to emitting light.

Other Materials

Restriction endonucleases and tissue culture materials were purchased from Life Technologies. Cyclosporin A was obtained from Sandoz Pharmaceuticals Corp. (East Hanover, NJ). Doxycycline was obtained from Fluka Chemical Corp. (Milwaukee, WI).

Data Statistics

All experiments were performed at least three times independently. Results in this study are a representative from a single experiment. Data were plot by SigmaPlot program. Shown are the mean values of triplicate cultures. Error bars represent standard error of three samples.

CHAPTER III

RESULTS

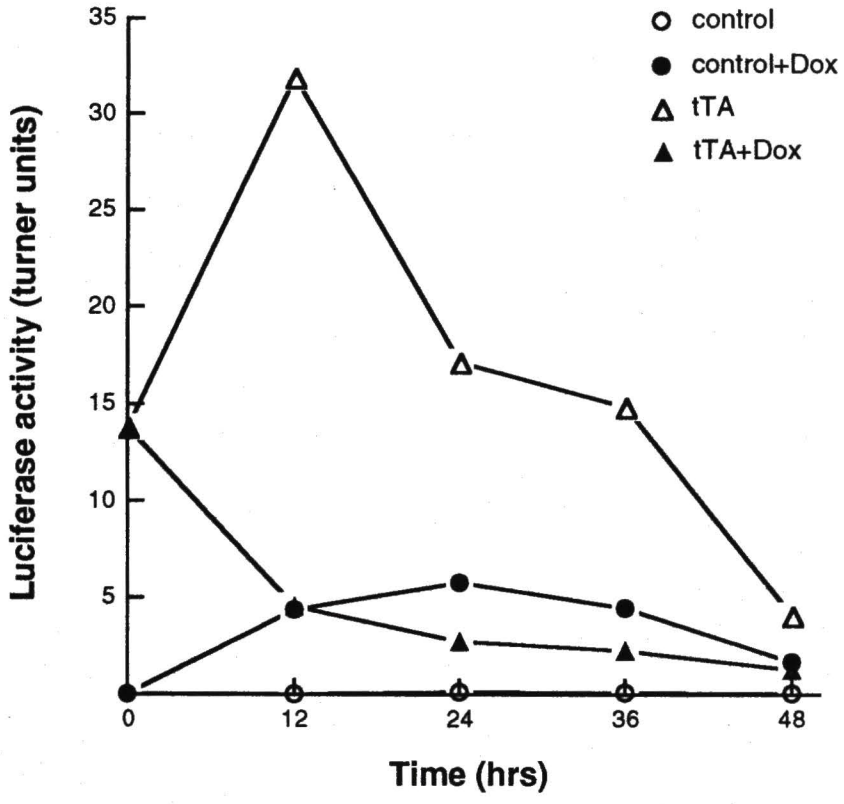
Regulation of an exogenous target gene transcription by the tetracycline-dependent transactivator (tTA) expression system in primary neonatal rat cardiomyocytes.

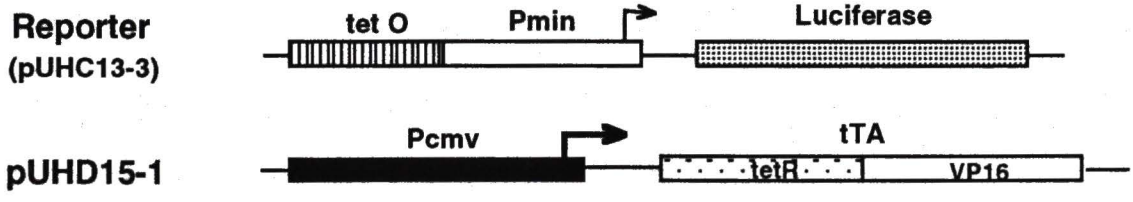
The firefly luciferase gene as a reporter gene was under control of the chimeric tet operon/CMV minimal promoter in pUHC13-3. When this construct and tTA expression plasmid pUHD15-1 were co-transfected into cells, in the absence or presence of the effector substance--doxycycline, the changes in luciferase activity would indicate the ability of tTA system to regulate transcription of the target gene. To test whether a tetracycline-regulated gene expression system functions in primary rat cardiomyocytes, the cell cultures were co-transfected with tTA expression plasmid pUHD15-1 (0.25ug) and tTA-stimulated luciferase gene expression construct pUHC13-3 (0.25ug). As a control, same amounts of reporter plasmid pUHC13-3 plus an empty vector pUHD10-3 were transfected in the cardiomyocytes from same cell preparation. After transfection, the luciferase activities in cell extracts were examined at different time points. In the absence of tTA, only very low levels of reporter gene activity were detected during 48 hour posttransfection. tTA expression resulted in a dramatic increase in reporter activity during this period; and the target gene activities reached the

maximum at 12 hour after transfection (Figure 8). At that time, the luciferase activities in the tTA-transfected cells were over 30-fold higher than those in the cell without tTA transfection (Figure 8). When the cells were treated with 1ug/ml doxycycline after transfection, the activation of the target gene was markedly repressed. However, the doxycycline treatment itself caused a transiently low level expression of the target gene (It was about 4 fold increase at 12 hour) (Figure 8), suggesting that doxycycline repression to the target gene in primary cardiomyocytes is not complete. It is possible that exogenous gene expression in transient transfection is not under tight control of naive gene regulation machinery in cells. Doxycycline treatment might cause a weak signal to stimulate the low level expression of this target gene. Therefore, doxycycline is not best effector to regulate cardiac gene transcription in our system.

Both the tTA and rtTA systems were further tested in primary neonatal rat cardiomyocytes. Similarly, the inducible luciferase reporter plasmid pUHC13-3 and either tTA expression construct pUHD15-1 or rtTA plasmid pUHD17-1 were co-transfected into the cardiomyocytes. As controls, the reporter alone or together with same amounts of an empty vector pUHD10-3 were transfected into same cell preparation. Following transfection, the cultures were treated with 1 ug/ml doxycycline or untreated. At 12 hour after transfection, the expression of target gene in each culture was detected by luciferase assay. Compared with controls, the expression of target gene was increased over 40 fold by tTA. Doxycycline significantly repressed the tTA induction of the target gene transcription, but doxycycline inhibition of target gene

Figure 8. Time course of tTA-dependent target gene transcription in primary rat cardiomyocytes. Neonate rat myocardial cells were isolated and cultured as described under "Materials and Methods". Two days after plating, cardiomyocytes were co-transfected with the tTA expression plasmid pUHD15-1(0.25 ug), and either a tTA-dependent luciferase expression construct pUHC13-3 (0.25 ug) or an empty vector pUHD10-3 (0.25 ug) as a control. After 16 hour transfection, the cells were incubated in either media 199/0.5% FBS or same media with 1 ug/ml doxycycline as indicated. At different time points following transfection, the cultures were harvested and extracted. Luciferase assay was carried out as described in the Methods. Values for luciferase activity are means of duplicate cultures.


control
control+Dox
tTA
tTA+Dox
Luciferase activity (turner units)
35
30
25
20
15
10
5
0
12
24
36
48
Time (hrs)

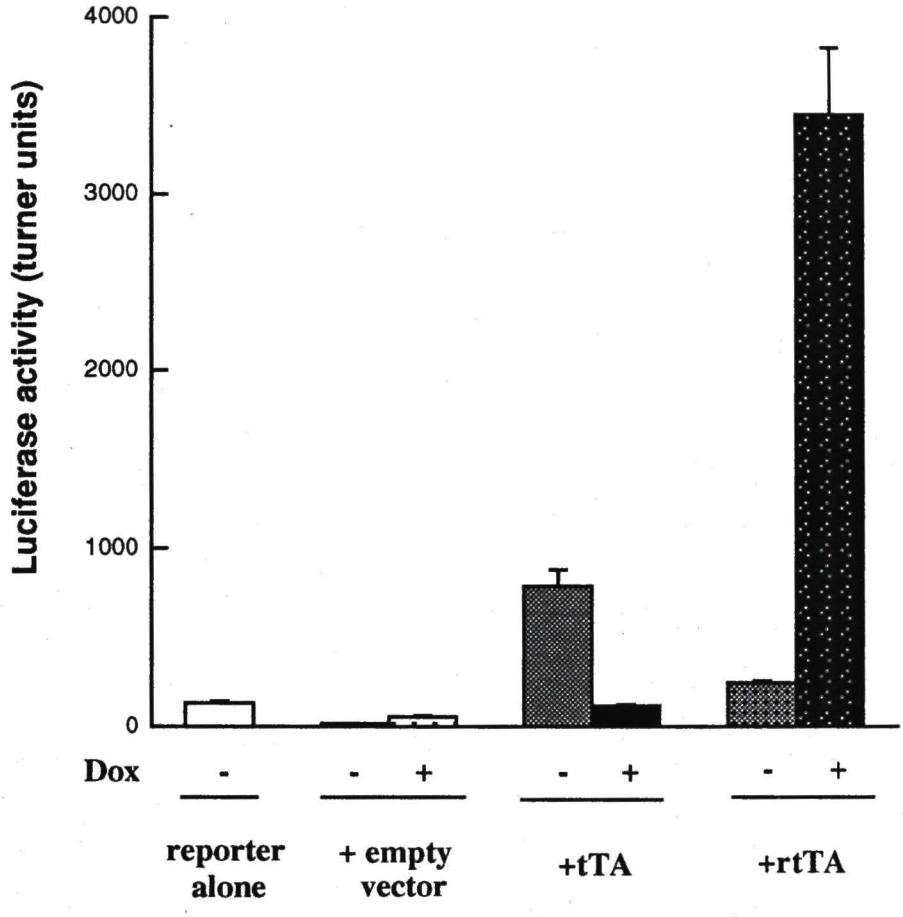

Reporter
(pUHC13-3)
tet O
Pmin
Luciferase
pUHD15-1
Pcmv
tTA
tetR
VP16

expression was incomplete (Figure 9); Moreover, in the presence of doxycycline, rtTA dramatically induced the transcription of the target gene, the luciferase activity in rtTA-transfected cultures were about 200-fold higher than those in the cultures without rtTA expression (Figure 9). Obviously, tTA and rtTA system are able to efficiently regulate the transcription of an exogenous target gene.

Transcription of the hypertrophy-sensitive genes induced by tTA-regulated calcineurin expression system depends on calcineurin gene dose.

Previous data from our laboratory have shown that the constitutively expressed active calcineurin activates the transcription of ANF reporter gene in primary rat cardiomyocytes (Zeng et al, unpublished data). Also, our preliminary results have indicated that the transcription of cardiac α-actin reporter gene were induced by tTA-stimulated active calcineurin expression in Pac-1 smooth muscle cells (Data not shown). To detect the effects of active calcineurin on the cardiac gene transcription in primary cardiomyocytes and investigate optimal concentration of calcineurin construct in transfection to induce cardiac gene expression, the different amounts of tTA-dependent active calcineurin expression construct p10-3CaN as indicated in Figure 10, 0.25 ug tTA expression plasmid pUHD15-1 and 0.25 ug cardiac α-actin promoter-reporter plasmid CardA-Luc were transfected into primary rat cardiomyocytes. The empty vector, pUHD10-3 plasmid were used as control to substitute p10-3CaN or complement total DNA to 0.75ug/per well. At 24 hour after transfection, the expression

Figure 9. Effects of tTA or rtTA system on the regulation of target gene transcription in primary rat cardiomyocytes. Neonate rat myocardial cells were isolated and cultured as described under "Materials and Methods". Two days after plating, cardiomyocytes were transfected either a tTA-dependent luciferase expression construct pUHC13-3 (0.25 ug) alone, or co-transfected same amount of this reporter together with the tTA expression plasmid pUHD15-1(0.25 ug) or rtTA expression plasmid pUHD17-1 (0.25 ug) or an empty vector pUHD10-3 (0.25 ug) as a control. After 16 hour transfection, the cells were incubated in either media 199/0.5% FBS or same media with 1 ug/ml doxycycline as indicated. At 12 hour after transfection, the ability of tTA or rtTA to regulate the transcription of the target gene and the effect of doxycycline on the reporter gene expression were shown and compared by luciferase assay. Shown are the mean values ± standard error (SE), n=3 cultures. Data are a representative of three identical experiments.


Luciferase activity (turner units)
4000
3000
2000
1000
0
Dox
- - + - + - +
reporter alone
+ empty vector
+tTA
+rtTA

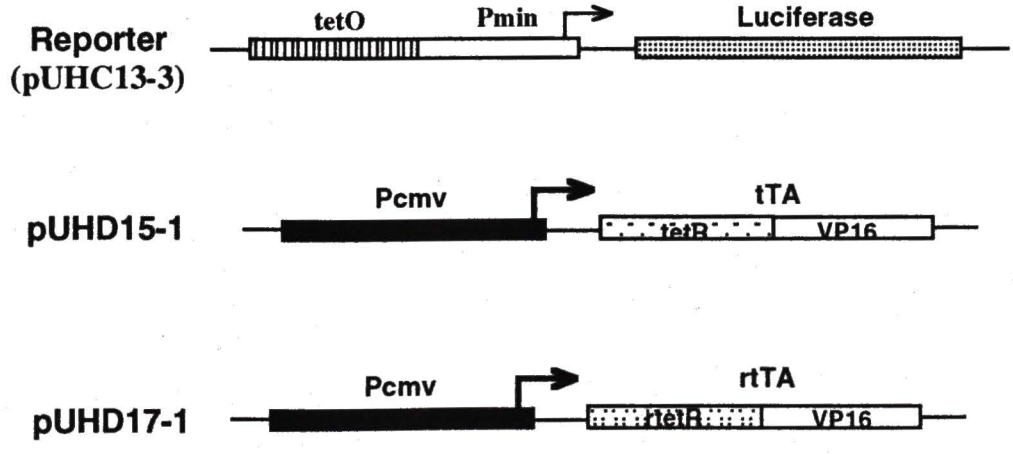
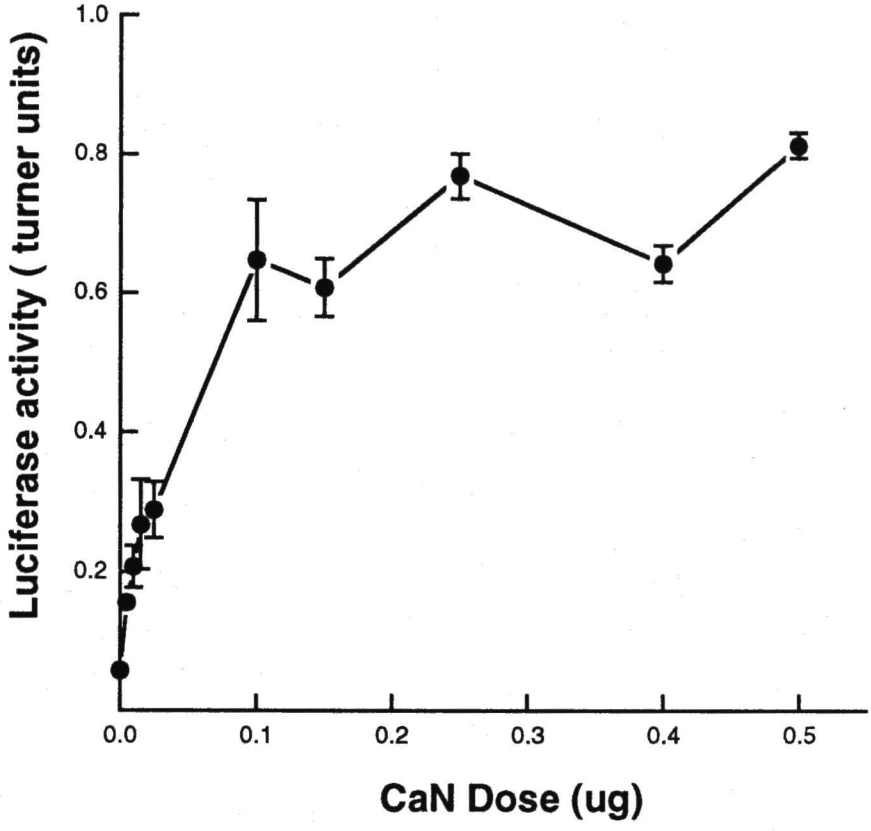
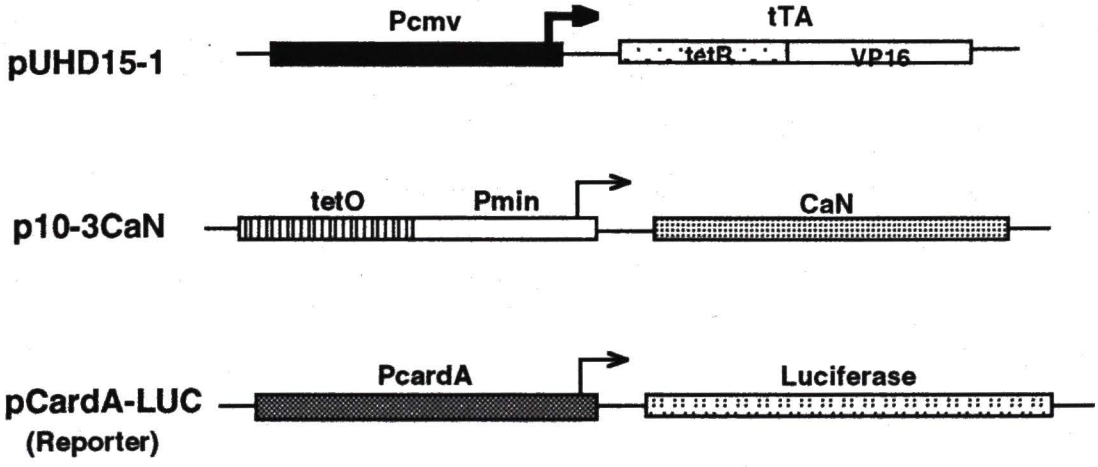

Reporter (pUHC13-3)
tetO
Pmin
Luciferase
pUHD15-1
Pcmv
tTA
tetR
VP16
pUHD17-1
Pcmv
rtTA
tetR
VP16

Figure 10. Active calcineurin gene dose-dependency of the transcription of hypertrophy-sensitive genes in myocardial cells. Primary rat cardiomyocytes were cultured in Media 199/10% FBS for 2-3 days. Then 0.25 ug tTA expression construct pUHD15-1, 0.25 ug cardiac α-actin promoter-reporter plasmid CardA-Luc, and different amounts of tTA-dependent calcineurin expression construct p10-3CaN (0--0.5 ug) were co-transfected into the cells. The empty vector pUHD10-3 DNA were used to adjust total amount of transfected DNA to 1 ug in each transfection. After 24 hour incubation following transfection, the cells were harvested and lyzed. Luciferase activities in each cultures were measured, respectively. Protein contents in each transfected cultures were also examined by BCA protein assay (Kit and Protocol from PIERCE, Rockford, IL) to confirm the consistence of total protein level. Data were representative of six independent experiments that product similar results. Values in this Figure are reported as means ± SE, n=3 cultures.


Luciferase activity (turner units)
1.0
0.8
0.6
0.4
0.2
0.0
0.1
0.2
0.3
0.4
0.5
CaN Dose (ug)


pUHD15-1
Pcmv
tTA
tetR
VP16
p10-3CaN
tetO
Pmin
CaN
pCardA-LUC
(Reporter)
PcardA
Luciferase

levels of cardiac α-actin reporter gene were analyzed by the luciferase assay. As shown in Figure 10, in the absence of exogenous active calcineurin, the level of reporter gene expression was quite low; whereas the transcription of cardiac α-actin reporter gene were gradually enhanced by increased dose of transfected p10-3CaN. When the amount of calcineurin DNA was up to 0.25 ug, the expression of reporter gene was increased near to a related maximum level. Obviously, the expression of cardiac α-actin gene is active calcineurin gene dose-dependent. 0.25 ug p10-3CaN would be used as the optimal dose in the following experiments.

Induced expression of active calcineurin form rapidly activates the transcription of three hypertrophy-sensitive genes in myocardial cells.

To further assess the ability of calcineurin to regulate the cardiac gene expression in myocardial cells, we studied the time courses of expression of three hypertrophic response gene under the induction of active calcineurin expression. Cardiac α-actin, skeletal α-actin, and ANF promoter-Luc plasmids were respectively used as a reporter, the primary neonatal rat cardiomyocytes were co-transfected with either reporter, pUHD15-1, and p10-3CaN, or empty vector pUHD10-3 instead of p10-3CaN, together with pUHD15-1 and reporter plasmids. The amount of each plasmid was all 0.25 ug. At different time points after transfection, the luciferase activities of cell extracts were measured. In each experiment, the highest level of luciferase activity was used as the maximum response of reporter gene, all data were normalized to a percentage of the maximum response. In the absence of the expression of exogenous

active calcineurin, the transcription levels of three cardiac genes were very low. However, active calcineurin expression markedly induced the transcription of three cardiac genes (Figure 11). The expressions of cardiac α-actin, skeletal α-actin, and ANF genes were all significantly increased near to their maximum levels after 48 posttransfection hours (Figure 11 and data not shown), suggesting that active calcineurin rapidly activates the transcription of these hypertrophy response gene in primary cardiomyocytes.

When same cardiac cell preparations were respectively co-transfected with same amounts of reporter, tTA, and calcineurin plasmid DNA as indicated in Figure 12, the expression levels of three cardiac genes were detected at 48 hour after transfection. Compared with the controls in which only empty vector pUHD10-3 instead of calcineurin plasmids, together with reporter and tTA plasmid, were transfected into the cardiomyocytes, calcineurin strongly activated the transcription of cardiac α-actin by about 8-10 fold, and moderately activated the ANF and skeletal α-actin expression by about 5-fold and 3-fold (Figure 12). These results not only indicated the stimulatory effect of calcineurin on the transcription of hypertrophy-sensitive genes, but also showed a utility of the inducible gene expression system. The ability of active calcineurin to induce transcription of hypertrophy response genes in the cardiomyocytes is consistent with the result from transgenic mice overexpressing the constitutively active form of calcineurin (Molkentin et al., 1998). For example, the mRNA level of skeletal α-actin gene in the heart of calcineurin transgenic mice was about 5-fold higher than that in normal mouse heart. The increased ANF gene expression induced by

Figure 11. Time courses of the transcription of hypertrophy response genes induced by active calcineurin in myocardial cells. Three cardiac promoter-luciferase expression constructs, cardA-Luc, ANF-Luc, and SkA-Luc were respectively used as a reporter. Myocardial cells were co-transfected with pUHD15-1, reporter plasmid, and either p10-3CaN or empty vector pUHD10-3 plasmid as control. Amount of each plasmid was 0.25 ug. Total DNA in each transfection was 0.75 ug. At various time points following transfection, expression levels of reporter genes were detected by luciferase assay. In each experiment, the highest level of luciferase activity was thought as the maximum response of this cardiac gene expression, thus all data were normalized as a percentage of the maximum response. Results from a single experiment are representative of three independent experiments and values are means ± SE, n=3 cultures.

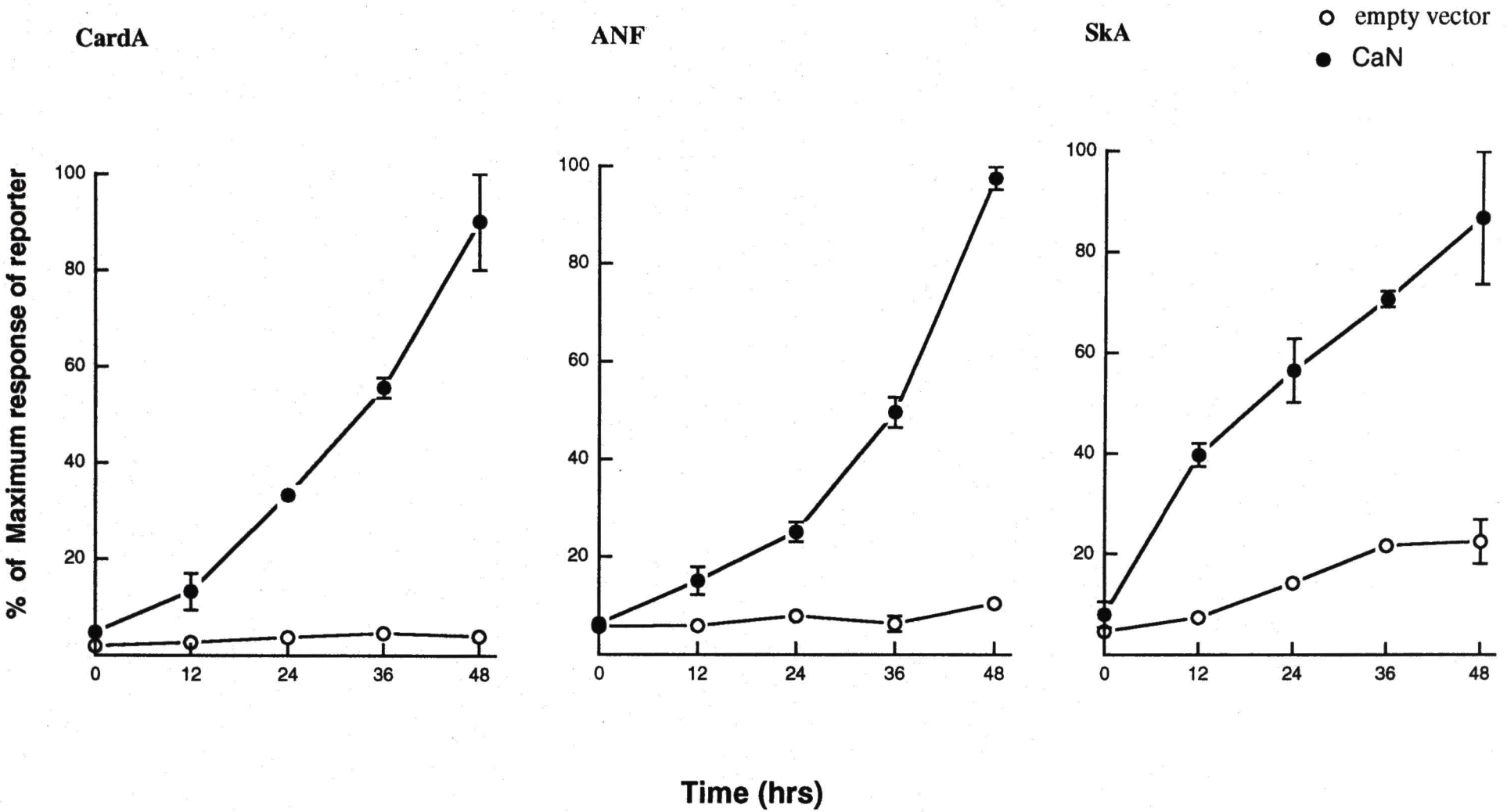
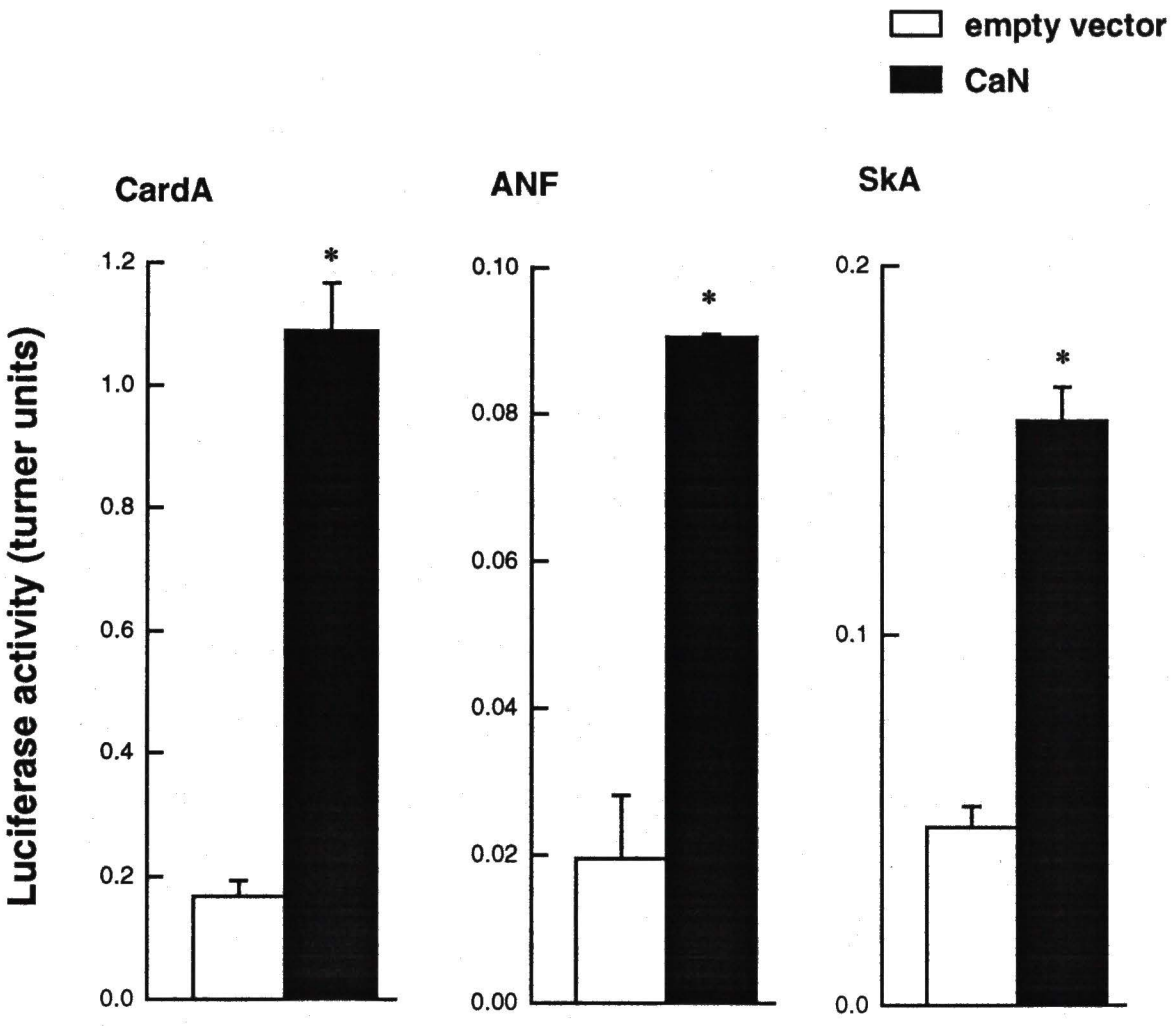

CardA
ANF
SkA
empty vector
CaN
% of Maximum response of reporter
100
80
60
40
20
0
12
24
36
48
Time (hrs)

Figure 12. Effects of active calcineurin on the transcription of hypertrophy response genes in myocardial cells. CardA-Luc, ANF-Luc, and SkA-Luc construct were respectively used as a reporter. Primary cardiomyocytes were co-transfected with pUHD15-1 (0.25 ug), reporter construct (0.25 ug), and either p10-3CaN (0.25 ug) or empty vector pUHD10-3 (0.25 ug) as control. After 48 hour of incubation following transfection, the cells were extracted, and then the transcriptional levels of each cardiac gene were evaluated by luciferase assay. Shown are a representative of three identical experiments that indicated similar results. Values are means ± SE, n=3 cultures. Data were analyzed based on *t* test. The values of calcineurin induction for three gene expression are all significantly different from their controls (*, $p< 0.001$).


empty vector
CaN
CardA
ANF
SkA
Luciferase activity (turner units)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.10
0.08
0.06
0.04
0.02
0.00
0.2
0.1
0.0
*
*
*

calcineurin in our inducible expression system is similar to that of BNP gene activation in calcineurin transgenic mice. However, the increase in transcription level of cardiac α-actin gene in our system is higher than that in transgenic mice overexpressing calcineurin.

Repression of calcineurin induction for hypertrophic response gene expression by cyclosporin A.

Cyclosporin A, an immunosuppressive agent, has been known to bind its cytosolic receptors called cyclophilin upon entering the cell. In hematopoietic cells, the cyclosporin-cyclophilin complex has been shown to specifically inhibit lymphokine gene transcription via inhibition of calcineurin activity (Liu, 1993). This finding that calcineurin is the molecular target for cyclosporin provides us an important pathway to regulate calcineurin activity and study cardiac gene functions in response to the changes in calcineurin activity.

To evaluate whether the transcriptional activation of hypertrophy-sensitive genes by activated calcineurin could be inhibited by CsA, a specific inhibitor of calcineurin, firstly, both the myocardial cell cultures transfected with either p10-3CaN construct or empty vector pUND10-3, together with tTA and CardA-Luc reporter plasmids as described in Figure 11, were treated with different concentrations of CsA or without CsA treatment from 0 hour after transfection. At three various time points, the luciferase activities in those cells were assayed and compared. In the control group which had no calcineurin transfection, the CsA treatment had no significant effect on

the transcription level of cardiac α-actin gene in comparison with no treatment (Figure 13). However, cardiac α-actin gene expression stimulated by active calcineurin was significantly inhibited by all those low concentrations of CsA (Figure 13), which further confirms the conclusion that the expression of these cardiac hypertrophic response gene is activated by a calcineurin-dependent signal transduction pathway. At 72 hour after transfection, the cardiac α-actin gene activity induced by calcineurin was inhibited over 80% by 100nM or 150nM CsA treatment (Figure 13), indicating that the very low concentrations of CsA can inhibit the transcriptional activation of cardiac α-actin gene induced by calcineurin. Therefore, 100nM would be chosen as the optimal concentration of CsA in the following experiments.

To further demonstrate the effect of CsA on the transcription of hypertrophy-sensitive genes induced by calcineurin, we analyzed how the expression of cardiac α-actin promoter-reporter gene under active calcineurin stimulation responds to CsA. Primary cardiomyocytes were co-transfected with either tTA, cardiac α-actin reporter constructs, and calcineurin, or tTA, cardiac α-actin reporter gene and empty vector as the control. CsA treatment (100 nM) was initiated right away after transfection. The cardiac α-actin gene activities in cardiac cell cultures were examined by luciferase assay and results were normalized to a percentage of the maximum response as described in Figure 11. In the cells transfected with the empty vector, the expression of cardiac α-actin gene was low and there was no marked difference in the gene activity between the CsA treated and untreated cells (Figure 14). But CsA treatment lead to a dramatic reduction in the cardiac gene transcription in active

Figure 13. Calcineurin induction of the hypertrophic response gene transcription is inhibited by low concentrations of cyclosporin in myocardial cells. Myocardial cells were cotransfection with tTA expression construct pUHD 15-1 (0.25 ug), CardA-Luc reporter (0.25 ug), and either p10-3CaN (0.25 ug) or empty vector pUHD10-3 (0.25 ug) as control. Following transfection, the cells were either treated with different concentration of CsA or untreated as indicated in the diagram. At three different time points, the cells were harvested and luciferase activities in these cells were examined by luciferase assay. Shown are means values of duplicate cultures.

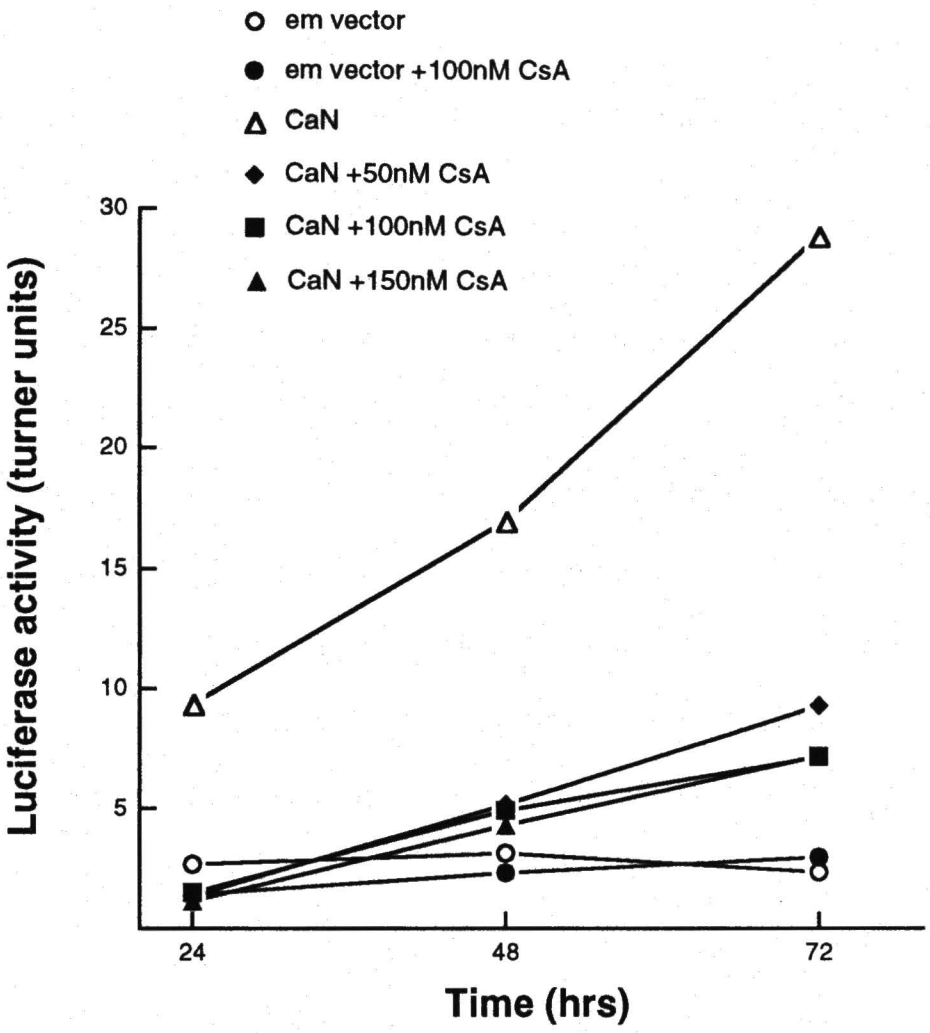
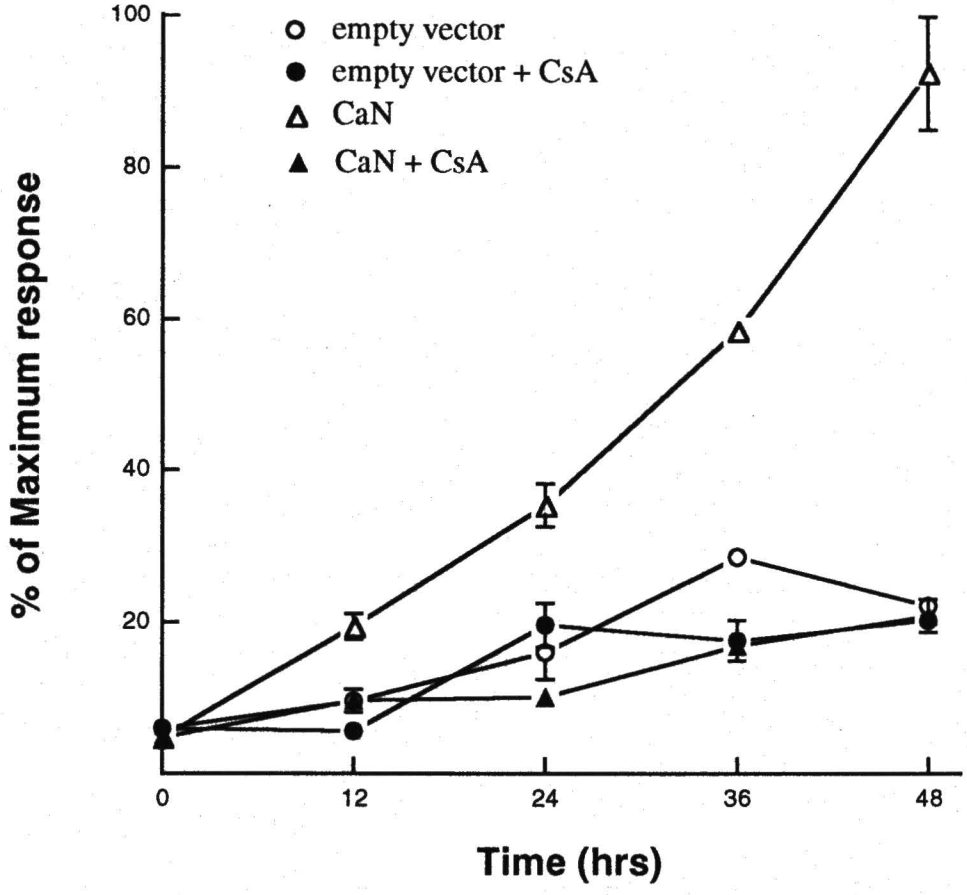

em vector
em vector +100nM CsA
CaN
CaN +50nM CsA
CaN +100nM CsA
CaN +150nM CsA
Luciferase activity (turner units)
30
25
20
15
10
5
24
48
72
Time (hrs)

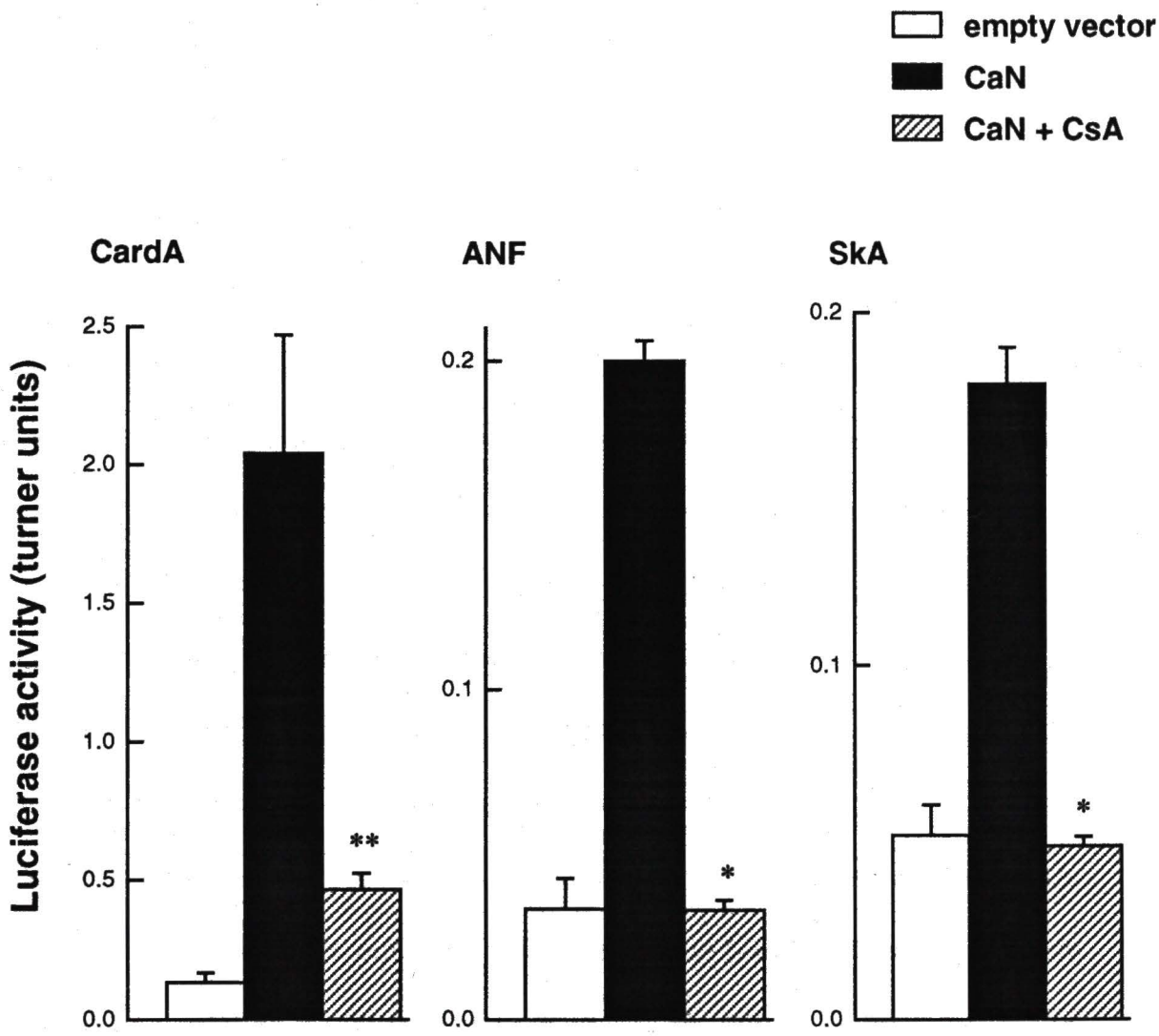
Figure 14. Time course of CsA inhibition for calcineurin induction of hypertrophy-sensitive gene expression in myocardial cells. Primary cardiomyocytes were co-transfected with CardA-Luc reporter, tTA expression construct pUHD15-1, and either p10-3CaN or empty vector pUHD10-3. Amounts of each plasmid were 0.25 ug. After transfection, the cell were cultured in media 199/0.5% FBS or same media with 100 nM CsA for different time. Then the cells were extracted and the luciferase activities in each cultures were examined. The highest level of luciferase activity in the experiment was used as the maximum response of the cardiac gene. All data were normalized to a percentage of the maximum response. Date presented are a representative of three independent experiments. Shown are means values of each treatment ± SE (n=3).


empty vector
empty vector + CsA
CaN
CaN + CsA
% of Maximum response
100
80
60
40
20
0
12
24
36
48
Time (hrs)

calcineurin-transfected cells. After 48 hour treatment, the expression of cardiac α-actin gene activated by calcineurin was decreased near to its basal level by CsA treatment (Figure 14).

Based on the results above, we tested the effects of CsA on the calcineurin-mediated transcriptional activation of ANF, cardiac α-actin and skeletal α-actin genes. The same amounts of each reporter construct together with tTA and p10-3CaN DNA were respectively co-transfected into the cells coming from same primary culture preparation. The empty vector pUHD10-3 instead of p10-3CaN was used as the control. After transfection, the cells transfected with active calcineurin were cultured in either the media with 100nM CsA or no CsA media for 48 hours. The cells transfected with empty vector were only cultured in no CsA media for same time. Then these cardiomyocytes were lyzed to analyze the activities of these cardiac hypertrophic response genes. The results are shown in Figure 15. The transcriptional activation of ANF and skeletal α-actin genes mediated by active calcineurin were completely blocked by CsA in primary rat cardiomyocytes; about 80% of transcriptional activity of cardiac α-actin gene up-regulated by the action of calcineurin was inhibited by CsA. Consequently, CsA treatment has a remarkable effect on the hypertrophic response mediated by active calcineurin. CsA may be a good pharmacological agent to negatively control hypertrophy response gene expression in this system. Our results are consistent with the role of CsA in reducing increased heart size of transgenic mice overexpressing calcineurin transgenics (Molkentin et al., 1998). At a cellular level, it

Figure 15. Effects of CsA treatment on the transcriptional activation of hypertrophic response gene by calcineurin in myocardial cells. Three promoter-luciferase constructs, CardA-Luc, ANF-Luc, and SkA-Luc, were respectively used as a reporter. Primary cardiomyocytes were co-transfected with reporter construct, tTA plasmid pUHD15-1, and either p10-3CaN or empty vector pUHD10-3 as the control. Amounts of each plasmid were 0.25 ug. Following transfection, the cells were either treated with 100 nM CsA or untreated as indicated. After 48 hour incubation, the cells were harvested and lyzed. Then the luciferase assay was carried out. Shown are a representative of three identical experiments that indicated similar results. Values are means ± SE, n=3 cultures. The values for CsA treatment in each experiment are significantly different from those of no treatment (*, $p< 0.001$; **, $p< 0.01$, *t* test).

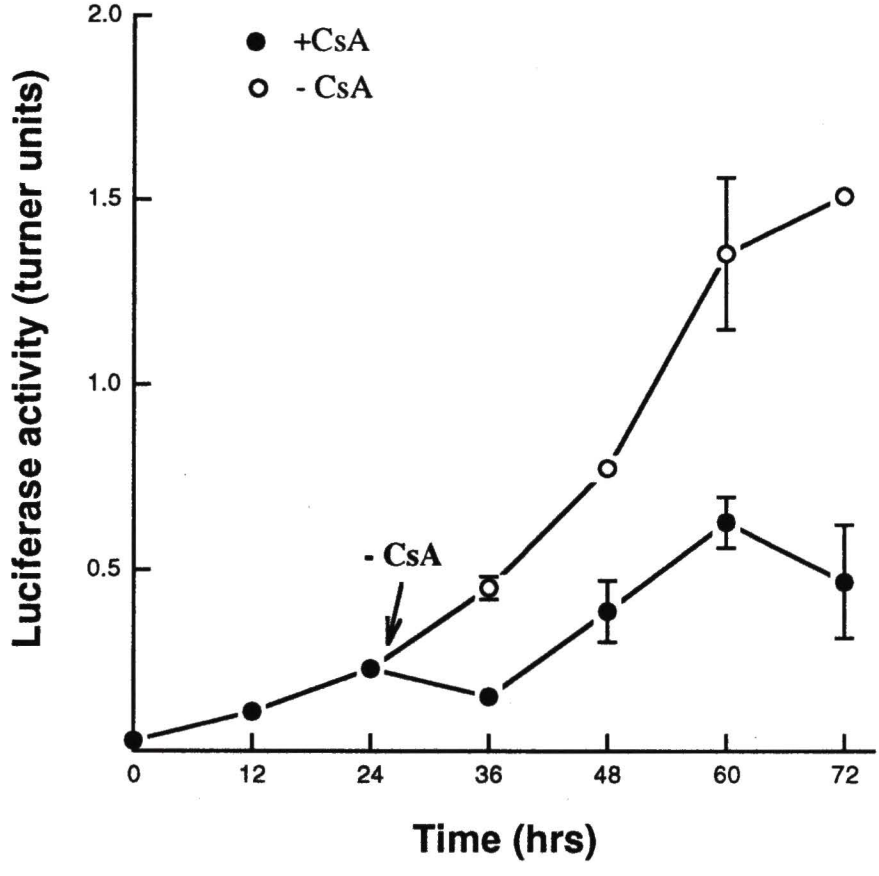

empty vector
CaN
CaN + CsA
CardA
ANF
SkA
Luciferase activity (turner units)
2.5
2.0
1.5
1.0
0.5
0.0
**
0.2
0.1
0.0
*
0.2
0.1
0.0
*

has been shown that CsA largely inhibited the hypertrophic response of cardiomyocytes in the calcineurin transgenics (Molkentin et al., 1998).

Repression of the transcription of hypertrophy-sensitive genes by CsA treatment could be reversibly regulated.

Due to practical requirements in complex studies, the ability to bidirectionally regulate the expression of exogenous gene would have a great significance in the studies of gene function as well as in gene therapies. To evaluate whether the repressed expression of the exogenous hypertrophic response genes in our system could be reversibly controlled by removing CsA from cell culture media, primary cardiomyocytes from same preparation were transfected with tTA, calcineurin construct, and cardiac α-actin reporter plasmids. After transfection, all cells were treated with 100nM CsA. 24 hours later, a part of the cells were continuously cultured in CsA media; whereas other part of the cells were switched to the media without CsA. At different time points, the reporter gene expression in these cardiomyocytes were examined by luciferase assay. As shown in Figure 16, during 72 hour period, the reporter gene activities in CsA-treated cells were low. However, the expression of the cardiac gene was rapidly elevated after discontinuing CsA treatment. 48 hour after removing CsA from media (72 h point), cardiac α-actin reporter gene activity had increased about 2.5-fold compared with that in cells treated with CsA. The data demonstrated that transcription of the cardiac gene repressed by CsA could be rapidly reversed by simply removing CsA out of system. Therefore, based on these results

Figure 16. Reversible regulation of the transcriptional repression of hypertrophic response gene expression by CsA manipulation in myocardial cells. Cardiomyocytes were co-transfected with pUHD15-1 (0.25 ug), CardA-Luc (0.25 ug), and p10-3CaN (0.25 ug). After transfection, the cells were immediately cultured in the media with 100 nM CsA. Following 24 incubation, half cultures were continuously treated with 100 nM CsA, the others were switched into no CsA media. At various time as indicated, the luciferase activities for each culture was detected by luciferase assay. Data from a single experiment are representative of three identical experiments. Shown are the mean values ± SE, n=3 cultures.


2.0
1.5
1.0
0.5
+CsA
- CsA
- CsA
Luciferase activity (turner units)
0
12
24
36
48
60
72
Time (hrs)

shown above, the expression of exogenous hypertrophy-sensitive genes in primary cardiomyocytes, under the experimental conditions used, could be rapidly regulated in either positive or negative way. We may conclude that this system would be useful for further studies of the cardiac gene function and signal transduction.

CHAPTER IV

DISCUSSION

Hypertrophic growth of cardiac myocytes is largely due to enhanced expression of cardiac hypertrophic response genes. Recent study has shown that overexpression of constitutively active form of calcineurin in the heart of transgenic mice activates hypertrophy-sensitive gene transcription and finally results in the development of cardiac hypertrophy (Molkentin et al., 1998). In the present study, first, the expression of active calcineurin in primary cardiomyocytes leads to a marked increase in reporter gene expression under the control of the promoters of cardiac α-actin, ANF, or skeletal α-actin genes (Figure 11; Figure 12). Second, the transcriptional activation of cardiac α-actin, ANF, and skeletal α-actin gene mediated by calcineurin was blocked by a specific inhibitor of calcineurin, cyclosporin A (Figure 14; Figure 15). These results indicated that calcineurin significantly induced the activation of several important hypertrophic response genes and their expression in primary rat cardiomyocytes. These findings are consistent with those from the studies of transgenic mice overexpressing active calcineurin (Molkentin et al., 1998). Under certain experimental conditions, while the amounts of the calcineurin construct for transfection was gradually increased, the expression of several cardiac hypertrophic response genes were correspondingly elevated up to their maximum levels (Figure 10). Moreover, the

transcription of the hypertrophic response genes were gradually elevated up to their maximum responses depending on the prolongation of calcineurin expression. These data further demonstrate that the transcriptional activation of these hypertrophic response gene was active calcineurin plasmid dose as well as time-dependent.

We have also shown that the expression levels of these hypertrophy-sensitive genes in the calcineurin-transfected cells were dramatically inhibited and rapidly decreased near to their basal levels by a low concentration of CsA treatment (Figure14; Figure 15), indicating the ability of CsA to efficiently block the calcineurin induction of hypertrophy response gene expression. More importantly, transcription of hypertrophic response genes can be regulated by controlled manipulation of CsA treatment in our inducible calcineurin system (Figure 16). To our knowledge this is the first report to address the expresssion pattern of hypertrophic response gene in response to controlled expression of active calcineurin in primary cardiomyocytes. It also documents the effects of CsA on the transcription activation of those cardiac genes mediated by active calcineurin in primary cardiomyocytes. The present study demonstrates that this system can rapidly and efficiently regulate the expression of hypertrophic response genes, suggesting the potential value of the inducible calcineurin expression system in the studies of hypertrophic signal transduction and gene function.

Our experiments revealed that the basal activity of the hCMV minimal promoter in primary rat cardiac myocytes are quite low, but can still be detected (Figure 8; Figure 9). A previous study has shown that the basal activities of the hCMV minimal promoter in the tTA-regulated reporter pUHC13-3 after transient transfection of various

cell lines were different (Ackland-Berglund and Leib, 1995). A possible reason for this phenomenon is that the expression of exogenous genes in transient transfection could not be tightly controlled by gene regulatory machinery of cardiomyocytes. Possibly, stable transfection would be a better approach to control the basal expression of inducible gene (Gossen and Bujard, 1995). When the tTA-responsive promoter is stably integrated into chromosome, its basal activity would be lower than that in transient transfection, because the effective copy number per cell is low and basal promoter activities are repressed after chromatin assembly. Particularly, if the inducible minimal promoter is integrated into transcriptionally silent but activatable region, which has no transcriptional activation from neighboring regions, tightly regulatable cells would be obtained. Alternatively, less amounts of inducible construct for transient transfection could be used for study objective, lower background would be observed.

The pattern of inducible target gene expression detected by the luciferase reporter has shown that the basal level of the target gene transcription was very low in primary cardiomyocytes. tTA could rapidly induce an over 30 fold increase in the target gene expression in our experiments. If we alter the dose ratio of induced target gene plasmid to tTA construct in transfection, we may finely regulate the expression level of inducible target gene in the primary cardiac myocytes. Thus, the expression of active calcineurin in the cardiomyocytes could be actually modulated to a given level by this partial induction technique. Almost complete inhibition of calcineurin activity by CsA and reversible regulation of CsA manipulation to calcineurin effects would allow us to temporally control the intracellular level of active calcineurin. According to objectives

of actual studies, calcineurin activity could be increased or decreased at different time and even be kept silent. Through these strategies, we could study the effect of quantitative aspects of calcineurin activity on downstream effectors and precise mechanisms of calcineurin-dependent signal transduction pathways. This system is also quite useful for studying the effects of other cardiac active enzymes and gene regulators on signal transduction and gene regulation during cardiac hypertrophy. Since induced expression levels of interest genes could be regulated to a normal level or pathological ranges, this inducible system has great advantages in the studies of gene function under physiological or pathological conditions compared to the studies in knockout gene or gene overexpression, and it would have wide applicability in gene therapy.

In our system, the constitutively active expression of tet-regulated transactivator (tTA) protein is triggered by the human virus promoter, P_{CMV} , which is very strong and might have side effects on cellular metabolism. However, our experiments showed that the tTA expression had no effect to the transcription of cardiac α-actin, ANF, or skeletal α-actin reporter genes in primary cardiomyocytes (Data not shown). Furthermore, by use of tissue-specific or developmentally regulated promoters such as the promoter of αMHC gene, tTA expression could be regulated in temporal and spatial way. The tissue-specific tTA expression construct could also be stably integrated into chromosome. Recently, Dr. Glenn Fishman's laboratory have generated transgenic mice overexpressing tTA in the heart (Passman and Fishman, 1994). Additionally, *in vivo* gene transfer technique has been developing in cardiac research. In the near further, through developing *in vivo* inducible calcineurin gene expression

system, it would be possible to study the mechanisms of hypertrophic signal transduction and gene regulation *in vivo*.

In the adult rat cardiac myocytes, the endogenous ANF and skeletal genes are actually silent. Without exogenous active calcineurin induction, the transcription levels of ANF and skeletal α-actin reporter genes in our system were very low, but they can still be examined by the sensitive luciferase assay. Because only truncated upstream proximal regions of these cardiac gene promoters were taken advantage of in our system, the effects of distant cis-elements and neighbor promoters on the gene activities are actually overlooked. Additionally, the basal activities of these transient transfected genes can not be repressed by chromatin assembly. The transcription levels of ANF and skeletal α-actin gene stimulated by active calcineurin were respectively increased about 5-fold and 3-fold in our system. These changes in ANF and skeletal α-actin gene transcription mediated by active calcineurin in primary cardiomyocytes are consistent with those in the transgenic mice overexpressing active calcineurin gene. It has been known that endogenous cardiac α-actin gene is stably expressed at a certain level during whole development period. Thus, it is reasonable that the basal transcriptional level of cardiac α-actin reporter gene in primary cardiomyocytes was high. However, the increased fold in the expression of cardiac α-actin reporter gene mediated by active calcineurin was higher than that in calcineurin transgenic mice (Molkentin et al., 1998). It is possible that other cellular regulators might also control the transcription of cardiac α-actin through distant cis-elements in promoter regions. Alternatively, the neighbor

environment of cardiac α-actin gene in chromosome or chromatin assembly may affect the expression of cardiac α-actin.

Cyclosporin A was first proved to be an extremely powerful therapeutic immunosuppressive agent. The immunosuppressive properties of CsA can largely be ascribed to its potent inhibition of the T cell-activation-dependent transcription of T cell growth factor (IL-2) and other immunologically important cytokine genes, including IL-3, IL-4, and GMCSF. It has been known that CsA neither does interfere with initial signaling events at the T cell membrane, such as Ca^{2+} mobilization, or PKC activation, nor directly inhibit the action of the specific transcription factors that are involved in lymphokine gene transcription (Emmel et al., 1989). Studies have shown that CsA binds the immunophilins cyclophilin, forming a complex, which can specifically bind the catalytic domain of calcineurin and inhibit calcineurin activity, and cyclophilin-cyclosporin A would home in on a single target, calcineurin (Liu, 1993). Our results show that CsA blocks the activation of hypertrophic response gene transcription mediated by active calcineurin in the cardiomyocytes. The ability of CsA to regulate the expression of hypertrophic response genes in primary cardiomyocytes would allow it to be a very good effector in this inducible system. In our system, CsA treatment which significantly reduced the responses of hypertrophy-sensitive genes is successful. Therefore, CsA treatment could become a novel therapy for certain cardiac diseases.

Although it was shown that doxycycline treatment is not good as CsA treatment to regulate transcription of three cardiac genes in our inducible calcineurin expression system (Data not shown), doxycycline is still considered as an effector to

modulate tTA-responsive gene expression in cardiac myocytes. The low level activity of tTA-responsive promoter stimulated by doxycycline treatment itself might be due to the activation of other tissue-specific transcription mediators which respond to doxycycline-induced signaling. It would be possible to reduce the effect of doxycycline on the tTA-regulated promoter through stable transfection of the inducible gene.

In this inducible system, both the induction of calcineurin gene expression and the responses of three cardiac genes to calcineurin stimulation are quite rapid. The activity of induced gene reached maximum at 12 hour following transfection. About 36 hours after that, the transcription of hypertrophic response genes mediated by active calcineurin was up to maximum. Obviously, the expression patterns of both calcineurin gene and hypertrophic response genes are corespondent and time-dependent.

It is well known that the increase in intracellular Ca^{2+} is associated with the initiation of cardiac hypertrophic phenotypes (Hongo et al., 1995). Previous studies have shown that the overexpression of calmodulin in the heart of transgenic mice results in hypertrophy (Gruver et al., 1993). Calcineurin is a serine/threonine protein phosphatase that contains a 59 Kda calmodulin-binding catalytic A subunit and a 19 Kda Ca^{2+} -binding regulatory B subunit. It has been shown that transient Ca^{2+} fluxes are not sufficient to activate the calcineurin. Because only two of calmodulin's four calcium-binding sites were occupied under this condition, calmodulin can only bind to calcineurin to form an inactive enzymatic complex in the *in vitro* binding assay. When a sustained Ca^{2+} plateau occurs, calcineurin is activated by calmodulin of which four Ca^{2+}

binding sites have fully been occupied. Thus, this feature indicates calcineurin is uniquely suited to mediate the prolonged hypertrophic response of a cardiomyocyte to Ca^{2+} signaling. Our results suggest that calcium/calmodulin signaling acts through calcineurin to transduce a positive hypertrophic response signal into the nucleus. The elevation of intracellular calcium activates calmodulin/calcineurin complex, leading to the enhancement of active calcineurin form. Activated calcineurin dephosphorylates its downstream substrates, which allows these downstream effectors to translocate into the nucleus. Subsequently, dephosphorylated substrates act as transcription factors to further activate the transcription of hypertrophic response genes. Therefore, calcineurin is a critical positive regulator of hypertrophic response gene expression.

In the cardiac myocytes, NFAT3 (cardiac form of NFAT family) within the cytoplasm is dephosphorylated by active calcineurin, enabling it to translocate into the nucleus. It has been known that NFAT can bind to the specific sequences on the promoter region of multiple hypertrophy-sensitive genes, and the transgenic mice overexpressing NFAT3 has shown marked hypertrophy phenotypes in the heart (Molkentin et al., 1998). Thus, NFAT could be a major effector of calcineurin pathway. However, due to the existence of multiple consensus sequences on the promoter regions in many hypertrophic response genes, it might require multiple transcription factors to synergistically act. One possibility is that upstream principle regulators such as calcineurin might control multiple effectors which may cooperatively activate the transcription of hypertrophic response genes. Since calcineurin is a ubiquitous protein phosphatase, it is reasonable that there are other substrates of calcineurin in addition to

NFAT. So far new substrates of calcineurin have not been identified. The inducible calcineurin expression system has a potential utility to identify downstream pathway(s) and molecular mechanism(s).

Alternatively, multiple signal pathways could cooperatively regulate transcription of hypertrophic response genes. As mentioned in the introduction, many transcription factors may involve in the initiation of hypertrophy-sensitive gene transcription. Multiple receptors and multiple intracellular signal pathways such as Ras-MAP kinase pathway, PKA, and PKC, can respond to the hypertrophic stimuli. These signalings may simultaneously be transduced into the nucleus and activate different transcription factors, which correspondingly regulate the transcription of a subset of hypertrophic response genes. Some negative transcription regulators may be activated. For example, it has been reported that a novel form of cardiac myocyte hypertrophy induced by IL-1β is characterized by an increase in protein content, but an absence of the fetal program of skeletal α-actin or βMHC gene expression (Patten et al., 1996). It has been known that IL-1β can activate both the JNK and p38 MAP kinase cascades (Saklatvala et al., 1996). In fact, IL-1β may induce the activation of the negative transcription factor YY1 which results in the repression of skeletal α-actin and βMHC gene expression (Patten et al., 1996). It is likely that different signaling pathways activated by individual stimuli or several extracellular stimuli could cross-talk and cooperatively modulate hypertrophy-sensitive gene transcription in the cardiomyocytes. Therefore, it is necessary to investigate the roles of calcineurin or other intracellular

regulators, as well as the responses of different cardiac gene under complex physiological or pathological conditions.

CHAPTER V

CONCLUSION

Cardiac hypertrophy, which leads to heart failure, is a major cause of morbidity in the United States. A calcineurin-dependent signaling pathway plays a critical role in the induction of cardiac hypertrophy. The elucidation of the quantitative characterizations of calcineurin would be crucial for understanding the pathogenesis of hypertrophy. In this present study, an inducible expression model system was successfully developed for manipulating calcineurin expression in primary cardiomyocytes. In response to the changes of active calcineurin expression, the expression of hypertrophy-sensitive genes can be up or down-regulated by this system at different time. Thus, the effects of calcineurin can be quantitatively and temporally investigated.

Because of the promising ability for this system to regulate gene expression, this system would be very useful for not only studying the roles of calcineurin but also investigating the effects of other cardiac regulators. Further works will be required on the improvement of this system, and the establishment of *in vivo* inducible system. We believe that the functions of more genes as well as the effects of their products upon cellular normal physiological activities or pathological changes would be conveniently studied by this regulatory approach in the future.

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