SIGNALING PATHWAYS IN MYOCYTE HYPERTROPHY
Role of GATA4, mitogen-activated protein kinases and protein kinase C

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Abstract

Cardiac myocytes react to increased workload and hypertrophic neurohumoral stimuli by increasing protein synthesis, reinitiating expression of fetal forms of structural genes, α-skeletal actin (α-SkA) and β-myosin heavy chain (β-MHC), and by increasing expression and secretion of atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP). Initially, the response is beneficial, but when prolonged, it leads to pathological cardiomyocyte hypertrophy. In this study, cardiomyocyte hypertrophy was initiated by hypertrophic agonists, endothelin-1 (ET-1) and phenylephrine (PE), and by increased stretching of atrial wall.

Transcription factor GATA4 was studied to identify the mechanism leading to increased gene expression of BNP. In BNP promoter, GATA4 binds to cis elements mediating hypertrophic response. Eliminating GATA4 binding by using the decoy approach, basal BNP gene expression was reduced. To identify mechanisms regulating GATA4, the roles of mitogen-activated protein kinases (MAPKs) were studied. Activation of p38 MAPK increased GATA4 binding to BNP gene and led to increased GATA4 dependent BNP gene expression. p38 MAPK was required for ET-1 induced GATA4 binding, whereas extracellular signal-regulated kinase (ERK) was required for maintaining basal GATA4 binding activity. PE and ET-1 activated protein kinase C (PKC) signaling in cardiac myocytes. Antisense oligonucleotide inhibition of PKCα markedly reduced PE induced ANP secretion and ET-1 induced BNP secretion, whereas gene expression of natriuretic peptides was not affected. Antisense PKCα treatment inhibited PE induced expression of α-SkA, while increased protein synthesis or β-MHC gene expression were not affected. Stretching of the perfused rat atria increased BNP, c-fos and BNP gene expression via mechanism involving p38 MAP kinase activation of transcription factor Elk-1. In cultured neonatal rat atrial myocytes stretch induced BNP gene expression was dependent upon transcription factor Elk-1 binding sites within the BNP gene promoter.

In conclusion, hypertrophic signaling in cardiac myocytes involves multiple signaling cascades. Activation of p38 MAPK is required for the development of ET-1 induced hypertrophic phenotype and GATA4 mediated BNP gene expression in cultured ventricular myocytes, and for stretch induced Elk-1 dependent BNP gene expression in atrial myocytes. PKCα is involved in PE induced hypertrophic response and PE induced switch in gene programming inducing expression of α-SkA, the fetal form of cardiac α-actin.

Keywords: endothelin-1, GATA4, hypertrophy, mitogen-activated protein kinase, protein kinase C
To my family
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AM</td>
<td>adrenomedullin</td>
</tr>
<tr>
<td>Ang II</td>
<td>angiotensin II</td>
</tr>
<tr>
<td>ANP</td>
<td>atrial natriuretic peptide</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>α-SkA</td>
<td>skeletal α-actin</td>
</tr>
<tr>
<td>ATF-2</td>
<td>activating transcription factor-2</td>
</tr>
<tr>
<td>β-MHC</td>
<td>β-myosin heavy chain</td>
</tr>
<tr>
<td>BNP</td>
<td>B-type natriuretic peptide</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CHF</td>
<td>congestive heart failure</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CNP</td>
<td>C-type natriuretic peptide</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporin A</td>
</tr>
<tr>
<td>EBS</td>
<td>ETS binding sequence</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ET</td>
<td>endothelin</td>
</tr>
<tr>
<td>ET_{α}</td>
<td>endothelin receptor subtype</td>
</tr>
<tr>
<td>FOG</td>
<td>friend of GATA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>G-proteins</td>
<td>guanine nucleotide binding proteins</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GSK3</td>
<td>glycogen synthase-3 kinase</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HIF-1</td>
<td>hypoxia-inducible factor-1</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin-1</td>
</tr>
<tr>
<td>IP_{3}</td>
<td>inositol-1,4,5-triphosphate</td>
</tr>
<tr>
<td>IR</td>
<td>immunoreactive</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
</tbody>
</table>
LV  left ventricular
LVH  left ventricular hypertrophy
MAPK  mitogen-activated protein kinase
MAPKK  mitogen-activated protein kinase kinase
MAPKKK  mitogen-activated protein kinase kinase kinase
MBP  myelin basic protein
MEF2  myocyte-specific enhancer-binding factor-2
MHC  myosin heavy chain
MI  myocardial infarction
MKP  mitogen-activated protein kinase phosphatase
MLC  myosin light chain
mRNA  messenger ribonucleic acid
NFAT  nuclear factor of activated T-cells
NEP  neutral endopeptidase
NPR  natriuretic peptide receptor
PCR  polymerase chain reaction
PE  phenylephrine
PKC  protein kinase C
PMA  phorbol myristate acetate
PI3K  phosphatidylinositol-3-OH kinase
RAS  renin-angiotensin system
RIA  radioimmunoassay
RT-PCR  reverse transcriptase polymerase chain reaction
SAPK  stress-activated protein kinase
SD  Sprague-Dawley
SEM  standard error of mean
SRE  serum response element
SRF  serum response factor
STAT  signal transducers and activators of transcription
TGF-β  transforming growth factor-beta
TnC  troponin C
TNF-α  tumor necrosis factor-alpha
TnI  troponin I
TnT  troponin T

α  alpha
β  beta
γ  gamma
δ  delta
ε  epsilon
η  eta
ι  iota
θ  theta
ζ  zeta
λ  lambda
List of original papers

This thesis is based on the following articles, which are referred to in the text by Roman numerals:


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1 Introduction

Primary and acquired structural heart diseases are the leading causes of mortality in the industrialized world (World Health Organization 2002). Acquired structural heart disease most often results from myocardial infarction (MI) due to coronary artery disease, hypertension and hypertensive cardiomyopathy (Berne & Levy 1993). Under stress compromising cardiac function, the heart attempts to maintain normal contractile function and cardiac output by undergoing a process of cardiac hypertrophy and remodeling. Initially, increase in myocyte size and thickening of left ventricle wall is beneficial, but when prolonged, it leads to pathological hypertrophy (for review, see Lorell & Carabello 2000). Increased stretching of cardiac myocytes is the main factor inducing hypertrophic growth, but circulating substances, such as endothelin-1 (ET-1) and angiotensin II (Ang II) either directly or indirectly also induce hypertrophic growth of cardiac myocytes (for review, see Sussman et al. 2002). It is not clear whether a primary stimulus for hypertrophy is the mechanical stretch itself or a concurring increase in neural and humoral factors. Cardiomyocytes respond to extracellular signals by hypertrophic growth of individual cells initiated by a specific set of mechanisms that regulate and modulate gene expression (for review, see Sadoshima & Izumo 1997). Multiple signaling molecules, many of them same as those that regulate proliferation of cancer cells or immune cells, are assembled between the signal and the gene to participate in signaling regulating growth of cardiomyocytes (for review, see Sugden & Clerk 1998b).

A set of protein kinase cascades has been in the focus of attention in studies on molecular mechanisms regulating hypertrophic response of cardiac myocytes evoked by external stimulus. Mechanical stretch of cardiomyocytes has been shown to activate second messengers, such as protein kinase C (PKC), mitogen-activated protein kinase cascades (MAPK) and calcineurin, which ultimately affects nuclear factors and the regulation of a number of genes, including atrial natriuretic peptide (ANP), skeletal α-actin (α-SkA) and β-myosin heavy chain (β-MHC) (for review, see Sugden & Clerk 1998b, Molkentin & Dorn II 2001). It is evident that there is no isolated signaling cascade for each stimulus or response, but the multitude of signaling molecules rather forms a network of cascades with numerous elements facilitating cross-talk between the cascades. In this manner it is evident that blockade of any of the intracellular signaling
pathways is not sufficient to efficiently prevent the hypertrophic growth of the cardiomyocyte. Similarly, activation of any of the pathways is predicted to activate hypertrophic growth of cardiomyocyte by cross-talking and activating other signaling networks.

The aim of the present study was to evaluate the role of GATA4 transcription factor in hypertrophic cardiomyocyte growth and gene expression, and to study the significance of the signaling pathways involved in cardiomyocyte stretch and hypertrophic agonist induced cardiomyocyte growth. Significance of GATA4 binding to B-type natriuretic peptide (BNP) gene promoter was studied by using decoy oligonucleotide approach. Mechanisms leading to GATA4 phosphorylation and activation were further studied by using specific inhibitors of MAP kinases and plasmids overexpressing MAPK pathway kinases. Role of PKCα in the regulation of cardiomyocyte hypertrophy was studied by using antisense oligonucleotides to inhibit PKCα.
2 Review of literature

2.1 The genetic response of heart to cardiac overload

The mammalian myocardium undergoes a period of hypertrophic growth during the postnatal maturation, which is characterized by an increase in size of individual cardiac myocytes without cell division (Lorell & Carabello 2000). The pattern of developmental hypertrophy is reinitiated in the adult heart in response to diverse mechanical, hemodynamic, hormonal and pathologic stimuli (Hunter & Chien 1999). Increased mechanical and neurohumoral load, such as hypertension, ischemic heart disease, valvular insufficiency and cardiomyopathy, increases wall thickness and results in concentric hypertrophy (Lorell & Carabello 2000). At the cellular level, cardiac myocytes respond to diverse types of biomechanical stress by initiating several different processes that via activation of transcription factors lead to hypertrophic gene expression and growth of individual myocytes (Fig. 1). Initially, the response is beneficial, but when prolonged, it leads to pathological myocyte hypertrophy. The first genetic response to increased load is activation of a pattern of early response, or immediate early genes: c-fos, c-myc and c-jun (Yamazaki et al. 1995b; Table.1). This is followed by induction of certain genes, such as ANP, BNP, β-MHC and α-SkA, accompanied by development of hypertrophic phenotype characterized by an increase in cell surface area, protein concentration and protein to DNA ratio (Sadoshima & Izumo 1997).
Fig. 1. A simplified model of a signal transduction pathway initiated by mechanical stretch or neurohumoral agonists in cardiac myocytes. The arrows provide the directional information of the signals forming a hierarchic system between the signaling molecules inside the cell. GPCR, G-protein-coupled receptor; PE, phenylephrine; MAPKKK, mitogen-activated protein kinase kinase; NFATc4, nuclear factor of activated T-cells c4; FOG-2, friend of GATA-2

2.1.1 Natriuretic peptides

ANP, BNP and C-type natriuretic peptide (CNP) are the known members of the mammalian natriuretic peptide system. Studies concerning ANP go back to 1981, when it was found that intravenous administration of atrial extracts into intact rats causes diuresis and natriuresis (de Bold et al. 1981). Soon thereafter, ANP molecule was purified and sequenced (Atlas et al. 1984). A few years later BNP and CNP were discovered from the porcine brain (Sudoh et al. 1988, Sudoh et al. 1990). ANP and BNP are cardiac hormones, and involved in the regulation of blood pressure and fluid homeostasis (Nakao
et al. 1992a). CNP is mainly found in the central nervous system and vascular endothelial cells (Chen & Burnett, Jr. 1998), where it increases cGMP levels and causes vasorelaxation (Furuya et al. 1990). Natriuretic peptides share a common structural motif, consisting of a 17-amino acid loop formed by an intramolecular disulphide linkage between two cysteine residues (for review, see Yandle 1994). The biologically active human ANP shares a high homology with other species, as does the active CNP. In contrast, the sequence variability of BNP between species is large, and the predominant circulating forms of BNP are 26, 45 and 23 amino acid peptides in pigs, rats and humans, respectively (Nakao et al. 1992a).

ANP and BNP are present as single-copy genes and are organized into three exons and two introns (Greenberg et al. 1984, Seidman et al. 1984, Argentin et al. 1985, Seilhamer et al. 1989). The ANPs are derived from a common precursor, named preproANP, which contains 149–152 amino acids depending on the species. Each preproANP molecule contains a signal peptide sequence at its amino terminal end. ANP is stored in atrial secretory granules mainly as a 126-amino acid peptide, proANP1-126, which is formed by cleavage of the signal peptide (Thibault et al. 1987). Upon release of ANP1-126 from secretory granules, the hormone is further split into an amino-terminal fragment, proANP1-98, and the biologically active carboxy-terminal peptide, ANP 99-126 (Schwartz et al. 1985, Thibault et al. 1985). The mRNA of BNP and CNP are also translated to preproBNP and preproCNP (Nakao et al. 1992a), which are further reduced by the cleavage of the signal peptide (for review, see Nakao et al. 1992a). The major storage form of BNP in the heart is cleaved mature peptide (Saito et al. 1989, Kambayashi et al. 1990, Ogawa et al. 1990, Mukoyama et al. 1991).

In the normal adult heart, ANP is mainly synthesized in the atria, but small quantities of ANP mRNA have also been found in normal adult ventricular myocytes, where the levels are approximately 0.5 to 3% of that in the atria (Ruskoaho 1992). Expression of atrial ANP is initially low and increases through development. In contrast, ventricular myocytes from neonatal hearts contain substantial amounts of ANP mRNA which declines rapidly after birth (Ruskoaho 1992). The ventricular expression and secretion of ANP is substantially elevated in cardiac hypertrophy. The major stimulus regulating ANP release is myocyte stretch (Lang et al. 1985, Ruskoaho et al. 1986). The secretion of ANP from the granules is also induced by a number of hormones and vasoactive peptides (for review, see Ruskoaho 1992).

BNP is expressed in both atria and ventricles, but is mainly released from the ventricles (Hosoda et al. 1991, Ogawa et al. 1991). BNP transcripts have also been detected in the central nervous system, lung, thyroid, adrenal gland, kidney, spleen, small intestine, ovary, uterus, and striated muscle (Gerbes et al. 1994). In studies with rat and man, the distribution of BNP has been strong in the spinal cord, while only small amounts are detected in the brain (Aburaya et al. 1989, Aburaya et al. 1991, Nakao et al. 1992a). In all peripheral tissues, the level of both natriuretic peptide transcripts are approximately 1–2 orders of magnitude lower than in cardiac ventricular tissues (Gerbes et al. 1994). Ventricular levels of BNP are substantially increased in response to chronic cardiac overload in the human heart (Hosoda et al. 1991, Takahashi et al. 1992).

CNP is a 22-amino-acid peptide, structurally related to, but genetically distinct from ANP and BNP (Minamino et al. 1991, Komatsu et al. 1991). Although initially identified in porcine brain, CNP immunoreactivity has been found in human vascular endothelial

Three natriuretic peptide receptors (NPRs) have been described that have different binding capacities for ANP, BNP and CNP (for reviews, see Espiner et al. 1986, Ruskoaho 1992, Anand-Srivastava 1997, Levin et al. 1998). Two of the receptors, NPR\(_A\) (CG\(_A\)) and NPR\(_B\) (CG\(_B\)), are guanylyl cyclases–linked receptors, and mediate the biologic actions, whereas NPR\(_C\) is not coupled to guanylyl cyclase (Chang et al. 1989, Chinkers et al. 1989, Schulz et al. 1989, Jamison et al. 1992). The natural ligands of NPR\(_A\) are ANP and BNP, whereas that of NPR\(_B\) is CNP (Jamison et al. 1992). The rank order of ligand selectivity for NPR\(_A\) receptor is ANP ≥ BNP > CNP, and for NPR\(_B\) receptor CNP > ANP ≥ BNP (Nakao et al. 1992b). The third receptor, NPR\(_C\), binds all three natriuretic peptides. Its messenger RNA lacks the guanylyl cyclase sequence present in the mRNA of the other natriuretic peptide receptors, suggesting that the principal function of NPR\(_C\) is to remove natriuretic peptides from the circulation (for review, see Anand-Srivastava & Trachte 1993). Natriuretic peptides, CNP in particular, are also subject to degradation by the ectoenzyme neutral endopeptidase 24.11 (NEP), which is widely distributed in the kidney, lung, heart, and endothelial cells (Kenny et al. 1993, Brandt et al. 1997). Natriuretic peptide receptor binding sites have been localized to atrial and ventricular endocardium, as well as aorta, pulmonary arteries and epicardial mesothelium (Rutherford et al. 1992). The significance of natriuretic peptide receptors has been mainly revealed in studies with transgenic mice. NPR\(_A\) deficient mice display an elevated blood pressure resulting in severe cardiac hypertrophy, whereas introduction of the NPR\(_A\) transgene in the cardiac myocytes reduced cardiac myocyte size in both wild-type and originally NPR\(_A\) null mice (Kishimoto et al. 2001).


<table>
<thead>
<tr>
<th>Early response</th>
<th>Intermediate response</th>
<th>Late response</th>
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<tbody>
<tr>
<td>c-fos</td>
<td>ANP</td>
<td>Cardiac (\alpha)-actin</td>
</tr>
<tr>
<td>c-myc</td>
<td>(\beta)-MHC</td>
<td>CT-1</td>
</tr>
<tr>
<td>c-jun</td>
<td>Skeletal (\alpha)-actin</td>
<td>NCX</td>
</tr>
<tr>
<td>BNP</td>
<td>AM</td>
<td>MMPs</td>
</tr>
<tr>
<td>Egr-1</td>
<td>Phospholamban</td>
<td>SERCA</td>
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</table>

Natriuretic peptides have been used in the treatment of diseases, with the most experience coming from intravenous BNP in the treatment of congestive heart failure (CHF) (McMurray & Pfeffer 2002, Mills et al. 2002). Another pharmacological approach being used is the inhibition of natriuretic peptide metabolism by NEP inhibitor drugs, which are currently being investigated as treatment for CHF and systemic hypertension (Corti et al. 2001).
2001, Weber 2001, McMurray & Pfeffer 2002). Increased blood levels of natriuretic peptides, ANP and BNP, have been found in certain disease states, suggesting a role in the pathophysiology of those diseases, including CHF, systemic hypertension, and acute MI (Ruskoaho 1992, Swynghedauw 1999). Elevated levels of ANP have been identified as indicators of adverse prognosis in the setting of CHF and MI (Rossi et al. 2000). BNP has proved to be a useful tool in the diagnosis of heart failure in the community and in the detection of asymptomatic left ventricular (LV) dysfunction (Daly et al. 2002). In addition, BNP plasma level values have been suggested as a guidance tool for pharmacotherapy in heart failure (Nicholls et al. 2001).

Studies with different in vivo and in vitro models have revealed that BNP gene expression is regulated by transcriptional and post-transcriptional mechanisms (Tokola et al. 2001, Suo et al. 2002). Transcriptional regulation of the BNP gene involves binding of transcription factors, such as GATA4, AP-1 (activator protein-1), transcription enhancer factor-1, Nkx-2.5 and NFATc4, to the 5'-flanking sequence of the BNP gene promoter (Grepin et al. 1994, Thuerauf et al. 1994, Thuerauf & Glembotski 1997, Durocher et al. 1997). The signaling cascades responsible for activation of transcription factors are not fully elucidated, but they involve activation of intracellular protein kinases and phosphatases, such as PKC, MAPKs and calcineurin, as discussed later in detail (for reviews, see Sugden & Clerk 1998b, Molkentin & Dorn II 2001).

2.1.2 Sarcomeric proteins

Increased ventricular load requires enhanced cardiac myocyte contractile performance, which is preceded by an increase in force-generating units (sarcomeres) in the myocyte (Lorell & Carabello 2000). The sarcomere consists of thick and thin filaments, which are organized to form contractile units (Murphy 1996). The thick filament proteins are two myosin heavy chain proteins (α-MHC and β-MHC), two myosin light chain proteins (MLC-1 and MLC-2) and C-protein (Hoh et al. 1978, Whalen & Sell 1980, Kasahara et al. 1994). β-MHC is the dominant isoform of the heavy chains in the ventricles of humans throughout the development (Schier & Adelstein 1982, Wessels et al. 1991, Mercadier et al. 1983). In response to mechanical load, genes from various striated muscle types respond by inhibiting expression of α-MHC, and increasing expression of β-MHC (Swynghedauw 1999). In experimental aortic stenosis model increased levels of β-MHC mRNA are observed two days after operation, peaking at days 4 to 6 (Izumo et al. 1987). β-MHC gene expression is also elevated in cultured rat cardiac myocytes by mechanical stress and by neurohumoral activators, such as ET-1 and α1-adrenergic agonists (Waspe et al. 1990, Shyu et al. 1995, Finn et al. 2001). Studies with rat β-MHC promoter have revealed that β-MHC gene can be induced by various agonists, including constitutively active PKC-β isoforms and activated MAPKs (Kariya et al. 1991, Finn et al. 2001). During embryogenesis, the β-MHC gene is expressed as part of the cardiac myogenic program under the control of NKX-2.5, myocyte-specific enhancer-binding factor-2C (MEF2C), and GATA4 (Hasegawa et al. 1997, Skerjanc et al. 1998). There is also evidence that calcineurin and NFATc4 are involved in the regulation of β-MHC gene
expression (Delling et al. 2000). MLC-2 mRNA levels are also upregulated in response to hypertrophic stimuli by α₁-adrenergic agonist PE, phorbol myristate acetate (PMA) and insulin growth factor-1 (Lee et al. 1988a, Shubeita et al. 1992, Ito et al. 1993).

The thin filament proteins include α- and β-tropomyosin genes, slow-twitch skeletal and cardiac troponin I genes, troponin C, slow-twitch skeletal and cardiac troponin T genes and three α-actin genes; α-cardiac, α-skeletal and smooth muscle α-actin, of which the α-cardiac actin predominates in the adult heart (Cummins & Perry 1973, Toyota & Shimada 1981, Mayer et al. 1984, Carrier et al. 1992, Jin et al. 1992, Murphy 1996). Changes in myocardial contractility and relaxation appear to involve changes in troponin isoform expression and phosphorylation (Solaro & Rarick 1998). The skeletal mRNA isoform of α-actin (α-SkA) has been found transiently expressed during pressure overload in rats (Schwartz et al. 1986, Izumo et al. 1988). The activation of vascular smooth muscle α-actin has also been suggested to occur during aortic constriction induced cardiac hypertrophy (Black et al. 1991). Levels of α-SkA are elevated at day 2 and reach peak levels 4 days after the onset of coarctation (Izumo et al. 1988). Hypertrophic agonists, such as PE and ET-1, have been shown to induce α-SkA gene expression via pathways involving MAPKs, calcineurin and PKC (Ito et al. 1991, Komuro et al. 1991, Fuller et al. 1997, Bueno et al. 2002b).

2.2 Signaling pathways in cardiac hypertrophy

The hypertrophic phenotype of cardiomyocytes is initiated by multiple endocrine, autocrine and paracrine factors that stimulate receptors bound to cell membrane. The activation of receptors affects multiple cytoplasmic signaling pathways, which regulate the activity of transcription factors, and finally regulate gene expression. The major contribution to studying the signaling pathways activated in cardiac hypertrophy was the development of a cell culture model with cardiac myocytes from neonatal rat hearts (Simpson & Savion 1982, Simpson et al. 1982). Initially, α₁-adrenergic agonists were shown to induce hypertrophic growth of a myocyte (Simpson 1985). Later, mechanical stress and several autocrine/paracrine growth factors and neurotransmitters, such as ET-1 and Ang II, were shown to promote a hypertrophic phenotype (Shubeita et al. 1990). For reviews, see Sugden & Clerk 1998b, Molkentin & Dorn II 2001. There is evidence that the action of these ligands on cultured neonatal cardiomyocytes mimics cardiac hypertrophy seen in vivo. During recent years development of kinase inhibitors and usage of adenoviral vectors and different transgenic models, have made it feasible to study cardiac load induced hypertrophic signaling in vivo as well.

2.2.1 Small GTP-binding proteins

Small GTP-binding proteins provide a critical link between receptors at the cell surface and kinase cascades that regulate a variety of cellular processes (Fig. 2). Studies have identified five different subfamilies: Ras, Rho, ARF, Rab and Ran (Sugden & Clerk
When these proteins are GTP-ligated, they are in active state, and when GDP-ligated, they return to inactive state. To date, participation of members of Ras and Rho families of small GTP-binding proteins has been described in hypertrophic signaling in cardiac myocytes (Sugden & Clerk 2000, Nicol et al. 2000).

Fig. 2. Schematic presentation of myocyte responses by small GTP-binding proteins Ras, RhoA and Rac1 (modified from Clerk & Sugden 2000).

2.2.1.1 Ras family

Ras is a low molecular weight (21 kDa) guanine nucleotide-binding protein, which is activated by GDP to GTP exchange initiated by membrane bound receptors. In cardiac myocytes GTP loading of Ras proteins can be induced by a number of hypertrophic agonists, such as PE, ET-1 and phorbol esters (Sugden & Clerk 2000). Activation of Ras can promote activation of Raf-1, phosphatidylinositol-3-OH kinase (PI3K) and Ral.GDS (Vojtek & Der 1998, Sugden & Clerk 1998b, Molkentin & Dorn II 2001). Ras has been shown to primarily promote ERK activation, but it can also activate c-Jun N-terminal kinase (JNK) and p38 MAPKs indirectly through Rac/Cdc42 (Sugden & Clerk 1998b).

Transfection of constitutively activated Ras into cardiac myocytes is sufficient to promote the hypertrophic phenotype, including increased c-fos, α-SkA and ANP gene expression and increased sarcomeric organization (Thorburn et al. 1993, Hoshijima et al. 1998, Fuller et al. 1998). In contrast, dominant negative Ras can block PE induced activation of ERK and ANP gene expression (Thorburn 1994). In vivo cardiospecific activation of activated Ras in mice leads to development of cardiac hypertrophy (Hunter
et al. 1995). Some studies suggest that Ras is a global regulator of gene expression rather than an activator of the specific hypertrophic program (Sugden & Clerk 2000).

2.2.1.2 Rho family

Members of the Rho family of small GTP-binding proteins are Rac, RhoA and Cdc42 (Sugden & Clerk 1998b). Rac1 can be activated by active Ras, which stimulates PI3K and increases levels of phosphatidylinositol-1,4,5-triphosphate leading to activation of Rac1 (Sugden & Clerk 2000). Another mechanism involved is Ras induced activation of p120GAP and p190GAP leading to Rho activation (Vojtek & Der 1998). RhoA and Rac have both been implicated in the hypertrophic growth response, mediating both morphological changes and the changes in gene expression (Clerk et al. 2001). Expression of activated Rac1 has been shown to stimulate the hypertrophic program, while expression of dominant negative Rac1 has been inhibitory (Aikawa et al. 1999, Pracyk et al. 1998). Rac1 has been shown to stimulate the ERK cascade either by promoting the phosphorylation of c-Raf or by increasing MEK1/2 association with c-Raf to facilitate MEK1/2 activation (Clerk et al. 2001). Rac1 may also promote JNK activation, whereas activation of p38 MAPK by Rac1 has not been observed.

A role for RhoA in myocyte hypertrophy is supported by several studies demonstrating the effects of activated and dominant negative forms of RhoA on hypertrophic gene expression (Aikawa et al. 1999, Hoshijima et al. 1998, Sah et al. 1996, Thorburn et al. 1997). Interestingly, cardiac specific expression of constitutively active RhoA was not sufficient to induce hypertrophic phenotype, but it did induce atrial enlargement associated with decreased heart rate and sinus and atrioventricular nodal dysfunction (Sah et al. 1999). The effects of RhoA on the morphological aspects of hypertrophy are not clear, since the results are contradictory (Thorburn et al. 1997, Hoshijima et al. 1998). RhoA does not stimulate JNK or p38 activity, but it may regulate gene expression by a mechanism involving Rho-dependent kinase ROK (Nicot et al. 2000, Clerk et al. 2001).

2.2.2 Protein kinase C

Protein kinase C represents a family of at least 12 serine/threonine kinases that participate in signal transduction in response to multiple stimuli (Nishizuka 1992). PKC is a single polypeptide with an N-terminal regulatory region and C-terminal catalytic region (Hug & Sarre 1993, Mellor & Parker 1998). Different isoforms can be divided into three groups based on differences in their structure, substrate specificity and calcium responsiveness: conventional or classic (cPKCs; α, β1, β2 and γ), novel (nPKCs; δ, ε, η and θ) and atypical PKCs (aPKCs; ζ and λ/ι) (Fig. 3). PKC isoforms are widely distributed in mammalian tissues and some isoforms are restricted to specific tissues. For example, PKCγ is restricted mainly to the central nervous system and spinal cord, and PKCθ to skeletal muscle and hematopoietic cells (Nishizuka 1992, Kanashiro & Khalil 1998). Cardiac
myocytes express members of all three subfamilies of PKC: one classic isoform (PKCα), two novel isoforms (PKCδ and PKCε), two atypical isoforms (PKCζ and PKCλ/ι) and probably PKCβI and PKCβII (Disatnik et al. 1994, Rybin et al. 1997, Clerk & Sugden 2001, Mackay & Mochly-Rosen 2001). Within a single cell, there are also differences in distribution between PKC isoforms before and after their activation. For example, in rat cardiac myocytes activated PKCα has been shown to translocate from the cytosolic fraction to the perinuclear region, whereas PKCε is translocated from the nucleus to myofibrils (Disatnik et al. 1994). Activation and translocation of the PKC isoforms to distinct subcellular sites is believed to confer specific physiological actions for each PKC isoform. The translocation of PKC isozymes involves binding to specific anchoring proteins, named RACKs (receptors for activated C kinases) (Disatnik et al. 1994, Mochly-Rosen 1995). Binding of PKC to RACK is saturable and isoform specific, and within a cell there may be multiple RACKs for each PKC in multiple cellular compartments (Schechtman & Mochly-Rosen 2001).

Fig. 3. Domain structure of the PKC subfamilies (modified from Mellor et al. 1998). The figure shows a comparison of the protein architecture of the various subgroups of the PKC subfamily, all consisting of constant (C) and variable (V) regions.
2.2.2.1 Conventional PKCs

Conventional isoforms of PKC are Ca\(^{2+}\)-dependent and activated by both phosphatidylserine and the second messenger diacylglycerol (Nishizuka 1992, Mellor & Parker 1998). Phorbol esters such as PMA are widely used as substitutes for diacylglycerol since they are not rapidly metabolized and produce a robust and long lasting activation of cPKCs and nPKCs. In the heart, PMA rapidly and irreversibly translocates cPKCs from the cytosolic fraction to cell membrane (Rybin & Steinberg 1994, Clerk et al. 1995). Both ET-1 and PE stimulate the hydrolysis of the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, leading to formation of IP3 and diacylglycerol, the physiological activator of cPKCs and nPKCs (Steinberg et al. 1989, Hilal-Dandan et al. 1992).

PKC\(\alpha\) is the major calcium-dependent PKC isozyme present in neonatal cardiac myocytes. In resting cells PKC\(\alpha\) is located in soluble fraction, and increase in calcium concentration translocates PKC\(\alpha\) to the particulate fraction (Goldberg et al. 1997, Muth et al. 2001). Exposure of cardiac myocytes to PMA or \(\alpha\)-adrenergic receptor agonist norepinephrine (NE) has been shown to translocate PKC\(\alpha\) to the perinuclear membrane (Disatnik et al. 1994). In contrast, translocation of PKC\(\alpha\) is not detected following exposure of cardiac myocytes to ET-1 or PE (Clerk et al. 1994). PKC\(\beta_1\) is located in the cytoplasm and around the nucleus in resting cells, but activation with PMA translocates PKC\(\beta_1\) into nucleus (Disatnik et al. 1994). Activated PKC\(\beta_1\) translocates from cytoskeletal elements to perinuclear region and cell periphery (Disatnik et al. 1994).

PKC\(\alpha\) is detected in extracts from fetal and neonatal rat cardiac myocytes (Rybin & Steinberg 1994). In contrast, only a small amount of PKC\(\alpha\) is detected in the adult rat hearts and no PKC\(\alpha\) immunoreactivity is found in adult ventricular myocytes, suggesting that in the adult rat heart PKC\(\alpha\) is present in nonmyocyte elements (Rybin & Steinberg 1994). However, 2- and 4-month-old transgenic mice overexpressing \(\alpha\)-type calcium channel showed increased membrane association of PKC\(\alpha\) (Muth et al. 2001). PKC\(\alpha\) can be constitutively activated by removing the autoinhibitory pseudosubstrate domain and activating the diacylglycerol binding domain in the N-terminal regulatory region. Transfection of this constitutively activated mutant of PKC\(\alpha\) activates MLC-2 and ANP gene expression (Muramatsu et al. 1989, Shubeita et al. 1992). Transfection of cardiac myocytes with PKC\(\beta_1\), which has been similarly constitutively activated, induces \(\beta\)-MHC gene expression and activates AP-1 dependent transcriptional activity (Kariya et al. 1991). Furthermore, cardiospecific overexpression of PKC\(\beta_1\) induces activation of the hypertrophic gene programme (Bowman et al. 1997). On the other hand, studies with PKC\(\beta_1\) knockout mice demonstrate that PKC\(\beta_1\) is not necessary for the development of cardiac hypertrophy nor does it attenuate the hypertrophic response (Roman et al. 2001). In transgenic mice PKC\(\beta_2\) expression under truncated \(\alpha\)-MHC promoter results in the development of hypertrophic phenotype, including increase in myocyte size and increased expression of \(\beta\)-MHC, c-fos, ANP and TGF\(\beta_1\) (Wakasaki et al. 1997). Finally, in samples taken from end-stage human dilated cardiomyopathy, the activity of the classical isoforms, PKC\(\alpha\) and PKC\(\beta_{1,2}\), is increased, whereas PKC\(\epsilon\) activity remains unchanged.
(Bowling et al. 1999). Studies with PKCβ1 and PKCβ2 translocation inhibitors have been shown to selectively inhibit phorbol ester induced translocation of PKCβ isozymes, but not PKCδ or PKCe, demonstrating their potential in studying PKC signaling (Ron et al. 1995).

2.2.2 Novel PKCs

Novel isoforms of PKC are Ca2+-independent and activated by both phosphatidylserine and diacylglycerol. In resting cells, the largest proportion of PKCδ and PKCe is located in the soluble fraction, and exposure to ET-1 and PE increases their association with the particulate fraction (Clerk et al. 1994). Activated PKCδ translocates to the perinuclear region, and PKCe to myofibrils (Disatnik et al. 1994). PKCδ is more abundant in fetal and neonatal than in adult cardiac myocytes (Rybin & Steinberg 1994). PKCe, which is present in adult cardiac myocytes, is the major novel isoform associated with hypertrophic cardiomyocyte growth (Sugden & Clerk 1998b). PKCe has been associated with cardiac hypertrophy, since PKCe selectively translocates to the particulate fractions in response to acute and chronic pressure overload and Ang II (Gu & Bishop 1994, Schunkert et al. 1995, Paul et al. 1997b). PKCδ in turn is activated in vivo by ischemia and diabetes (Koya & King 1998, Kawamura et al. 1998). Results using the recently identified PKCδ inhibitor peptide show that cell damage induced by simulated ischemia is caused, at least in part, by activation of PKCδ (Chen et al. 2001). In neonatal cardiac myocytes adenoviral overexpression of constitutively active PKCe is sufficient to increase ANP and β-MHC mRNA levels, but it has no effect on cell size or protein to DNA ratio (Strait et al. 2001). However, adenovirus-mediated expression of dominant negative PKCe is not sufficient to block ET-1 induced increase in ANP or β-MHC levels (Strait et al. 2001). In transgenic mice expression of PKCe inhibiting peptides results in increased α-SkA gene expression, increased myocyte size and impaired left ventricular contractile function (Mochly-Rosen et al. 2000). In contrast, high expression of PKCe binding peptide ψεRACK, an analogue of the anchoring and activation protein for PKCe, is associated with increased β-MHC gene expression, decreased myocyte cell size and normal left ventricular function (Mochly-Rosen et al. 2000). PKCe has also been shown to regulate activity of other signaling pathways, such as ERK, providing an example of cross-talk between the signaling molecules (Strait et al. 2001). Stress related factors, such as UV irradiation, ischemia and oxidants have been observed to activate PKCe and stress activated MAPKs, JNK and p38 MAPK, in parallel (Chen et al. 1999, Brodie et al. 1999, Ping et al. 1999). Taken together, activation and downstream signaling via the PKCe and PKCδ cascade may be necessary for the induction of hypertrophic gene programme.
2.2.2.3 Atypical PKCs

Atypical isoforms of PKC are Ca\(^{2+}\)-independent and do not require diacylglycerol for activation, yet phosphatidylserine regulates their activity. PKC\(\zeta\), the atypical isoform present in cardiac myocytes, is located around the cytosol and the nucleus, and exposure of cells to PMA translocates PKC\(\zeta\) to perinuclear region (Disatnik et al. 1994). No translocation of PKC\(\zeta\) is detected following exposure to PE or ET-1 (Clerk et al. 1994). PKC\(\zeta\) is most abundant in fetal and neonatal myocytes, whereas in the adult rat heart PKC\(\zeta\) resides in nonmyocyte elements (Rybin & Steinberg 1994). PKC\(\zeta\) has also been connected with cardiac hypertrophy, since in guinea-pigs aortic banding increases expression of PKC\(\zeta\) isozyme, and overexpression of constitutively active PKC\(\zeta\) is sufficient to induce ANP promoter activity in cultured cardiac myocytes (Decock et al. 1994, Jalili et al. 1999).

2.2.3 Mitogen-activated protein kinases

There are multiple MAPK pathways in all eukaryotic cells, which allow the cells to respond differently to divergent inputs. MAPK signaling cascades are usually divided into three parallel pathways: ERK, JNK and p38 MAPK pathways. All MAPK pathways include three signaling levels, i.e. MAPKKK activating MAPKK, which in turn activates MAPK. Activation of MAPKKKs results from translocation, oligomerization and phosphorylation by upstream kinases (Marshall 1995, Luo et al. 1996, King et al. 1998). Active MAPKKKs phosphorylate serine and threonine residues in MAPKKs, which in turn activate tyrosine and threonine residues in the activation loop of the MAPKs. Most physiological substrates of the MAPKs possess specific binding sites for MAPKs that allow strong interactions with selectivity for MAPK subfamilies (Kallunki et al. 1996, Yang et al. 1998a, Deacon & Blank 1999, Tanoue et al. 2000). MAPKs, in turn, also possess complementary docking sites, which allow them to interact with MAPK binding domains on substrate proteins (Kallunki et al. 1994, Tanoue et al. 2000). The signaling mechanism is coordinated by the interaction of components of the protein kinase cascade with scaffold proteins, such as JNK interacting protein-1 and MEK1 partner (Dickens et al. 1997, Teis et al. 2002).

2.2.3.1 Extracellular signal-regulated kinase

The ERK family of MAPKs (ERK1,2), also known as p42/44 MAP kinase, is activated by mitogenic stimuli and generally associated with cell growth and survival. It was the first of the MAPK pathways proposed to participate in hypertrophic signaling in cardiac myocytes (Bogoyevitch et al. 1993, Sugden 2001). ERKs and the upstream activators of the MEKs were first identified from yeast (for review, see Levin & Errede 1995, Herskowitz 1995; Fig. 4). The kinases in all levels are activated by G\(_q\)/G\(_{11}\) protein
coupled receptors and PMA in the heart (Bogoyevitch et al. 1994). Activation of c-Raf and A-Raf occurs in response to PMA, ET-1 and PE, involving activation of PKC (Bogoyevitch et al. 1995). In fact, several studies have found ERK as a downstream target of PKC (Goldberg et al. 1997, Ho et al. 1998, Sugden & Clerk 1998b, Molkentin & Dorn II 2001). PMA and ET-1 activate also MEKs and ERKs, whereas activation by \(\alpha\)-adrenergic agonist PE is less effective (Bogoyevitch et al. 1993, Bogoyevitch et al. 1994, Bogoyevitch et al. 1996a).

![Diagram of MAP kinase cascades](image)

**Fig. 4.** Schematic overview of MAP kinase cascades activated by diverse hypertrophic stimuli and regulating phosphorylation and activation of numerous cardiac transcription factors (modified from Sugden 1999). TNF-\(\alpha\), tumor necrosis factor-\(\alpha\); IL-1\(\beta\), interleukin-1\(\beta\); ATF-2, activating transcription factor-2.

As noted, ERKs are activated by various neurohumoral stimuli, and also by myocyte stretching in cultured cardiac myocytes and in vivo (Yamazaki et al. 1993, Sadoshima & Izumo 1993, Yamazaki et al. 1995a). Transfection of constitutively active MEK1 into cultured cardiac myocytes results in increased ANP promoter activity, whereas inhibition of ERK cascade with dominant negative mutant of MEK1 abolishes PE induced ANP promoter activity (Gillespie-Brown et al. 1995). Transgenic mice with cardiac restricted expression of activated MEK1 demonstrated concentric hypertrophy with an approximately 25% increase in heart to body ratio (Bueno et al. 2000, Bueno & Molkentin 2002). Antisense oligonucleotide inhibition of ERK1 and ERK2 has been shown to attenuate PE induced sarcomeric organization and increase in cell size, the morphological changes seen in hypertrophy, as well as PE induced increase in ANP promoter activity and ANP mRNA levels (Glennon et al. 1996). Since then, inhibitors of ERK pathway, PD 98059 and U0126 have become commercially available (Favata et al. 1998, Dudley et al. 1995). ERK inhibition with PD 98059 has been shown to attenuate the myofibrillar organization induced by PE or ET-1, supporting the role of ERKs in the
development of cardiac hypertrophy (Clerk et al. 1998b). However, contradicting data also exist, showing that dominant-negative ERK1 or ERK2, or PD 98059 are not sufficient to block PE induced ANP promoter activity or ET-1 induced increase in protein synthesis (Post et al. 1996, Choukroun et al. 1998). Although the exact role of ERKs in cardiac hypertrophy is not fully clear at present, there are convincing data indicating involvement of ERK in mechanical stretch induced increase in c-fos and α-SkA gene expression, and hypertrophic agonist induced increase in ANP promoter activity (Komuro et al. 1991, Yamazaki et al. 1993, Yamazaki et al. 1995a, Gillespie-Brown et al. 1995, Sadoshima & Izumo 1997). In fact, ERKs were the first MAPKs shown to phosphorylate transcription factor c-Jun, although later studies proved JNKs to be principally responsible for c-Jun phosphorylation (Pulverer et al. 1991, Karin 1995; Fig. 4). Recent studies have also shown a role for ERKs in regulation of transcription factor GATA4 phosphorylation (Liang et al. 2001b; Table 2). Previously, Elk-1 transcription factor has been shown to contain a phosphorylation motif for ERK2, and ERK pathway has also been implicated in the regulation of Elk-1 dependent transcriptional activation (Yang et al. 1998b, Babu et al. 2000).


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<tr>
<th>Name</th>
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2.2.3.2 c-Jun N-terminal kinase

Molecular cloning of p54, JNK2, has revealed a family of SAPKs or JNKs, encoded by at least three genes (Kyriakis et al. 1994, Derijard et al. 1994, Sluss et al. 1994; Table 2). JNK activity is induced by a number of upstream kinases (Kyriakis & Avruch 2001; Fig. 4). The JNKs are serine/threonine kinases, which also, similarly to ERKs, require tyrosine and serine/threonine phosphorylation for their activation (Kyriakis & Avruch 1990, Kyriakis et al. 1991). Interest in these kinases was heightened when they were found to phosphorylate c-Jun, a component of the AP-1 transcription factor (Pulverer et
al. 1991, Karin 1995). JNKs are preferentially activated by cellular stresses, such as UV-light and osmotic stress, and by inflammatory cytokines, such as TNF-α and IL-1β (Galcheva-Gargova et al. 1994, Hibi et al. 1993, Kyriakis et al. 1994, Derijard et al. 1994). In the heart, activation of JNKs, primarily JNK1 and JNK2, results from myocyte stretch or activation of GPCRs (Ito et al. 1999, Cook et al. 1999, Choukroun et al. 1999).

A number of studies have implicated JNKs in the regulation of cardiac hypertrophy in vitro and in vivo. Adenovirus mediated gene transfer of dominant negative mutant of MKK4, an upstream kinase of JNKs, blocks ET-1 induced increase in protein synthesis, sarcomere organization and ANP mRNA levels (Choukroun et al. 1998). Activated MEKK1 has been shown to stimulate ANP reporter gene expression, while a dominant negative MEKK1 mutant inhibits PE induced ANP expression (Ramirez et al. 1997, Thorburn et al. 1997). Promoter studies have further revealed that activated MEKK1 induces β-MHC, ANP and α-SkA expression in cultured neonatal ventricular myocytes (Bogoyevitch et al. 1996b). The results from studies using dominant negative or activated MEKK1 are not clear, since MEKK1 has also been shown to induce activation of both ERK and p38 MAPK pathways (Zechner et al. 1997, Thorburn et al. 1997). Crosstalk between MAPK pathways is also known to exist at other levels, i.e. MEKK3, a MAPKKK of ERK pathway, activating MAPKKs in ERK, JNK and p38 MAPK pathways, as well as MKK4 activating JNKs and p38 MAPKs (Derijard et al. 1995, Kyriakis & Avruch 2001; Fig. 4). Also, specific activation of p38α by adenovirus-delivered constitutively active MKK3β has been shown to result in potent inhibition of the activity of ERK1,2 and its upstream activator MEK1,2 (Westermarck et al. 2001). There are also contradictory results concerning the role of JNKs in hypertrophic signaling. Activated MEKK1 induced ANP expression was inhibited by activation of JNK pathway, whereas in an earlier report MEKK1 induced ANP expression was JNK dependent (Thorburn et al. 1997, Nemoto et al. 1998). Recently, studies with MEKK1 deficient mice showed that JNK signaling via MEKK1 is not required for aortic banding induced cardiac hypertrophy and ANP expression, but protects the heart from apoptosis (Sadoshima et al. 2002). Adenovirus mediated gene transfer of dominant negative MKK4 has been shown in vivo to inhibit pressure overload induced cardiac hypertrophy, characterized by a decrease in LV thickness, LV to body weight ratio and ANP expression (Choukroun et al. 1999). Primary targets for JNKs are transcription factors c-Jun, Elk-1 and ATF-2 (Gupta et al. 1995, Livingstone et al. 1995, Gupta et al. 1996, Sugden & Clerk 1998a; Fig. 4, Table 2). ATF-2 and c-Jun have been shown to form heterodimers that transactivate at cAMP response element -like consensus sequences within various promoters (van Dam et al. 1993, Sugden & Clerk 1998a). In the future, studies with selective inhibitors are expected to further enlighten the JNK dependent mechanisms of hypertrophic gene expression.

2.2.3.3 p38 mitogen-activated protein kinase

The p38 MAPK was also identified as a protein kinase cascade activated by IL-1β and physiologic stress (Freshney et al. 1994). Like other MAPKs, p38 is activated by dual
tyrosine/threonine phosphorylation in catalytic domain (Freshney et al. 1994, Rouse et al. 1994). Four p38 isoforms have been described to date: p38α, p38β, p38γ and p38δ (for review, see Kyriakis & Avruch 2001). The MAPKKs for p38 are MKK3 and MKK6, which specifically activate p38 MAPKs (Derijard et al. 1995, Raingeaud et al. 1996). As mentioned, MKK4, a MAPKK for JNK pathway, also phosphorylates and activates p38 in vitro (Derijard et al. 1995). The primary MAPKK is MAPKKK5 (ASK1), which also activates MKK4 and MKK7 in the JNK pathway (Kyriakis & Avruch 2001). MEKK1 is also capable of inducing the p38 pathway, but not until MEKK1 expression robustly exceeds that required for maximal JNK activation (Yan et al. 1994; Fig. 1). The p38 MAPKs are activated by a variety of stimuli, including chemical stress, physical stress, osmotic stress and radiation, and by GPCR-agonist activation (Paul et al. 1997a).

In cardiac myocytes p38α and p38β are the most important isozymes, whereas p38γ and p38δ are hardly detectable (Jiang et al. 1997). In the heart, p38 MAPKs are activated by GPCR ligands, such as ET-1, PE, Ang II and by myocyte stretch (Sugden & Clerk 1998a, Liang et al. 2000). Activated p38 isoforms are shown to phosphorylate and activate a number of transcription factors, including MEF2, ATF-2, ATF-6, nuclear factor-kappaB (NF-κB) and Elk-1 (Raingeaud et al. 1996, Han et al. 1997, Thuerauf et al. 1998, Liang & Gardner 1999, Yang et al. 1999a; Fig. 4, Table 2). In addition to transcription factors, p38 MAPKs phosphorylate and activate several other protein kinases. These include MAPK-activated protein kinases 2 and 3 (MAPKAP-K2 and MAPKAP-K3) and p38-regulated/activated kinase, which phosphorylates the small heat shock protein Hsp25/27 (Clifton et al. 1996; Table 2) (Clerk et al. 1998a, New et al. 1998). MAPKAP-2 is expressed in adult cardiac ventricular myocytes as 66 kd alpha and 61 kd beta subunits (World Health Organization 1998). Other known substrates for p38 MAPKs are Mnk1 and Mnk2, and mitogen- and stress-activated protein kinases (Kyriakis & Avruch 2001).

In cultured cardiac myocytes p38 is rapidly activated by ET-1, PE and stretching of the myocytes (Liang et al. 2000). In vivo pressure overload activates p38 MAPK, and increased p38 activation has also been observed in human hearts with failure secondary to coronary artery disease (Wang et al. 1998, Cook et al. 1999). In vitro overexpression of activated MKK3 or MKK6 induces hypertrophic growth of myocytes and increased expression of hypertrophic genes, such as ANP, BNP and α-SkA (Zechner et al. 1997, Nemoto et al. 1998, Wang et al. 1998). Adenoviral overexpression of wild type p38β results in increased cell size and ANP mRNA levels (Wang et al. 1998). Two relatively specific inhibitors exist for p38α and p38β, SB203580 and SB202190 (Lee et al. 1993, Goedert et al. 1997, Lee et al. 1994). In vitro pharmacological inhibition of p38 with SB 203580 (20 µM) blocks PE induced increase in ANP and BNP promoter activity, cell size and sarcomeric organization (Zechner et al. 1997). SB 202190 at 20 µM concentration is sufficient to suppress PE induced ANP promoter activity and ET-1 induced increase in cell size and sarcomeric organization (Nemoto et al. 1998). Role of p38 is not fully clear, since 10 µM concentration of SB 203580 failed to inhibit ET-1 and PE induced increase in β-MHC expression and cell size, and only partially inhibited agonist induced myofibrillar organization (Clerk et al. 1998b).
2.2.4 Phosphatases

2.2.4.1 Calcineurin

Calcium/calmodulin–dependent phosphoprotein phosphatase calcineurin, also known as protein phosphatase-2B (PP2B), is a heterodimer composed of two subunits, calcineurin A containing the catalytic site and calcineurin B containing the calcium binding regulatory domain (Bueno et al. 2002a). There are three different mammalian genes encoding calcineurin A, designated α, β and γ, of which calcineurin Aα and calcineurin Aβ are present in the heart in multiple alternatively spliced forms (Bueno et al. 2002a). Calcineurin B is encoded by two genes, each having alternatively spliced isoforms (Ueki et al. 1992). Calcineurin physically interacts with NFATc family members and dephosphorylates serine residues within these proteins (Rao et al. 1997). Once dephosphorylated by calcineurin, NFATc transcription factors translocate to the nucleus and directly activate immune response genes, usually in collaboration with AP-1 like transcription factors (Flanagan et al. 1991, Loh et al. 1996a, Loh et al. 1996b). Nuclear export of NFATs, in turn, is dependent upon rephosphorylation by glycogen synthase-3β (GSK3β), JNK or p38 MAP kinases (Chow et al. 1997, Gomez et al. 2000, Neal & Clipstone 2001). Using a yeast-two-hybrid screening for search of interacting factors for transcription factor GATA4, a specific interaction was observed between zinc finger domains of GATA4 with the DNA binding domain of NFATc4 (NFAT3). This interaction was sufficient to induce synergic activation of BNP transcription (Molkentin et al. 1998). Inhibition of calcineurin by cyclosporin A (CsA) or FK506 reduces PE and AngII induced increase in cell size and sarcomeric organization (Molkentin et al. 1998). Transgenic mice overexpressing the α-MHC driven catalytic subunit of calcineurin in the heart demonstrate a vast increase in heart size and activation of expression of hypertrophic genes β-MHC, α-SkA and BNP. Furthermore, treatment with CsA is sufficient to prevent the dysfunction developed in the transgenic mice (Molkentin et al. 1998). At present there are numerous studies supporting the hypothesis that calcineurin is involved in hypertrophic signaling in cardiac myocytes (Wilkins & Molkentin 2002). Recent studies have utilized naturally occurring inhibitors of calcineurin, so-called calcipressins, or focused on inhibiting calcineurin involved transgenic overexpression of the calcineurin inhibitory protein MCIP1 (myocyte-enriched calcineurin-interacting protein-1) to genetically modify calcineurin mediated hypertrophic response (Taigen et al. 2000, Görlach et al. 2000, Rothermel et al. 2001). Recent findings suggest proteolysis as a mechanism regulating calcineurin phosphatase activity. Abundance of the calcineurin A C-terminus containing the autoinhibitory domain was decreased in patients with hypertrophic obstructive cardiomyopathy and aortic stenosis leading to increased NFAT2 electrophoretic mobility (Ritter et al. 2002, Williams 2002). In addition to NFATcs, also MEF2 transcription factor is directly regulated by calcineurin (Passier et al. 2000). Activation of calcineurin signaling is also associated with activation of other signaling pathways, such as certain PKC isoforms, JNK and Akt (De Windt et al. 2000a, De Windt et al. 2000b). Inhibition of calcineurin blocks β-adrenergic agonist induced activation of ERKs and ET-1 gene expression (Zou et al. 2001, Morimoto et al. 2001).
2.2.4.2 Mitogen-activated protein kinase phosphatases

Other phosphatases suggested to participate in hypertrophic signaling in cardiac myocytes involve mitogen-activated protein kinase phosphatases (MKPs). So far 11 MKPs have been identified, including MKP-1, MKP-2, hVH3/B23, MKP-3, MKP-X, MKP-4, MKP-5, hVH-5/M3/6, PAC-1, MKP-6 and MKP-7 (Haneda et al. 1999, Masuda et al. 2001). MKPs differ in terms of substrate specificity, tissue distribution, subcellular localization and target gene regulation. In vitro, cultured neonatal cardiomyocytes infected with a calcineurin-expressing adenovirus and stimulated with PE demonstrate reduced p38 phosphorylation and increased MKP-1 protein levels. Inhibition of calcineurin with CsA results in decreased expression of MKP-1 protein levels, further supporting the interconnection between the pathways (Lim et al. 2001). Transgenic mice overexpressing MKP-1 lack activation of all three MAPK branches, ERK, JNK and p38, although p38 and JNK are preferred substrates over ERK (Franklin & Kraft 1997, Bueno et al. 2001). These mice are characterized by reduced developmental hypertrophy resulting in severe ventricular dilatation and neonatal lethality (Bueno et al. 2001). In addition, Ang II treatment has been shown to increase MKP-1 mRNA levels, and thereby negatively regulate MAPKs (Fischer et al. 1998, Hiroi et al. 2001).

Myocardial hypertrophy is associated with increased basal glucose metabolism, characterized by increased expression of glucose transporter-1 mRNA. Transfection of myocytes with MKP-3 has been shown to decrease TPA and PE induced increase in Glut1 mRNA levels (Montessuit & Thorburn 1999). MKP-3 shares 36% identity with MKP-1, which efficiently blocks phosphorylation and activation of ERK2 (Muda et al. 1996). MKP-2 can also be found in adult myocardium, where its levels are increased during myocardial failure (Communal et al. 2002). Possible phosphatases worth further investigation include PAC1 and M3/6, which have been shown to dephosphorylate p38 in vivo (Mackay & Mochly-Rosen 2000).

2.3 Cardiac transcription factors

Cardiac gene expression is controlled via transcriptional and several posttranscriptional mechanisms (Darnell, Jr. 1982). Several proteins inside the cell serve as regulatory transcription factors, which recognize and bind specific DNA sequences in promoters regulating gene transcription. Several transcription factors regulating the expression of cardiac genes have been identified, many of those induced during cardiogenesis as well as during hypertrophic cardiomyocyte growth (Srivastava 2001). To date, numerous transcription factors have been identified within the promoters of cardiac genes that are upregulated in response to increased hemodynamic load. Analysis of mechanisms controlling the expression of natriuretic peptide genes, ANP and BNP, has revealed many key regulatory elements, among which there are binding sites for transcription factors GATA4, NFATc4 and Elk-1 (Nemer & Nemer 2001, Pikkarainen et al. 2003).
2.3.1 GATA family of transcription factors

Six different GATA factors have been identified, and they can be divided into two subfamilies, GATA1, -2 and -3, and GATA4, -5 and -6. GATA1, -2 and -3 are preferentially expressed in hematopoietic cells, whereas GATA4, -5 and -6 are expressed in various tissues including the heart, liver, lung, gonad and gut (Arceci et al. 1993, Kelley et al. 1993, Laverriere et al. 1994, Morrisey et al. 1996, Morrisey et al. 1997a, Suzuki et al. 1996, for review, see Orkin 1998). Members of the GATA family of transcription factors contain a highly conserved DNA binding domain with two zinc fingers. This domain specifically interacts with DNA cis elements containing a consensus (A/T)GA TA(A/G) sequence. Mouse GATA4, -5 and -6 encode proteins of 48, 42 and 45 kDa, respectively (Arceci et al. 1993, Morrisey et al. 1996, Morrisey et al. 1997a). The proteins are 85% identical to each other at the amino acid level within the DNA binding region containing the zinc finger and basic regions (Molkentin 2000b). It has been reported that only the C-terminal zinc finger and the adjacent basic domain are necessary for specific DNA binding in vitro (Yang & Evans 1992, Omichinski et al. 1993, Visvader et al. 1995). Using nuclear magnetic resonance-technique, the central and N-terminal portions of GATA-1 were shown to interact with the major groove of DNA, whereas the C-terminal zinc finger made site-specific interactions within the minor groove. In fact, the N-terminal zinc finger of GATA1 has been shown to interact with adjacent GATA DNA sequence elements and with other cooperating transcription factors (Trainor et al. 1996, Weiss et al. 1997). Deletion analysis of GATA4 revealed that the C-terminal zinc finger is necessary and sufficient also for GATA4 DNA binding (Morrisey et al. 1997b). Deletion analysis also suggested that GATA4 has a nuclear localization signal in the basic domain adjacent to the C-terminal zinc finger (Morrisey et al. 1997b). On the other hand, the N-terminus of GATA4 protein contains two separate transcriptional activation domains, which are also partially conserved in GATA5 and GATA6, suggesting a similar mechanism of transcriptional activation (Morrisey et al. 1997b).

Binding motifs for GATA-factors have been identified within the promoters of a number of cardiac-expressed genes, including β-MHC (Hasegawa et al. 1997), α-MHC (Molkentin et al. 1994, Huang & Liew 1997), cardiac troponin C (Ip et al. 1994), ANP (Grepin et al. 1994), BNP (Thuerauf et al. 1994), cardiac troponin-I (Murphy et al. 1997, Di Lisi et al. 1998, Bhavsar et al. 2000) and natrium-calcium exchanger (Nicholas & Philipson 1999, Cheng et al. 1999). Studies with α- and β-myosin heavy chain promoters have shown a preferential utilization of GATA4 over GATA6 (Charron et al. 1999). On the other hand, the same study revealed that GATA6 participates in the regulation of cardiac gene expression by regulating ANP and BNP promoter activities in cooperation with GATA4 (Charron et al. 1999). Analysis of β-MHC promoter implicated that the proximal region of the promoter containing the GATA binding sites directs gene expression in response to pressure overload (Hasegawa et al. 1997). Similarly GATA4 has been shown to regulate pressure overload induced angiotensin type-1A receptor and BNP promoter activity (Herzig et al. 1997, Marttila et al. 2001). More recently, adenoviral overexpression of GATA4 in cultured neonatal cardiac myocytes enhanced sarcomeric organization, induced an increase in cell surface area and resulted in a significant increase in total protein accumulation (Liang et al. 2001a). Transgenic mice
with 2.5-fold overexpression of GATA4 within the adult heart demonstrate an increase in heart to body weight ratio, histological features of cardiomyopathy, and activation of hypertrophic genes, such as ANP, BNP and α-SkA, clarifying the functional role of GATA4 in the development of hypertrophic phenotype (Liang et al. 2001a). In cultured cardiac myocytes electrical pacing induced hypertrophy was associated with an increase in GATA4 mRNA levels, further supporting the role of GATA4 in the development of hypertrophic phenotype (Xia et al. 2000).

GATA4 participates in the regulation of cardiac gene expression in response to diverse hypertrophic stimuli, including pressure overload, isoproterenol, PE, ET-1, Ang II and PMA (Hasegawa et al. 1997, Morimoto et al. 2000, Hautala et al. 2001, Liang et al. 2001b, Kitta et al. 2001, Clement et al. 2002). Hypertrophic agonists activate GATA-dependent gene expression by increasing GATA4 binding or transactivating activity, but the signaling pathways involved are not clear (Liang & Molkentin 2002). Previous studies in hematopoietic progenitor cells and in Cos cells have indicated an ERK-dependent mechanism phosphorylating GATA2 (Towatari et al. 1995). In cardiac myocytes PE induced ET-1 promoter activity was associated with phosphorylation of GATA4 via ERK-dependent mechanism (Morimoto et al. 2000). The phosphorylation site for ERK was later identified as Ser 105, which is situated at a site potentially regulating GATA4 DNA binding. Phosphorylation of GATA4 by GSK3β results in cytosolic localization and decreased activity of GATA4 (Morisco et al. 2001).

In addition to phosphorylation and increased gene expression, GATA4 is regulated by interactions with other transcriptional cofactors. A number of transcription factors and nuclear cofactors have been shown to interact with GATA4 to activate or repress cardiac gene expression. GATA4 directly interacts with cardiac transcription factor NKX2.5 to regulate expression of ANP and α-actin promoters (Durocher et al. 1997, Sepulveda et al. 1998, Lee et al. 1998). GATA factors also regulate developmental expression of NKX2.5, suggesting a strengthened regulatory mechanism between these transcription factors (Molkentin 2000b). GATA4 physically interacts with NFATc4 and MEF2 regulating cardiac gene expression (Molkentin et al. 1998, Morin et al. 2000). Interaction of the N-terminal zinc finger of GATA4 with the transcriptional modifying protein FOG-2 has also been described (Lu et al. 1999, Svensson et al. 1999, Tevosian et al. 1999).

### 2.3.2 NFATc family of transcription factors

A recently discovered intracellular pathway linking extracellular stimuli to hypertrophic gene expression employs the calcineurin dependent downstream activator NFATc4 (Molkentin et al. 1998). Cardiac myocytes contain four members of NFATc proteins: NFATc1 (also named as NFAT2 or NFATc), NFATc2 (NFAT1 or NFATp), NFATc3 (NFAT4 or NFATx) and NFATc4 (NFAT3). NFATs are cytoplasmic proteins, which are translocated into the nucleus in response to stimulation that promotes Ca²⁺ mobilization and activates calcineurin (Rao et al. 1997). Activated calcineurin interacts physically with cytoplasmic NFAT proteins and dephosphorylates multiple serine residues within the N-terminal regulatory domains, resulting in nuclear translocation of the NFATc proteins
Phosphoserines within these residues are believed to mask nuclear localization sequences (NLS). Dephosphorylation exposes the NLS and results in rapid nuclear import of NFATc proteins. In addition to calcineurin, constitutively activated Ras results in increased NFAT activity and nuclear translocation of NFATc3 (Ichida & Finkel 2001). Ras appears to function upstream of calcineurin, since CsA treatment blocks Ras induced NFAT-dependent gene expression and nuclear localization of NFATc4 (Ichida & Finkel 2001). In the nucleus NFATc proteins can bind the DNA sequence GGAAAT as monomers or dimers, and they can also form complexes with other transcription factors to bind composite DNA sequences in the regulatory regions of their target genes. In the nucleus NFATc proteins can bind the DNA sequence GGAAAT at the regulatory regions of their target genes, usually in cooperation with AP-1-like factors binding to adjacent sites (Jain et al. 1992, Boise et al. 1993, Macian et al. 2000).

Discovery of phosphoserine residues within N-terminus of NFATc controlling nuclear export of NFATcs gave rise to search for kinases controlling the translocation (Beals et al. 1997a). Purification of kinases able to phosphorylate these functional sites, but not other sites within NFATc protein, led to identification of glycogen synthase-3 kinase (Beals et al. 1997b). Later on it was discovered that GSK3β negatively regulates not only nuclear translocation of NFATc, but also DNA binding activity of NFATc (Neal & Clipstone 2001). Other kinases shown to oppose NFATc localization into the nucleus include protein kinase A, p38 MAPK and JNK, which control the export of NFATc2, NFATc3 and NFATc4 (Beals et al. 1997b, Chow et al. 1997, Gomez et al. 2000, Yang et al. 2002). It has also been found recently that activation of cGMP-dependent protein kinase type 1 by NO/cGMP suppresses NFATc transcriptional activity, BNP induction and myocyte growth in response to α1-adrenergic stimulation (Fiedler et al. 2002). In contrast to other known NFATc kinases, Pim-1 kinase has been shown to enhance NFATc-dependent transactivation by phosphorylating NFATc1 on several serine residues distinct from the negative regulatory sites (Rainio et al., 2002). Evidence for both positive and negative feedback loops regulating NFATC activity has also become evident. In T cells, NFATc1 expression is stimulated upon calcineurin-induced binding of NFATc2 to the NFAT site within the NFATc1 promoter (Zhou et al. 2002). In muscle cells calcineurin and NFATc activate MCIP1, an endogenous inhibitor of calcineurin, creating negative feedback control (Yang et al. 2000). The upregulation of MCIP1 expression in cardiomyocytes is mediated by a cluster of tandem NFAT binding sites in MCIP1 gene promoter (Yang et al. 2000). Recent studies indicate that MCIP1 is also a substrate for ERK and GSK3 (Vega et al. 2002).

Involvement of NFATc signaling in the regulation of hypertrophic cardiomyocyte growth was discovered by Molkentin et al., who found that transgenic mice with cardiac-selective overexpression of either calcineurin or NFATc4 developed severe cardiac hypertrophy (Molkentin et al. 1998). This suggests that, differently from the T-cells, NFATc4 activation alone without other signaling elements can activate hypertrophy and expression of its target genes. N-terminal deletion mutant of NFATc4 that lacks the sites for calcineurin dephosphorylation is constitutively localized into the nucleus. The observation that N-terminal deletion mutant of NFATc4 can induce cardiac hypertrophy when overexpressed in vivo confirmed that dephosphorylation of NFATc4 is sufficient to induce cardiac growth (Crabtree & Olson 2002). In addition to GATA4, MEF2 has also
been shown to associate with NFATc4 and control hypertrophic response genes (Blaeser et al. 2000). NFATs together with cofactors have been implicated in the regulation of BNP, β-MHC, ET-1 and MCIP1 gene expression (Wilkins & Molkentin 2002). Calcineurin induced nuclear translocation of NFATc proteins is blocked by immunosuppressive drugs FK506 or CsA, which block calcineurin activation. Pharmacological studies employing the calcineurin inhibitors CsA or FK506 in different rodent models of heart disease have produced largely controversial results, with some studies reporting complete rescue from hypertrophy and others reporting either partial effects or no effects at all (Olson & Molkentin 1999, Molkentin 2000a, Rothermel et al. 2001). It has also been reported that hypertrophy can be exacerbated by CsA treatment in mice with a mutation in α-MHC gene resembling a mutation common in humans (Fatkin et al. 2000).

Several genetic approaches involving calcineurin/NFATc signaling have been applied in recent years. Transgenic mice expressing a constitutively active form of GSK3β under control of α-MHC promoter suppressed calcineurin induced cardiomyocyte growth, with reduced nuclear localization of NFATc (Antos et al. 2002). Overexpression of MCIP1 in the heart under control of α-MHC promoter inhibits cardiac hypertrophy in response to activated calcineurin, pressure overload and exercise (Rothermel et al. 2001). While dephosphorylated NFATc4 is able to induce hypertrophy when overexpressed in the heart, there is no evidence that hypertrophic gene expression requires direct binding of NFATc4 to target gene promoters.

2.3.3 The ETS-domain transcription factor family

The mammalian ETS-domain family of transcription factors is composed of nearly 30 members (Maroulakou & Bowe 2000). They were originally identified on the basis of a region of primary sequence homology with the protein product of the v-ets oncogene encoded by E26 (E twenty six) avian erythroblastosis virus (for review, see Karim et al. 1990). The ETS-factors share a unique structure of the 85 amino acid ETS domain that has been described as a winged helix-turn-helix (Donaldson et al. 1994). The DNA binding of ETS-factors is also conserved, with all members recognizing a core enhancer sequence (C/G)GA(A/T) (Woods et al. 1992, Gupta et al. 1998). In many cases ETS-factors regulate gene expression by co-operating with other transcription factors, such as AP-1, and ternary complex factors (TCFs), such as SRF (Sharrocks et al. 1997).

There is a TCF subfamily of ETS-domain transcription factors, composed of three members: Elk-1, SAP-1 and SAP-2 (Net) (Sharrocks et al. 1997). These proteins contain four conserved domains; an N-terminal ETS DNA-binding domain; the B-box, which binds directly to SRF; the D domain, which represents a docking site for MAPKs; and the C-domain, which acts as an MAPK inducible transcriptional activator domain (Fig. 5) (Treisman 1996, Sharrocks et al. 1997, Yang et al. 1998a, Li et al. 2000). In fact, the TCF subfamily of ETS-factors represents a key nuclear target for MAPK pathways. There is evidence that Elk-1 is activated by ERK, JNK and p38 MAPK pathways (Treisman 1996, Yang et al. 2001). Elk-1 is phosphorylated at multiple sites within the C-terminal
transcriptional activator domain, resulting in both enhanced DNA-binding activity and transactivation (Sharrocks et al. 1997). Phosphorylation of Elk-1 also results in change of mobility of DNA-bound complexes, suggesting a conformational change in the complex. Elk-1 has been proposed to exist in two conformations, such that either phosphorylation by MAPKs or SRF binding to the B-box is believed to convert Elk-1 to an open conformation that can efficiently bind DNA in both binary and ternary complexes (Yang et al. 1999b). It has recently been shown that MAP kinase phosphorylation-dependent activation of Elk-1 leads to activation of the co-activator p300, which may play a role in the regulation of stress responsive immediate early genes (Li et al. 2003). There are also studies demonstrating that interaction of Elk-1 with other transcription factors, such as histone deacetylase-1 and Sp1, can lead to transcriptional repression (Li et al. 2000, Yang et al. 2001).

Cardiac myocytes represent several targets for ETS factors. Pressure overload is known to induce expression of c-fos, a member of the AP-1 transcription factor complex. Deletion and point mutations in the SRE of the c-fos promoter result in loss of pressure-induced reporter gene expression, indicating that the SRE is necessary for pressure response (Aoyagi & Izumo 1993). The minimal c-fos promoter consisting of SRE alone is sufficient to confer pressure responsiveness, confirming that the pressure response element coincides with the SRE (Aoyagi & Izumo 1993). p38 MAPK mediated Elk-1 binding on ETS binding sequence (EBS) within the BNP promoter has recently been shown to regulate ET-1 induced BNP gene expression (Pikkarainen et al. 2003). The two palindromic EBSs within the α-MHC promoter have been shown to act as strong repressor elements for α-MHC gene expression (Gupta et al. 1998). In cultured chick ventricular myocytes Ets-1 has been shown to participate in the regulation of inducible nitric oxide synthase (iNOS) gene expression (Takahashi et al. 2001). Several cardiac genes contain potential binding sites for ETS-factors in their promoters, including α-SkA, cardiac α-actin, β-MHC, MLC-2, cardiac troponin T, cardiac troponin C and cardiac troponin I, which therefore serve as potential targets for ETS-factors (Gupta et al. 1998).
2.3.4 HIF-1

Hypoxia-inducible factor (HIF)-1 is a heterodimeric DNA binding transcription factor consisting of two subunits, 120 kDa HIF-1α and 91- to 94 kDa HIF-1β (Wang et al. 1995, Bertges et al. 2002). HIF-1β can dimerize with several different basic helix-loop-helix – PAS proteins, whereas HIF-1α is the oxygen regulated subunit responsible for HIF activity (Wang et al. 1995, Semenza 1999). The regulation of HIF involves changes at transcription level as well as posttranscriptional and posttranslational alterations in response to hypoxia (Jung et al. 2002). The most characteristic regulatory mechanism for HIF is its protein stabilization under hypoxic conditions, and its rapid degradation on reoxygenation. Hydroxylation of a proline residue within HIF-1α degradation domain by HIF1-α prolyl-hydroxylase under hypoxic conditions has been shown to regulate HIF1-α stability and HIF transcriptional cascade (Jaakkola et al. 2001).

Under normoxic conditions HIF-1 activity is mainly regulated by ubiquitination by multiple domains within the HIF-1 protein. In addition, there is an increasing body of evidence indicating that also changes in phosphorylation of HIF-1 affect HIF-1 expression and transactivation of target genes. HIF-1 serves as a substrate for various kinase pathways including PI3K, ERK, JNK and p38 MAP kinase (Minet et al. 2001). It has been reported that ectopically overexpressed c-Jun cooperates with HIF-1 to regulate hypoxic response element dependent reporter gene expression (Alfranca et al. 2002). HIF-1 preferentially binds to a TACGTGCT motif that first identified in 3’ enhancer element of erythropoietin gene (Semenza & Wang 1992). Functional HIF-1 binding sites have since been identified within promoters of a number of hypoxia responsive genes, such as vascular endothelial growth factor, glucose transporter-1 and lactate dehydrogenase A (Bertges et al. 2002). In cardiac myocytes HIF-1 has been shown to regulate the expression of AM, ET-1, iNOS and vascular endothelial growth factor (Cormier-Regard et al. 1998, Jung et al. 2000, Kim et al. 2002, Kakinuma et al. 2002). A recent study also revealed binding sites for HIF-1 in rat ANP promoter and showed that overexpression of HIF-1alpha was sufficient to enhance ANP promoter activity (Chun et al. 2003).
3 Aims of the research

The aim of the present study was to evaluate the role of GATA4 transcription factor in hypertrophic cardiomyocyte growth and gene expression, and to study the mechanisms regulating GATA4 function in response to GPCR stimulation. The second purpose was to study the roles of MAPK and PKC signaling pathways in regulating GPCR agonist and stretch induced cardiomyocyte growth and gene expression, and to identify nuclear factor elements targeting the regulatory modules of load induced cardiac genes.

Specifically the aims were:

1. To study the specific role of GATA4 binding on BNP gene expression and hypertrophic cardiomyocyte growth.
2. To define the role of MAPK pathway kinases in the regulation of ET-1 induced GATA4 binding to BNP gene promoter and GATA-dependent BNP gene expression.
3. To study the role of PKCα on PE and ET-1 induced hypertrophic cardiomyocyte growth and gene expression.
4. To characterize the role of ERK and p38 MAPK in regulating hypertrophic gene expression in response to increased load in isolated rat atrium.
4 Materials and methods

4.1 Materials

The chemicals and supplies used in this study were: formaldehyde and guanidine isothiocyanate (Fluka Chemie AG, Buchs, Switzerland), CsCl (Serva Feinchemica GmbH & Co, Heidelberg, Germany), LiCl (JT Baker Chemicals BV, Holland), agarose NA (Pharmacia LKB Biotechnology, Uppsala, Sweden), Hybond N+ nylon membrane (Amersham Life Science, Buckinghamshire, UK), Quick Prime Kit (Pharmacia, Sweden), ET-1 (Phoenix Pharmaceuticals Inc., CA, USA and Peninsula Laboratories, Belmont, CA, USA) and bovine myelin basic protein (MBP) (Upstate Biotechnology, Lake Placid, NY). [γ-32P] ATP, [α-32P] dCTP, [3H] leucine, ECL plus Western blotting reagent, Hyperfilm MP and p42/44 (ERK) kinase enzyme assay system were purchased from Amersham International (Amersham, Bucks, UK). p38 In vivo Kinase Assay Kit (Mercury™) was from Clontech (Palo Alto, CA). Luciferase and β-galactosidase reagents were purchased from Promega (Madison, WI), Fugene 6 transfection reagent from Roche Molecular Biochemicals (Mannheim, Germany), DOTAP from Boehringer Mannheim (Mannheim, Germany) and TFX-50 from Promega (Madison, WI). PhosphoPlus p38 MAP Kinase Antibody Kit was purchased from New England Biolabs Ltd (Hitchin, Hertfordshire, UK). GATA4, GATA5, GATA6 and HIF-1 polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphoserine antibody and anti-phosphotyrosine antibody were from Zymed laboratories Inc (San Francisco, CA). Anti-phosphothreonine antibodies were obtained from Alexis Corporation (San Diego, CA), Zymed laboratories Inc (San Francisco, CA) and Santa Cruz Biotechnology (Santa Cruz, CA). pCMV-Flag-p38α, pCDNA-Flag-JNK1 and pCDNA3-Flag-JNK1(APF) plasmids were kind gifts from Dr. R.J. Davis (Howard Hughes Medical Institute, University of Massachusetts Medical School). pMT2-mGATA4 plasmid was a gift from D.B. Wilson (Dept of Pediatrics, St. Louis Children’s Hospital). pCMV-MEK1 and pCMV-MEK1 plasmids were purchased from Clontech (Palo Alto, CA). pUC19 plasmid was obtained from New England Biolabs Ltd (Hitchin, Herts, UK). Other chemicals were from Sigma.
4.2 Experimental animals

Male 2-to-4-day-old and 8-week-old Sprague-Dawley (SD) rats were from the Center for Experimental Animals at the University of Oulu, Finland. The animals were housed in plastic cages with free access to tap water and normal rat chow in a room with controlled 40% humidity and temperature of 22 °C, where a 0600 h on, 1800 off environmental light cycle was maintained. The maintenance diet of the animals contained 0.65% sodium chloride (NaCl). The Animal Use and Care Committee of the University of Oulu approved the experimental design. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

4.3 Experimental protocols

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<td>PE and ET-1 induced hypertrophic myocyte growth Neonatal rat ventricular myocytes</td>
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4.3.1 Neonatal rat cardiac myocyte cell culture (I–IV)

Ventricular and atrial cells were prepared from 2-to-4-day-old SD rats using the collagenase dissociation method (Tokola et al. 1994). The hearts were perfused 6–8 times for 5 minutes with disaggregation medium (collagenase 1 g/l and CaCl$_2$ 25 µM in phosphate buffered saline (PBS) at 37°C). The cells were filtered and washed twice in DMEM/F-12 supplemented with 10% fetal calf serum and antibiotics (penicillin-streptomycin at 100 IU/ml). The cells were preplated onto 100 mm culture dishes for 15–45 min at 37°C in humidified air with 5% CO$_2$. The nonattached cells were collected and plated at the density of 2 × 10$^5$ /cm$^2$ onto Falcon wells from 15 to 60 mm in diameter. Following 16-hour incubation, the myocytes were subjected to liposome-mediated transfection with FuGENE 6, DOTAP (Boehringer Mannheim, Mannheim, Germany) or TFX-50 (Promega, Madison, WI) for 6–8 hours. In reporter gene analysis experiments, the transfection efficiency was controlled by co-transfecting the cells with Rous sarcoma virus or cytomegalovirus (CMV) promoter driven β-galactosidase gene plasmids. The antisense and decoy oligonucleotides (30 and 20 bases, respectively) used were phosphorothioated to increase nuclease resistance and support RNase H cleavage of hybridizing RNA. Sequence for GATA decoy oligonucleotide (ODN) was 5’-TGTGTCTGATAAATCAGAGATAACCCCACC-3’ and for control ODN 5’-TAAATTTGGCCAAGTGTAGCTCCGTTTGTGA-3’. Both ODNs with their complementary sequence ODNs were heated to +80°C and annealed at room temperature for 2 h. Sequence for antisense PKCα ODN (As1) was 5’-ATTATCTCTGAGTATTTTGGA 3’ targeting to 3’untranslated sequence on the rat PKCα cDNA, and for scrambled ODN: 5’-TGTATATGTGAATTTTC-3’. Two other antisense PKCα ODNs used were 5’-TAAACGTCAGCCATGGTCCC-3’ targeting to 5’untranslated sequence (As2) and 5’-TTAGCGATGACCAGCTGATC-3’ targeting to coding sequence (As3) on the rat PKCα cDNA. After transfection, cells were washed twice with DMEM/F-12 and cultured in complete serum-free medium (CSFM), which was DMEM/F-12 medium supplemented with 2.5 mg/ml bovine serum albumin, 1.0 µM insulin, 5.64 µg/ml transferrin, 32 nM selenium, 2.8 mM sodium pyruvate, 1 nM T$_3$ and 100 IU/ml penicillin-streptomycin. When appropriate, PE (100 µM) or ET-1 (100 nM) were added to culture medium on the third day of culture. The protein kinase inhibitors used (SB 203580, SB 202190 and PD 98059) were added to the culture medium 2–4 hours prior to exposure to PE or ET-1. Samples for RIA were taken before and after the transfection, and at 8–24 hour intervals after the transfection. At the end of the experiments, the cells were washed twice with ice cold PBS and either frozen at -70°C or collected by scraping for further analysis.

4.3.2 Isolated perfused atrial preparations (IV)

Male SD rats weighing 290–400 g were used. The rats were decapitated, and the heart from each rat was rapidly removed and placed in oxygenated (~10°C) buffer solution (in mM: 113.8 NaCl, 28.6 NaHCO$_3$, 2.5 CaCl$_2$, 5.0 HEPES, 1.1 MgCl$_2$, 1.2 KH$_2$PO$_4$, 4.7 L-
glutamic acid, 5.0 taurine, 5.0 creatine, 5.0 succinic acid, 5.0 glucose and insulin (0.06 nM); pH 7.4), which was also used at 37°C for superfusion of the atrium. The experimental model used in this study was the isolated rat atrial appendix (Laine et al. 1994). The left atrial auricle was attached to one of the four ends of a cross-branch polyethylene adapter, and the tissue was placed in a constant temperature (37°C) organ bath. A tube was attached to the opposite end of the cross-branch adapter, and another tube with a smaller diameter was inserted inside to carry the perfusate inflow into the lumen of the atrium. For the outflow, another tube was attached to one of the cross branches of the adapter. The same outflow tube was used to control the intra-atrial pressure by adjusting the height of the end of the tube. The fourth cross branch of the cross-branch adapter was connected to a pressure transducer (TCB 100, Millar Instruments) to record the pressure in the lumen of the atrium. Inflow and outflow (2 ml/min) to both the atrial lumen and the organ bath at a constant temperature were controlled by a peristaltic pump (7553-85, Cole-Parmer Instrument). Samples for RIA were taken from the perfusate beginning from 20 minutes of the start of the perfusion. For atrial stretch, the intra-atrial pressure inside the lumen was elevated from 2 to 8 cmH2O at 32 minute time point.

### 4.4 Isolation and analysis of cytoplasmic RNA (I, III–IV)

At the end of each experiment, the cultured cardiac myocytes were washed twice with ice cold PBS and stored at -70°C. The atrial tissue was blotted dry, weighed, immersed in liquid nitrogen and stored at -70°C until assayed. RNA was isolated by the guanidine thiocyanate-CsCl method (Chirgwin et al. 1979). Northern blot hybridization, in which the size and amount of specific mRNA molecules in total RNA preparations are determined, was performed after isolation of RNA. For the RNA Northern blot analyses, 5–10 µg of samples of the RNA were transferred to Amersham Hybond N+ nylon membranes. A 390-bp fragment of rat BNP complementary deoxyribonucleic acid (cDNA) probe (Ogawa et al. 1991) (a generous gift from Dr. K. Nakao, Kyoto University School of Medicine, Kyoto, Japan), full-length rat ANP cDNA probe (Flynn et al. 1985) (a generous gift from Dr. P. L. Davies, Queen's University, Kingston, Ontario, Canada), PCR amplified rat AM cDNA probe (nucleotides 287-736) (Romppanen et al. 1997), a 482 bp fragment of cDNA probe complementary to rat 18S ribosomal RNA (Lee et al. 1988b) were labeled with [32P]dCTP using T7 Quick Prime Kit (Pharmacia). cDNA probes for rat α-SkA, rat β-MHC and rat c-fos were made with the RT-PCR technique. Sequencing showed that the probes corresponded to bases 2950 to 3184 (GeneEMBL access number v01218), 5794 to 5923 (x15939) and 231-12 80 (x06769), respectively. The membranes were hybridized overnight at +42°C in 5 x SSC (sodium sodium citrate, 1 x SSC = 0.15 M NaCl, 0.015 M trisodium citrate, pH 7), 0.5% sodium dodecyl sulfate (SDS), 5 x Denhardt’s solution, 50% formamide and 100 µg/mL sheared herring sperm DNA (Tokola et al. 1994). After hybridization, the membranes were washed in 0.1 x SSC, 0.1% SDS three times for 20 min at +55°C and exposed to Phosphor screen (Molecular Dynamics, Sunnyvale, CA, USA) at room temperature. Phosphor screens
were scanned with Phosphor Imager (Molecular Dynamics) or Molecular Imager FX Pro MultiImager System (Bio-Rad laboratories). The hybridization signals of AM, ANP, BNP, c-fos, α-SkA and β-MHC mRNA were normalized to that of 18S RNA for each sample to correct for potential differences in loading and/or transfer.

PKCα, β-MHC and HIF-1 mRNA levels were measured by quantitative reverse transcription-PCR analysis with an ABI 3700 Genetic Analyzer using TaqMan chemistry (Applied Biosystems, Foster City, CA) as previously described (Majalahti-Palviainen et al. 2000). The RNA was extracted as previously described and a cDNA reaction was performed according to the manufacturer’s protocol (Gibco BRL), after which mRNA levels were measured by quantitative RT-PCR analysis. Forward and reverse primers for PKCα mRNA detection were AGACCACAAATTCTCATGCC and CAAAACCCAGATGAAGTCG, respectively. PKCα amplicon was detected using fluorogenic probe 5'-FAM-CCCACCTTCTGAGCCTAGCCTGAGC-TAMRA-3', the probe, forward and reverse primers for rat β-MHC were 5'-FAM-GGCAGGCCAGGCCAGAGGACTGAG-3', GCTACCCAACCCTAGGATGC and TCTGCTAAGGTGCATTCAA, respectively. For rat HIF-1α, the probe, forward and reverse primers were 5'-FAM-CCCGCCTCAGACCTGAGC-TAMRA-3', GATTCGCCATGGAGGCG and GACGTTCGACTCATCCTATTTT, respectively. The results were normalized to 18S RNA quantified from the same samples using the forward and reverse primers TGGTTGCAAAGCTGAAACTTAAAG and AGTCAAATTAAGCCGCAGGC, respectively. The probe for the 18S amplicon was 5'-VIC-CCTGGTGTTGCCCTCCGTCA-TAMRA-3'.

4.5 Radioimmunoassays (I, III–IV)

The medium samples were collected at 8–24 hour intervals in cell culture experiments and the perfusate samples at 4 min intervals in isolated atrial perfusion experiments. The immunoreactivity of ANP and BNP from cell culture medium and the immunoreactivity of ANP from perfusate samples were determined directly from the sample. For isolated atrial perfusate BNP radioimmunoassay, the 5 ml perfusate sample was extracted by Sep-Pak C18 cartridges, lyophilized and redissolved to 500 µl of RIA buffer. The extracts and unextracted perfusate and culture medium samples in duplicates of 100 µl were incubated with specific rabbit BNP (Ogawa et al. 1991) and ANP (Jougasaki et al. 1995, Vuolteenaho et al. 1985) antiserum. Synthetic rat BNP 51-95 (BNP-45) and synthetic rat ANP99-126 were incubated as standards. The tracers were prepared by chloramine-T iodination of synthetic rat [Tyr0]-BNP 51-95 and rat ANP99-126 followed by reverse phase high performance liquid chromatography purification. After incubation for 48 hours at +4°C, the immunocomplexes were precipitated with sheep antiserum directed against rabbit gammaglobulin in the presence of 500 µl of 8% Polyethylene Glycol 6000, pH 7, followed by centrifugation at 3000 g for 30 min. The sensitivities of the BNP and ANP assays were 2 fmol/tube and 1 fmol/tube, respectively. The intra- and inter-assay variations were less than 10% and 15%, respectively. Serial dilutions of perfusate and tissue extracts showed parallelism with the standards. The BNP antiserum did not
recognize ANP or CNP. The ANP antiserum recognized ANP and proANP with equal avidity, but did not cross-react with BNP or CNP.

4.6 Reporter gene assays (I–II, IV)

Rat BNP promoter fragment was generated by PCR, with rat genomic EMBL3-λ-clone as a template and using the following primers: sense 5’-GGGATTTGAACTCAGG -3’ with KpnI, MluI-linker; antisense 5’-CCTAGGAGCTAGCAACG -3’ with BamH1-linker. Subsequently, PCR-product was digested with KpnI and BamH1 and cloned to KpnI-BglII-site of pGL3-Basic vector (Promega) resulting in a (Δ-5kbp/+4) BNP promoter construct.

(Δ-5kbp/+4) BNP-pGL3 construct was used to produce a (Δ-538/+4) BNP-pGL3 by nested deletion (Pharmacia Biotech). Site-directed mutations to two adjacent (−91 and −80) GATA-sites of (Δ-538/+4) BNP-pGL3 were prepared (Stratagene) and the resulting construct is referred to here as BNP Gmut. The primers for mutagenesis were sense 5’-GGCAGGAATGTGTCTTGAAAATCAGATGAAACCCCACCCCTAC-3’; antisense 5’-GTAGGGGTGGGGTTGCATCTGATTTGCAAGACACATTCTCCTGCC-3’. A reporter plasmid containing three GATA element consensus sequences linked to pXP-1 vector (Grepin et al. 1994) was a kind gift from Dr. M. Nemer (Institut de Recherches Cliniques de Montréal, Montreal, Canada).

4.7 Gel mobility shift assys (I–II, IV)

Nuclear extracts from cardiomyocytes were prepared as described previously (Schreiber et al. 1989). Protein concentration from each sample was determined by using Bradford assay (Bradford 1976) (Bio-Rad Laboratories). Double stranded-oligonucleotide corresponding to GATA binding region (Δ-68/−97) of rat BNP promoter was used for analysis of GATA DNA binding activity and a previously described ODN for measurement of Octamer-1 (Oct-1) DNA binding activity (Kemler et al. 1989; Table 3). Both probes were sticky-end-labeled with [α−32P]dCTP by Klenow enzyme. For each reaction mixture (20 µl) 6 µg of nuclear protein and 2 µg of poly(dI-dC) was used in a buffer containing 10 mM HEPES (pH 7.9), 1 mM MgCl2, 50 mM KCl, 1mM dithiothreitol (DTT), 1mM EDTA, 10% Glycerol, 0.025% NP-40, 0.25mM phenylmethylsulfonyl fluoride (PMSF) and 1 µg/mL of each leupeptin, pepstatin and aprotinin. Reaction mixtures were incubated with a labeled probe for 20 min followed by non-denaturating gel-electrophoresis on 5% polyacrylamide gel. Subsequently, the gels were dried and exposed in a PhosphorImager screen and analyzed with ImageQuant (Molecular Dynamics) or Molecular Imager FX Pro Multimager System (Bio-Rad laboratories). To confirm DNA sequence specificity of the protein-DNA complex formation, competition experiments with 10-, 50- and 100-molar excesses of non-radiolabeled ODNs with intact or mutated binding sites were performed. For competition
and supershift experiments, appropriate ODNs or antibodies (Santa Cruz Biotechnology) were added to reaction mixture 20 minutes before addition of labeled probe.

Table 3. Probes for gel mobility shift analysis.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GATA (rat BNP)</td>
<td>for: TGTGTCTGATAAAATCAGAGATAAC</td>
</tr>
<tr>
<td></td>
<td>rev: GGGGGGTTATCTCTGATTTATCAGACACA</td>
</tr>
<tr>
<td>NFAT (human BNP)</td>
<td>for: AGAGCTATCCCTTTTTTTGCCATCTCTG</td>
</tr>
<tr>
<td></td>
<td>rev: GGGCCAGGATGGAAAAACAAAAAGGATAG</td>
</tr>
<tr>
<td>HIF-1 (mouse AM)</td>
<td>for: AGAGCCCGTGCAAACGTGTTC</td>
</tr>
<tr>
<td></td>
<td>rev: GGGOAAGACACGTCCACACCGG</td>
</tr>
<tr>
<td>Oct-1 (human HH2A)</td>
<td>for: GATCGAGCTTTACCTATTTGCTAAGCGATTGA</td>
</tr>
<tr>
<td></td>
<td>rev: GATCTCAATCGCTTATGCAAATAAGGTAAGCTCG</td>
</tr>
</tbody>
</table>

4.8 Western blot analysis (II–IV)

Cells were washed twice with ice cold PBS and collected by scraping into 150–500 µl of lysis buffer, which consisted of 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml aprotinin, 2 mM benzamidine, 1 mM PMSF, 2 mM and 50 mM NaF. The extracts were further lysed with sonication and supernatant was collected after centrifugation. For western blot analysis, cell lysates were matched for protein concentration (10–20 µg), loaded on SDS-PAGE and transferred to nitrocellulose filters. The membranes were blocked in 1–5% nonfat milk and then incubated with phospho-p38α, p38α, NFATc3, NFATc4, phospho-Elk-1, Elk-1, GATA4, anti-serine, anti-threonine, anti-tyrosine, PKCα, PKCβII, PKCδ, PKCγ or PKCe antibody overnight at 4°C. The filters were washed the following day and incubated for 1 hour with an HRP conjugated anti-mouse, anti-rabbit or anti-goat secondary antibody. The amount of protein was detected by enhanced chemiluminescence.

4.9 Kinase activity assays (II, III)

Following treatment with appropriate agents, myocytes (approximately 1 × 10^6) were washed with ice cold PBS at room temperature. Samples for PKC activity and ERK activity assays were collected by scraping into 100 µl of lysis buffer containing 10mM Tris (pH 7.5), 150 mM NaCl, 2 mM EGTA, 2 mM DTT, 1 mM Na3VO4, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 2 µg/ml pepstatin and 5 mM benzamidine. The extracts were sonicated and supernatant was collected after centrifugation. 15 µl of protein extract was incubated at 30°C for 15 minutes with 10 µl of substrate buffer containing specific
substrate peptide in the presence of 1 μCi (γ-32P) ATP. Each reaction was terminated and blotted on separate peptide binding paper discs, which were washed repeatedly with 75 mM orthophosphoric acid. The incorporated radioactivity was measured with a scintillation counter (Rackbeta II, LKB Wallac).

For immuno complex kinase assay endogenous p38 was immunoprecipitated with specific antibody (New England Biolabs) at 4°C overnight, followed by protein G-Sepharose precipitation. The immunoprecipitates were washed three times with buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 25 mM β-glycerophosphate, 25 mM NaF and 1% Triton X-100. Lysates were washed once more with kinase buffer containing 25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 1.0 mM Na3VO4 and 10 mM MgCl2. The activity of the immuno complex was assayed at 30°C for 15 minutes in 30 µl of kinase buffer in the presence of 2 μCi [γ-32P] ATP and 20 μg of MBP as substrate (Kumar et al. 1997). The reactions were terminated and the reaction contents electrophoresed on 15% SDS-polyacrylamide gels followed by PhosphorImager or Molecular Imager FX Pro analysis to determine the phosphorylation level of MBP. The effect of p38 inhibitor SB 203580 on p38 activity was measured by in vivo kinase assay.

4.10 Calcineurin activity assay (III)

A specific kit was used for measurement of calcineurin activity (Biomol, Plymouth Meeting, PA). According to the manufacturer’s instructions, the cells from neonatal rat ventricular cell culture were washed twice with ice cold PBS and collected by scraping into 300 µl of lysis buffer including a mixture of proteases inhibitors provided by manufacturer. Cell lysis was further centrifugated for 45 min at 15000 g. The supernatant was incubated with RII phosphopeptide, a known substrate for calcineurin, and the free phosphate released was detected based on the Malachite green assay (Martin et al. 1985). In cellular extracts RII phosphopeptide is also cleaved by other phosphatases; i.e. PP1, PP2A and PP2C. Hence, okadaic acid was used to inhibit PP1 and PP2A and, since EGTA inhibits calcineurin, okadaic acid and EGTA were used to reveal the PP2C activity.

4.11 Cytochemistry (I, III)

For immunocytochemistry, cardiomyocytes were grown on glass coverslips. Cells were washed in PBS, fixed in 4% paraformaldehyde containing 0.2% Triton X-100, washed three times in PBS and once with pure ethanol in −20°C. Cells were next placed in ice bath, washed three times with cold PBS and blocked in PBS containing 10% FBS and 0.02 M glycine. Sarcomeric organization was visualized by labeling α-actin filaments with Alexa Fluor 568 Phalloidin (Molecular Probes, Eugene, OR). PKCa was assessed using antibody against PKCa (Upstate Biotechnology, Lake Placid, NY) at a dilution of 1:400 in blocking solution. Subsequently, the cells were washed twice with cold PBS-
glycine, and anti-mouse FITC-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added at a dilution of 1:200 in blocking solution. Finally cells were washed twice with PBS, once with dH2O and subjected to fluorescence microscopy.

For calcium concentration transient measurements and transfection efficiency experiments the cardiac myocytes were cultured on glass bottom wells and, as desired, loaded with Fluo-3 (10 µM, Molecular Probes) or transfected with complexes of 3’-fluorescein isothiocyanate (FITC) labeled ODNs and FuGENE 6 as described above. The intracellular calcium transients and localization of 3’FITC labeled ODNs in spontaneously beating ventricular myocytes was examined using an LSM 510 laser scanning confocal microscope (Zeiss, Thornwood, NY) equipped with argon laser and attached to a Zeiss Axiovert 100 TV microscope (Williams 1990). Differential interference contrast and green fluorescence channels were used.

4.12 Protein synthesis (I–III)

[^3H]leucine-incorporation and total cellular protein content were measured from cells cultured and transfected in 24-well plates. On the third day in culture, medium was replaced with CSFM supplemented with[^3H]leucine (5 µCi/mL) and when appropriate, with ET-1 (100 nM) or PE (100 µM). After 24 hours cells were lysed and processed for measurement of incorporated[^3H]leucine (Amersham-Pharmacia) by liquid scintillation counter (Rackbeta II, LKB Wallac) and total cellular protein content by Bradford assay (Bradford 1976) (Bio-Rad Laboratories).

4.13 Statistical analysis

The results are expressed as mean±standard error of mean (SEM). Differences between data groups were evaluated for significance using Student’s t test of unpaired data or one-way analysis of variance and Bonferroni’s post-test. Repeated measures ANOVA was used for multivariate analysis. Differences at the 95% level were considered statistically significant.
5 Results

5.1 Role of GATA4 in the regulation of BNP gene expression (I)

5.1.1 GATA4 DNA binding activity

The proximal (Δ−68/−97) region of the rat BNP promoter contains two adjacent GATA sites, which control cardiac-specific expression of BNP (Grepin et al. 1994). ET-1 increased GATA binding to (Δ−68/−97) BNP promoter within 30 minutes, sustained for 3 hours. As a control for the specificity for GATA DNA binding activity, Oct-1 DNA binding activity remained unchanged. Supershift analysis with specific antibody to GATA4 confirmed the presence of GATA4 in the BNP promoter binding complex. Specific antibodies of GATA5 and GATA6 had no effect on the electrophoretic mobility of the BNP promoter binding protein complex.

In order to block GATA DNA binding, the proximal (Δ−68/−97) rat BNP promoter sequence was used to generate a 30 bp GATA4 factor binding cis-element decoy ODN. Transfecting the GATA decoy ODN to cardiac myocytes abolished both basal and ET-1-induced GATA4 DNA binding (> 95%) at 24 hours after transfection when compared to cells transfected with control ODN, and similar downregulation of GATA binding was detected at 48 hours after transfection. In vitro GATA4 DNA binding was totally abolished by preincubation of nuclear extracts of cardiac myocytes with 50-molar excess amount of the GATA decoy ODN but not with 50-molar excess of control ODN. Neither of the ODNs competed with Oct-1 DNA binding. Blockade of GATA element binding factors was specific and had no effect on Oct-1 DNA binding in ODN treated cardiomyocytes. A positive control plasmid containing three GATA response elements was used to verify the specific interaction of GATA with its target promoter. Co-transfection with GATA decoy ODN significantly decreased GATA-dependent promoter activity, whereas control ODN was without effect. When GATA4 binding to rat ANP promoter was studied, GATA4 also bound on (Δ-109/-131) of rat ANP promoter, and the
DNA binding was totally abolished by preincubation of nuclear extracts with 50-molar excess amount of the GATA decoy ODN, but not with control ODN.

5.1.2 Effect of GATA decoy treatment on ANP and BNP

To study the effect of GATA decoy ODN treatment on the hypertrophic pattern of gene expression, the expression of cardiac natriuretic peptides was evaluated. GATA decoy ODN treatment significantly decreased basal secretion and gene expression of ANP and BNP in cardiac myocytes (Fig. 6). Interestingly, ET-1 induced secretion of ANP and BNP was independent of blocked GATA4 DNA binding, and similarly the increase of ANP and BNP mRNA by ET-1 remained unaffected at 24 hours.

Fig. 6. Effect of GATA decoy ODN treatment on basal and ET-1 induced secretion of ir-ANP, ir-BNP and activation of a 5kb and 538bp rat BNP promoters in cultures neonatal ventricular myocytes. Open bars, untreated cells; solid bars, ET-1 treated cells. Results are mean ± SEM. *P < 0.01, †P < 0.001 compared with untreated control cells.

To study the rat BNP promoter region we introduced 5kb and 538bp BNP promoter luciferase constructs to the myocytes. Deletion of a –5kb to –538 bp upstream region of the BNP promoter did not affect basal promoter activity or ET-1-inducibility of the gene. The mutation of two proximal GATA binding sites at –91bp and –80 bp of the 538bp BNP promoter significantly decreased basal promoter activity, but it had no effect on ET-1 induced promoter activity (Fig. 6).
5.1.3 Effect of GATA decoy on protein synthesis and sarcomere assembly

Hypertrophic cardiomyocyte growth in response to stretch and exposure to various agonists involves increased protein synthesis and increased organization of contractile proteins to sarcomeric structures (Shubeita et al. 1990). To study the effect of GATA decoy ODN treatment on ET-1-induced de novo protein synthesis, incorporation of $^{3}\text{H}$-labeled leucine into the myocytes was studied. We found that GATA decoy ODN treatment had no effect on basal or ET-1 induced protein synthesis and total cellular protein content. Neither did GATA decoy ODN treatment affect ET-1 induced increased organization of sarcomeres.

5.2 Role of MAP kinases in GATA4 activation and BNP gene expression (II)

5.2.1 Activation of ERK and p38 MAP kinase by ET-1

p38 MAP kinase is activated in neonatal rat ventricular myocytes by various extracellular stimuli, such ET-1 and PE (Clerk et al. 1998b, Liang et al. 2000). Western blot analysis was used to confirm p38 activation by ET-1. ET-1 induced phosphorylation of p38 was noted at 15 minutes. The kinetics of p38 activation was measured by immuno complex kinase assay. Endogenous p38 was immunoprecipitated with anti-p38 antibody and its activity was measured using MBP as a substrate. ET-1 induced a rapid increase in p38 activity, which was maximal at 15–20 minutes. In vivo kinase assay, which uses ATF-2 as a substrate, was used to verify the inhibition of p38 by SB 203580 in cardiac myocytes. Treatment with ET-1 (100 nM) for 24 hours led to a 3.4-fold increase of p38 activity, and activity was totally inhibited by p38 inhibitor SB 203580 (20 $\mu$M), which also decreased basal activity of p38 MAP kinase by 50%. In contrast, treatment of myocytes with a potent MEK1 inhibitor PD 98059 increased basal p38 activity, but had no effect on ET-1 induced p38 activity.

Activation of ERK by GPRC agonists has been previously established (Sugden & Clerk 1997). ERK activity was measured by an assay which measures transfer of a phosphate group to a peptide highly selective for ERK. As reported previously (Bogoyevitch et al. 1993, Liang et al. 2000), ET-1 at the concentration of 100 nM was a strong activator of ERK. The response was maximal at 5 minutes and declined to almost basal level within 35 minutes. MEK1 inhibitor PD 98059 (20 $\mu$M) was sufficient to inhibit ET-1 induced ERK activation by 80% measured at 5 minutes.
5.2.2 Effect of ERK and p38 inhibition on ET-1 induced protein synthesis

To examine whether blockade of p38 MAPK or ERK with specific inhibitors is sufficient to attenuate ET-1 induced protein synthesis, incorporation of [3H]-labeled leucine in cardiac myocytes was examined. Treatment of myocytes with SB 203580 or PD 98059 at the dose of 20 µM had no effect on basal protein synthesis. ET-1 induced 2.5-fold increase in [3H]leucine incorporation was totally abolished by p38 MAP kinase inhibition with SB 203580 (20 µM), whereas ERK inhibition with PD 98059 (20 µM) had no effect.

5.2.3 Regulation of GATA4 by MAP kinases

To examine GATA4 activation by ET-1 and the role of MAP kinases in the activation of GATA4, gel mobility shift assay was applied. Activation of GATA binding to BNP promoter occurred rapidly in response to ET-1 (100 nM). ET-1 stimulated GATA4 binding was detectable in 15 minutes and was maximal in 60 minutes. GATA4 binding remained upregulated for 3 hours, and supershift analysis revealed that GATA4 was the major cardiac nuclear factor that binds to the BNP GATA site. Mutation of either of the GATA-binding sites showed that GATA4 binding was equal to both sites, but the binding activity was reduced to about half of the observed with a probe having both sites intact.

Next the myocytes were pretreated with p38 and ERK inhibitors (SB 203580 and PD 98059, respectively), or the cells were transfected with dominant negative form of JNK and then subjected to ET-1 treatment. The induction of GATA4 binding to BNP gene was completely inhibited by p38 inhibitor SB 203580, and moreover, this inhibition of GATA4 binding was dose-dependent (Fig. 7). Inhibition of ERK pathway with PD 98059 had no effect on ET-1 induced increase in GATA4 DNA binding, but basal GATA4 binding activity was significantly decreased (Fig. 7). Inhibition of JNK pathway with dominant negative form of JNK had no effect on basal or ET-1 induced GATA4 DNA binding.

To further elucidate the role of p38 MAP kinase in the induction of GATA4 DNA binding, the p38 protein levels were increased by transfecting the myocytes with a CMV promoter driven plasmid overexpressing p38α. ERK and JNK pathways were studied similarly by using CMV promoter driven plasmids overexpressing MEK1 and MEKK1. Myocytes transfected with pUC-19 were used as controls. Overexpression of p38 substantially evoked GATA4 binding to BNP gene as compared to control plasmid. Treatment with p38 inhibitor SB 203580 completely abolished the effect. Inhibition of ERK with PD 98059 (20 µM) resulted in a modest decrease in p38 induced GATA4 binding to BNP gene. Overexpression of MEK1 or MEKK1 had no effect on GATA4 DNA binding.
Fig. 7. Effect of p38 and ERK inhibitors (SB 203580 and PD 98059, respectively) on ET-1 induced GATA4 binding activity. The results are mean ± SEM. **P < 0.001, *P < 0.05 compared with untreated control cells, #P < 0.05, ##P < 0.001 compared with ET-1 treated cells.

To examine whether p38 induced increase in GATA4 DNA binding activity was due to changes in phosphorylation of GATA4, the myocytes were transfected with plasmids overexpressing p38α, MEK1, MEKK1 or pUC19 (control). Subsequently, GATA4 phosphorylation was examined by Western blot analysis. Immunoblotting with GATA4 antibody showed that GATA4 protein levels were unaffected. Overexpression of MEK1 and p38α exhibited a marked increase in serine phosphorylation of GATA4, whereas overexpression of JNK pathway (MEKK1) had no effect. p38 induced serine phosphorylation of GATA4 was inhibited by p38 inhibitor SB 203580 and also by ERK inhibitor PD 98059 consistently with the finding that p38α induced GATA4 binding was also suppressed by PD 98059. Forced expression of p38, MEK1, or MEKK1 did not induce threonine phosphorylation or tyrosine phosphorylation of GATA4.

To further investigate the role of MAP kinases in the regulation of GATA4, COS-1 cells transiently expressing GATA4 were cotransfected with plasmids overexpressing p38α, MEK1 or JNK1. Control cells, cotransfected with pUC19, showed modest GATA4 binding activity to BNP promoter. p38α overexpression resulted in a 4-fold increase in GATA4 binding activity, whereas MEK1 or JNK1 overexpression had no effect on GATA4 binding to BNP gene promoter. Oct-1 binding activity was not affected by transient expression of different plasmids.

5.2.4 p38 MAP kinase regulation of GATA-dependent promoter

To test whether p38 overexpression is sufficient to stimulate BNP promoter activity, the myocytes were co-transfected with (Δ–534bp/+4bp) BNP promoter plasmids and p38α expression plasmid or pUC19 plasmid (control). p38α overexpression resulted in a 4-fold increase in rat BNP promoter activity (Fig. 8). The mutation of two proximal GATA binding sites at –91 bp and –80 bp of the rat BNP promoter totally abolished p38 induced
increase in promoter activity. Cotransfection with a plasmid expressing either MEK1 or MEKK1 induced both the BNP and the mutated constructs similarly.

Fig. 8. p38 MAP kinase regulation of a luciferase linked 534bp rat BNP promoter with GATA binding sites intact or mutated (Gmut). The results are mean ± SEM. *P < 0.05 compared with basal -534BNP promoter activity, #P < 0.05 compared with p38 induced -534BNP promoter activity.

5.3 PKCα in the regulation of cardiomyocyte hypertrophy (III)

5.3.1 Antisense inhibition of PKCα

Transfecting cardiac myocytes has been found difficult compared to many other cell types. In the current study, three different cationic lipids were studied to optimize the transfection method and to ensure that the lipid treatment did not interfere with the results. In the experiments, highest transfection efficiency was achieved with FuGENE 6, which also showed least toxicity analyzed with microscope imaging of the cells. To examine the transfection efficiency of the antisense PKCα ODNs using the cationic liposome delivery system, fluorescent-tagged ODNs (500 nM) corresponding to PKCα were added with FuGENE 6, and the cells were subjected to confocal microscopy. Increased accumulation of fluorescein-tagged particles inside the myocytes revealed that both antisense and scrambled ODNs were effectively delivered into the cells with FuGENE 6. The proportion of fluorescent-tagged ODN transfected myocytes in the culture was ~60–80%. The strongest fluorescence was seen in the areas around the nucleus. On the second day after the transfection, fluorescence staining was decreased, but still substantial. To verify that the results were not influenced by the lipid treatment, the experiments were repeated using also DOTAP and TFX-50 as lipids. The most effective transfection ratio of ODN and FuGENE 6 was 1:2, whereas the most effective ratios for both DOTAP and TFX-50 were 2:3 (ODN:l lipid). The transfection efficiencies with DOTAP and TFX-50 were considerably lower than with FuGENE6.
light microscope imaging of the cells revealed striking morphological changes in DOTAP treated cells. In the absence of cationic liposome carrier, only minimal transfection efficiency was observed.

Several ODNs, each 20 bases in length, were designed to hybridize to different regions of rat PKCα mRNA. Treatment of myocytes with the PKCα antisense ODN (0.5 µM) targeted to 3′untranslated sequence (As1) on rat PKCα cDNA reduced PKCα mRNA levels by 50% compared with scrambled ODN. The levels of 18S RNA were unaffected by the PKCα antisense ODN treatment. In agreement with changes in PKCα mRNA levels, treatment with antisense PKCα ODNs reduced PKCα protein levels by more than 60%. Dose response studies revealed that maximal reduction in PKCα protein levels was achieved at 0.5 µM, whereas control ODN with the same base composition as the antisense ODN but with a scrambled sequence had no effect. Western blot analysis with antibodies selective to PKCδ, PKCε, PKCζ, and PKCβIII showed that antisense PKCα treatment had no effect on other PKC isoforms. In untreated control cells PKCα staining was diffuse around the cell, and treatment with PE (100 µM) for 5 minutes translocated PKCα to the perinuclear region. In antisense PKCα ODN treated cells, fluorescence staining for PKCα was dramatically decreased, whereas in control ODN treated cells a significant amount of PKCα was seen around the nucleus.

PKC activity was studied by measuring the transfer of a phosphate group to a peptide substrate highly selective for protein kinase C. PE treatment evoked a 2.8-fold increase in PKC activity at 4 minutes, which was significantly inhibited with pretreatment of cells with PKCα antisense ODNs. A similar result was also seen at 30 minutes, although the response of PKC to PE had already decreased markedly. The two other antisense PKCα sequences targeting to 5′ (As2) and coding (As3) sequence of the rat PKCα cDNA had no effect on the PE induced PKC activity.

5.3.2 Effect of antisense ODN inhibition of PKCα on hypertrophic phenotype

Expression of two hypertrophic genes, β-MHC and α-SkA, were measured to assess whether treatment of myocytes with PKCα antisense oligonucleotide was sufficient to influence cardiomyocyte hypertrophy. Treatment with PE for 48 hours induced a 1.6-fold increase in α-SkA and a 2.1-fold increase in β-MHC mRNA levels (Fig 9). Inhibition of PKCα was sufficient to inhibit α-SkA gene expression by 30%, whereas β-MHC gene expression was not affected (Fig. 9).

Effect of PKCα inhibition on PE induced calcineurin activity was determined next to examine whether PKCα is an upstream regulator of calcineurin. PE evoked a 4-fold induction in calcineurin activity (1.18 ± 0.12 vs 0.29 ± 0.08 µM PO₄ released). Transfecting the cells with PKCα antisense ODNs had no significant effect on calcineurin activity.

To examine the role of alpha subunit of PKC on PE induced ERK activity, an assay measuring transfer of phosphate by active ERK was used. While PE induced a rapid activation of ERK, antisense PKCα treatment did not have a significant effect on ERK
activity at 4 minutes or at 30 minutes. Interestingly, at 24 hours the PE induced increase in ERK activity was partially inhibited by antisense PKCα ODN treatment.

Fig. 9. Effect of antisense PKCα ODN treatment on α-SkA and β-MHC mRNA levels in cultured neonatal rat ventricular myocytes. Veh, vehicle; Scr, scrambled ODN. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.01 compared with untreated control cells, #P < 0.05 compared with scrambled ODN treated cells.

PE and ET-1 have been shown to increase intracellular calcium concentration transients in cultured neonatal cardiac myocytes (Eble et al. 1998, Furukawa et al. 1992). Using single-cell imaging of Fluo-3-loaded (10 µM) cultured cardiac myocytes, PE was found to induce a significant increase in intracellular calcium levels (218 ± 37%, p < 0.001). Antisense PKCα ODN treatment had no effect on PE or ET-induced calcium concentration transients or spontaneous myocyte beating rate.

To study the effect of PKCα inhibition on protein synthesis, incorporation of [3H] leucine incorporation into myocytes and total protein content of the myocytes were measured. Treatment of myocytes with PE for 24 hours induced a 2.7-fold increase in leucine incorporation and a 1.5-fold increase in total protein content. Treatment of cells with antisense PKCα ODNs had no effect on PE induced increase in protein synthesis or total protein content. In agreement with previous studies, ET-1 induced 2.5-fold increase in [3H] leucine incorporation, which was not affected by antisense PKCα ODN treatment. Neither did antisense PKCα ODN treatment have any effect on PE or ET-1 induced increase in myocyte size or myofilament organization.

5.3.3 Effect of antisense PKCα treatment on ET-1 and PE induced natriuretic peptide secretion and gene expression

Treatment of myocytes with antisense PKCα ODNs had no effect on basal ANP or BNP secretion. PE (100 µM) treatment induced an up to 32-fold and 15-fold increase in ANP and BNP release, associated with a 3.1-fold and 1.8-fold increase in ANP and BNP.
mRNA levels, respectively. Inhibition of PKCα with antisense ODNs significantly inhibited PE induced ANP release (Fig. 10), whereas it had no effect on PE induced BNP release (Fig. 10). Antisense treatment had no effect on PE induced increase in ANP or BNP mRNA levels.

To further characterize the significance of PKCα on hypertrophic signaling, effects of PKCα inhibition on ET-1 induced natriuretic peptide release and gene expression were also studied. ET-1 (100 nM) led to an up to 3-fold and 10-fold increase of ANP and BNP secretion, respectively. Interestingly, antisense inhibition of PKCα resulted in a marked decrease in both ET-1 induced ANP and BNP secretion (Fig. 10). Similarly to PE stimulus, inhibition of PKCα had no effect on ET induced increase in ANP or BNP mRNA levels.
Fig. 10. Effect of antisense PKCa (As1) or scrambled (Scr) ODN treatment on PE (A and B) or ET-1 (C and D) induced ANP and BNP secretion. Results are expressed as mean ± SEM. *P < 0.01, **P < 0.001 compared with untreated control cells, #P < 0.05, ##P < 0.001 compared with scrambled ODN treated cells.
5.4 Regulation of atrial gene expression by MAP kinases (IV)

5.4.1 Effect of MAPK inhibition on stretch induced natriuretic peptide gene expression and secretion

Adult rat heart was dissected and the left atrial auricle was attached to the end of a perfusion tube consisting of two lumens (Laine et al. 1994). The auricle was perfused with modified Krebs buffer for 30 minutes, thereafter the auricle was stretched by raising the end of the buffer outflow tube, and thereby increasing the pressure inside the auricle. p38 MAP kinase activity was increased to 3-fold following a 10-minute stretch. After a 30-minute stretch, the p38 MAPK activity had returned close to basal level. Stretch also induced a time-dependent increment in ERK activity that peaked at 10 minutes. Immunoblot analysis with anti-non-phosphospecific antibodies for p38 and ERK showed that comparable amounts of samples were quantitated in these experiments.

Stretching of atrial prepare for 90 minutes induced a 1.9-fold increase in BNP gene expression. Inhibition of p38 MAP kinase with SB 203580 (5 \( \mu \)M) resulted in a 30% decrease of both basal and stretch induced BNP gene expression (Fig. 11). Induction of the BNP gene expression in the stretched atria was accompanied by a 2.9-fold increase in atrial IR-BNP levels. Administration of p38 MAP kinase inhibitor SB 203580 (5 \( \mu \)M) resulted in a 40% decrease in BNP secretion. A 90 minute stretch was not sufficient to induce ANP gene expression, while a 3.2-fold increase was induced in ANP secretion. Inhibition of p38 with SB 203580 (5 \( \mu \)M) inhibited ANP secretion by 30% at the 8-minute time point. Administration of ERK inhibitor PD 98059 (5 \( \mu \)M) had no effect on ANP and BNP gene expression and secretion in the stretched atria.

![Figure 11](image.png)

**Fig. 11.** Effect of p38 MAP kinase inhibition on stretch induced increase in BNP mRNA levels. Results are expressed as mean ± SEM. **\( P < 0.001 \), *\( P < 0.01 \) compared with vehicle, #\( P < 0.05 \) compared with stretch.**
5.4.2 Effect of atrial stretch on cardiac gene expression

The increase in atrial stretch resulted in a transient increase in c-fos mRNA levels, which was not significant at the 90-minute time point (Fig. 12). Inhibition of p38 MAP kinase by SB 203580 (5 µM) decreased basal c-fos gene expression by 42% and in the stretched auricle, gene expression was attenuated by 36% (Fig. 12). β-MHC mRNA levels, in turn, were elevated by 73% in response to increased stretch. p38 inhibition by SB 203580 (5 µM) was sufficient to decrease the stretch induced increase in β-MHC mRNA levels by 60% (Fig. 12). Interestingly, p38 inhibition induced a slight, but not significant increase in β-MHC mRNA levels in non-stretched preparations (Fig. 12).

Fig. 12. Effect of p38 MAP kinase inhibition on c-fos and β-MHC gene expression in perfused rat atria. Results are expressed as mean ± SEM. **P < 0.001, compared with vehicle, #P < 0.05 compared with stretch.

5.4.3 Effect of stretch on transcription factors

Transcription factor Elk-1 has been shown to participate in the regulation of GPCR agonist induced c-fos gene expression (Babu et al. 2000). Therefore, possible involvement of transcription factor Elk-1 in stretch activated gene expression was also studied. Stretching of the auricle was found to induce an increase in serine (ser-383) phosphorylation of Elk-1. The increase in phosphorylation was already detectable after 5 minutes’ stretching and persisted until 15 minutes. Inhibition of p38 MAP kinase by SB 203580 (5 µM) significantly augmented the stretch induced serine phosphorylation of Elk-1. To examine whether increased serine phosphorylation also resulted in functional changes in Elk-1, gel mobility shift analysis was used to measure Elk-1 binding on EBS in rat BNP promoter (Pikkarainen et al. 2003). Similarly to Elk-1 phosphorylation, stretching of the auricle induced a rapid increase in Elk-1 binding to BNP promoter persisting until 30 minutes. At the end of the 90-minute experiment, Elk-1 binding had returned to basal levels. In accordance with Elk-1 phosphorylation, p38 MAP kinase inhibition with SB 203580 (5 µM) resulted in a significant decrease in Elk-1 binding to
BNP promoter. Inhibition of ERK with PD 98059 (5 μM) had no effect on Elk-1 binding activity. Stretch also induced a 35% increase in HIF-1α mRNA levels, which was totally abolished by administration of both PD 98059 (5 μM) and SB 203580 (5 μM).

5.4.4 Regulation of BNP gene expression via EBS

To further characterize the significance of Elk-1 binding on the BNP promoter, we introduced cultured atrial myocytes with a 534 bp BNP promoter construct with Elk-1 binding site mutated at -498 bp. Stretching of the myocytes for 24 hours activated the proximal rBNP construct, and the activation was inhibited by the mutation of EBS (Fig. 13). Basal rBNP promoter activity was not significantly affected by mutation of EBS.

Fig. 13. Effect of ETS binding site mutation on basal and stretch induced proximal (-534 bp) rat BNP promoter activity. Results are expressed as mean ± SEM. *P < 0.001, compared with basal -534BNP promoter activity, #P < 0.05 compared with stretch induced -534BNP promoter activity.
6 Discussion

6.1 Role of GATA4 in the regulation of myocyte hypertrophy

GATA4 transcription factor has been shown to play an important role in cardiac development and in the regulation of cardiac-specific gene expression (Orkin 1995, Charron & Nemer 1999). The six GATA members can be divided into two subfamilies; GATA1, -2 and -3, which are expressed in hematopoietic cells, and GATA4, -5 and -6, which are expressed in various tissues, including the heart. Binding motifs for GATA-factors have been identified within the promoters of most cardiac-expressed genes. Adenoviral overexpression of GATA4 enhanced sarcomeric organization, induced an increase in cell surface area and induced a significant increase in total protein accumulation in cultured neonatal cardiac myocytes (Liang et al. 2001a). Transgenic mice with 2.5-fold overexpression of GATA4 in the adult heart demonstrated an increase in heart to body weight ratio, histological features of cardiomyopathy, and activation of hypertrophic genes, such as ANP, BNP and α-SkA (Liang et al. 2001a).

It has been recently shown that pressure overload and hypertrophic agonists regulate gene expression via GATA4-dependent mechanism (Hautala et al. 2001). Hypertrophic agonists activate GATA4-dependent gene expression by increasing GATA4 binding or transactivating activity (Liang & Molkentin 2002). To assess whether GATA4 binding to DNA was sufficient and necessary for the development of hypertrophic phenotype and hypertrophic gene expression, we employed a novel decoy mechanism using GATA binding decoy oligonucleotides to block GATA4 DNA binding.

GATA decoy ODNs were transfected to cardiac myocytes with high efficiency compared to previous studies (I). Gel mobility shift analysis showed that GATA decoy ODNs efficiently blocked both basal and ET-1 induced GATA4 binding to BNP promoter. Blockade of GATA4 DNA binding resulted in a significant decrease of basal ANP and BNP secretion and gene expression. Surprisingly, ET-1 induced secretion and gene expression of both ANP and BNP remained elevated. Previously, GATA4 depletion by antisense strategy has been shown to downregulate baseline secretion of ANP to a similar extent (Charron et al. 1999). The decoy strategy used here not only blocks GATA4 binding to the promoter, but also prevents possible compensation by increased binding
activity of GATA5 and GATA6. This is a clear benefit over GATA4 antisense strategy, since it has been reported that GATA6 mRNA levels were increased in GATA4 deficient mice as a potential compensatory mechanism (Molkentin et al. 1997, Kuo et al. 1997). To study further the significance of GATA binding sites within the BNP promoter, a 538 bp BNP promoter luciferase construct was studied. The results were in accordance with those revealed by the decoy strategy. Mutation of two proximal GATA binding sites in the BNP promoter decreased basal BNP transcription activity, but it had no effect on ET-1 induced increase in promoter activity.

ET-1 treated cardiac myocytes exhibited a marked increase in sarcomeric assembly and protein synthesis, as seen in hypertrophied heart in vivo (Hunter & Chien 1999). Blockade of GATA4 binding by GATA decoy ODNs was not sufficient to prevent these changes. Adenoviral overexpression of GATA4 was previously shown to enhance sarcomeric organization and increase total protein accumulation (Liang et al. 2001a). This implies that GATA4 may participate in hypertrophic gene expression without direct binding to a gene promoter, probably by interacting with other transcription factors present in the nucleus, such as Ncx2.5, Nfat3, FOG-2 and MEF2. Results of the current study (I) demonstrate that blockade of GATA4 binding to a gene promoter has a specific effect on basal cardiac natriuretic peptide gene expression, but it does not compromise ET-1 induced natriuretic peptide gene expression. This suggests that direct interactions of GATA4 with GATA motifs within cardiac gene promoters are not required for ET-1 induced promoter activity. Other characteristics of hypertrophic phenotype induced by ET-1, such as sarcomeric organization and increased protein synthesis, do not appear to require direct interaction of GATA4 with gene promoters, either.

6.2 Distinct roles of MAP kinases in the regulation of myocyte hypertrophy

MAP kinases, ERK, JNK and p38, have been shown to regulate a broad range of biological functions in response to extracellular stimuli (Sugden & Clerk 1998b). Each MAP kinase pathway is a multistep cascade facilitating the amplification of the signal. On the other hand, MAP kinases are known to cross at multiple levels, leading easily to misinterpretation when studying the MAP kinases. There is an increasing body of evidence implicating involvement of GATA4 in the hypertrophic signaling in cardiac myocytes (Hasegawa et al. 1997, Molkentin et al. 1998, Morimoto et al. 2000, Liang et al. 2001a). GATA4 transcription factor has been shown to be a target for phosphorylation by ERK in cardiac myocytes (Morimoto et al. 2000). In fact, GATA4 protein has 7 serine residues, which are potentially phosphorylated by MAP kinases. The aim of the present study (II) was to examine the role of MAP kinase signaling in the development of hypertrophic phenotype induced by ET-1 in cardiac myocytes.

ET-1 induced a rapid activation of ERK and p38 MAP kinase, which were totally abolished by pretreatment of cells with specific inhibitors, PD 98059 and SB 203580, respectively (Clerk et al. 1998b). ET-1 induced de novo protein synthesis of neonatal rat ventricular myocytes was also inhibited by pharmacological blockade of p38 MAP
kinase, but not with blockade of ERK signaling. These results are rather different compared to a study which had previously suggested that p38 inhibition has no effect on ET-1 induced protein synthesis (Choukroun et al. 1998). Another interesting finding in the study (II) was the induction of p38 activity by ERK inhibitor PD 98059. Previously, a high dose of PD 98059 (50 µM) has been shown to increase basal levels of phosphorylated p38 MAP kinase (Clerk et al. 1998b). In turn, activation of ERK pathway by constitutive active MEK1 (an upstream kinase of ERK pathway) has been shown to inhibit p38 MAP kinase activity and p38 induced phosphorylation of TATA-binding protein (Carter & Hunninghake 2000). The inhibition was suggested to be mediated by MKP-1, which has been shown to block ET-1 induced activation of the MAP kinases (Carter & Hunninghake 2000, Bueno et al. 2001). Since ERK is necessary for the stimulation of MKP-1 mRNA expression, the blockade of ERK for 24 hours in the present experiments is likely to inhibit MKP-1 expression, and thus result in increased p38 activity (Haneda et al. 1999). Hypertrophic agonists are known to activate MKP-1, thereby explaining the lack of additive effect with PD 98059 on ET-1 induced p38 activity. Another mechanism involved may be the substrate specificity of MKP-1, since it has been shown to preferentially block the activation of p38 MAP kinase (Franklin & Kraft 1997).

A major finding in this study (II) was the differential regulation of GATA4 binding activity by MAP kinases. Blockade of ERK pathway led to decreased phosphorylation of serine residues in GATA4 and decreased basal binding activity, but it had no effect on ET-1 induced increase in GATA4 DNA binding. ERK overexpression led to phosphorylation of the serine residues of GATA4 protein, but it was not sufficient to increase GATA4 binding to BNP gene. Blockade of p38 pathway similarly decreased phosphorylation of serine residues in GATA4, and in contrast to ERK inhibition, totally abolished ET-1 induced GATA4 binding to BNP gene. It is noticeable that p38 overexpression not only phosphorylated serine residues in GATA4 protein, but also increased GATA4 binding to BNP promoter. The p38 MAP kinase was also shown to induce BNP promoter activity in GATA4 dependent manner, since mutation of two proximal GATA binding sites in the BNP promoter abolished p38 induced BNP promoter activity. Previously, these GATA binding sites have been shown to direct cardiac myocyte specific expression of rat BNP promoter and regulate basal promoter activity (Grepin et al. 1994, Thuerauf et al. 1994). Our results also revealed that overexpression of ERK or JNK pathway members also induced BNP promoter activation, but independently of GATA binding.

The present results show that blockade of p38 MAP kinase pathway abolishes hypertrophic agonist induced GATA4 binding to BNP gene, whereas inhibition of ERK pathway only disrupts GATA4 binding activity in nonstimulated myocytes. Inhibition of GATA4 binding also results in functional changes in BNP promoter activity. Taken together, results of the study (II) demonstrate that activation of p38 MAP kinase is necessary for hypertrophic agonist induced GATA4 binding to BNP gene and sufficient for GATA-dependent BNP gene expression.
6.3 PKCα signaling in myocyte hypertrophy

There are several previous studies indicating the involvement of PKC in the development of cardiac hypertrophy (Shubeta et al. 1992, Clerk et al. 1994, Sugden & Clerk 1998b, Braz et al. 2002), yet there are only limited data concerning the precise roles of different PKC isozymes in hypertrophic signaling. Translocation of PKC isozymes by pressure overload has provided indirect evidence of PKC participation in hypertrophic signaling. The objective of this study (III) was to evaluate the significance of PKCα in cardiomyocyte hypertrophy. Due to the lack of specific pharmacological inhibitors, studies concerning the role of different isozymes of PKC in the development of cardiac hypertrophy have been accomplished by using antisense strategy or dominant negative constructs (Sugden & Clerk 1998b, Molkentin & Dorn II 2001, Strait, III et al. 2001).

In the current study (III), antisense oligonucleotide targeting 3'-untranslated sequence on the rat PKCα cDNA was most effective in inhibiting PKCα protein synthesis, which is similar to findings in previous studies using antisense PKCα ODNs (Benimetskaya et al. 2001, Dean et al. 1994). Other cardiac isozymes of PKC were not affected by antisense PKCα treatment approving the specificity of the antisense. The PKC activity assay also revealed a significant decrease in agonist induced PKC activity in PKCα antisense ODN treated cells. Antisense PKCα treatment had no effect on PKC activity in resting cells, which is probably due to low basal PKC activity. PKCα protein expression was also studied by cytochemistry, which showed significantly decreased levels of PKCα in antisense PKCα treated cardiac myocytes.

Cardiac hypertrophy is associated with an increase in cell size and sarcomeric organization. In the present (III) study, inhibition of PKCα with antisense ODNs was not sufficient to prevent PE induced increase in cell size or sarcomeric organization. Interestingly, antisense PKCα treatment significantly decreased PE induced gene expression of sarcomeric protein α-SkA. It is therefore intriguing to speculate that a longer experiment time could well reveal attenuation in sarcomere assembly. In fact, a recent study using dominant negative form of PKCα showed a marked attenuation in PE induced increase in sarcomeric organization and cell surface area (Braz et al. 2002). Contrary to the present results, inhibition of PKCα with a dominant negative adenovirus also showed a marked decrease in PE induced leucine incorporation. The reason for these contradicting results may be the longer lasting effect on PKCα protein levels produced by dominant negative PKCα compared to antisense PKCα treatment.

Treatment of cardiac myocytes with dominant negative PKCα also resulted in a marked decrease in PE induced ANP staining in cardiac myocytes. In the current study (III), PE and ET-1 both produced a drastic increase in ANP and BNP secretion and gene expression. Antisense inhibition of PKCα was sufficient to decrease PE induced ANP secretion and ET-1 induced secretion of both ANP and BNP. Another interesting finding was that antisense PKCα treatment had no effect on PE or ET-1 induced increase in ANP or BNP mRNA levels. Cellular mechanisms regulating natriuretic peptide secretion are not fully understood, but there is a previous study suggesting that ANP secretion from atrial myocytes is stimulated both by the increase in calcium levels and activation of PKC (Suzuki et al. 1992). BNP secretion, in turn, is augmented by the activation of PKC, rather than the elevation in intracellular calcium levels (Suzuki et al. 1992).
Interconnection of PKCα with other signaling pathways was also studied. As previously reported, we found ERK as a downstream target for PKCα (Sugden & Clerk 1998b, Braz et al. 2002). There are also previous data demonstrating that activation of calcineurin signaling increases α-SkA mRNA levels. In the current study (III), PE induced a significant activation of calcineurin, which was not affected by inhibition of PKCα. Therefore it is more likely that calcineurin is an upstream regulator of PKC, as also previously suggested (De Windt et al. 2000a).

6.4 MAPK regulation of stretch induced cardiac gene expression

Multiple signaling pathways are involved in the regulation of cardiac gene expression during hemodynamic load, among them PKC, MAP kinases, calcineurin and intracellular calcium (Sugden & Clerk 1998b, Hunter & Chien 1999, Nicol et al. 2000, Molkentin & Dorn II 2001). Cardiomyocyte stretch is known to result in increased activity of several nuclear effectors leading to transcriptional activation of pattern of genes including structural proteins, such as α-SkA and β-MHC, and natriuretic peptides, ANP and BNP (Sadoshima & Izumo 1997). The factors transducing the signal from the cell membrane to the nucleus are not well characterized. The results of this study (IV) suggest that stretch induced activation of c-fos, β-MHC and BNP gene expression is regulated by p38 MAP kinase and transcription factor Elk-1 in the perfused atria.

Hemodynamic stress induces activation of ERK and p38 MAP kinase cascades and their downstream effectors in cardiac myocytes (Liang et al. 2000, Sugden 2001). In the current study, atrial stretch provoked a rapid activation of ERK and p38 MAP kinase cascades. Transcription factor Elk-1 represents a known target for MAP kinases. In fact, there is evidence that Elk-1 is activated by ERK, JNK and p38 MAPK pathways (Treisman 1996, Yang et al. 2001). Interestingly, each of the promoters, c-fos, β-MHC and BNP, possesses an ETS-binding domain. Previously, Elk-1 together with SRF has been shown to regulate c-fos gene expression (Aoyagi & Izumo 1993). In fact, minimal c-fos promoter with a point mutation at the p62TCF binding site of the SRE failed to respond to pressure overload (Aoyagi & Izumo 1993). In addition to synergic mechanism with SRF, ETS domain transcription factors have also been shown to act without adjacent SRE in the promoter to regulate cardiac gene expression (Gupta et al. 1998). Recently, p38 MAP kinase induced Elk-1 binding on EBS within the proximal rat BNP promoter was shown to mediate ET-1 induced BNP gene expression in cultured neonatal rat ventricular myocytes (Pikkarainen et al. 2003).

In the current study, atrial stretch induced phosphorylation of Elk-1 and increased Elk-1 binding activity on the rat BNP promoter. Inhibition of p38 MAP kinase, but not ERK, decreased stretch induced Elk-1 phosphorylation and Elk-1 DNA binding activity. To further characterize the significance of Elk-1 binding on the BNP promoter, cultured atrial myocytes were transfected with a proximal rat BNP promoter construct with the EBS site mutated. 24-hour cyclic stretch was sufficient to induce a 2.9-fold increase in the activity of intact BNP promoter. Mutation of EBS at -498 significantly decreased stretch induced BNP promoter activity. Previously, mechanical load induced BNP gene
expression has been shown to converge via ET-1 dependent mechanism in the atria, but not in the ventricle (Magga et al. 1997). Together with the current findings, this proposes a novel mechanism regulating atrial BNP gene expression in response to increased load.

6.5 Future perspectives

Findings of the current studies show that multiple signaling pathways are involved in hemodynamic load induced cardiac gene expression, and further help to define the roles and interrelationships among the regulatory pathways (Fig. 14). While the methods used here may prove useful in future studies, certain reservations need to be taken into consideration. Decoy oligonucleotides, which bind to DNA binding site of a transcription factor, are useful for studying the significance of a transcription factor binding to target genes, but still allow the transcription factor to control gene expression via interactions with other nuclear effectors. Antisense ODNs hybridizing to target mRNA, as well as decoy ODNs, have short half-lifes and their transfection efficiency may prove insufficient. Instead, post-transcriptional gene silencing by RNA interference would be useful for studying the roles of specific genes. Overexpression of MAPK pathway members could also be enhanced by simultaneous overexpression of a constitutively active upstream kinase, i.e. coexpression of constitutively active MKK3 with wild type p38α. However, overexpression of a specific kinase may result in a shortage of scaffold proteins assembling the kinase pathway, and therefore allow unwanted leaking of the signal to other kinase pathways. It is to be expected that additional experimental tools, such as usage of small interfering RNAs and adenoviral vectors expressing different kinases, will allow for cardiac gene regulation to be more fully explored.

Fig. 14. A schematic view of the results of current studies defining the roles of cellular signaling elements contributing to hemodynamic load induced cardiac gene expression.
7 Summary and conclusions

Cardiac hypertrophy is seen in both physiological and pathophysiological conditions. Myocardial infarction, essential hypertension, chronic heart failure and cardiomyopathies are associated with increased cardiac pressure and/or volume overload. Prolonged overload leads to pathologic cardiac hypertrophy, which involves changes at the level of gene transcription, protein synthesis and myofibrillar organization.

There are numerous intracellular signaling events contributing to hypertrophic gene expression. Direct myocyte stretch and GPCR agonists induce activation of intracellular signaling cascades, such as PKC and MAP kinase pathways, which ultimately affects activation of transcription factors, including GATA4 and Elk-1, and induces hypertrophic growth programme of individual myocytes.

1. GATA4 transcription factor is required for normal cardiac development. However, it is unknown whether GATA4 is an essential mediator of hypertrophic responses in the heart. The present study demonstrated that GATA decoy treatment efficiently blocks ET-1 induced GATA4 binding to BNP gene promoter. GATA decoy treatment significantly decreased basal ANP and BNP secretion and gene expression, but it had no effect on ET-1 induced natriuretic peptide secretion or gene expression. Nor did the depletion of GATA binding have any effect on ET-1 induced protein synthesis or sarcomeric organization. The present results emphasize that GATA4 DNA binding is not necessary for ET-1 induced development of hypertrophic phenotype.

2. All three MAPK pathways, ERK, JNK and p38, are activated by ET-1 in cultured cardiac myocytes. Pharmacologic inhibition of p38 MAP kinase attenuated ET-1 induced GATA4 binding to BNP gene promoter and serine phosphorylation of GATA4. Inhibition of ERK cascade with MEK1 inhibitor PD 98059 reduced basal and p38 induced GATA4 binding activity, but it had no significant effect on ET-1 induced GATA4 binding activity. Overexpression of p38 MAPK pathway, but not ERK or JNK pathways, activated GATA4 binding to BNP gene promoter and induced rat BNP promoter activity via proximal GATA binding sites. These findings demonstrate that activation of p38 MAPK is necessary for hypertrophic agonist induced GATA4 binding to BNP gene and sufficient for GATA-dependent BNP gene expression.
3. ET-1 and PE induce activation of PKCα in cultured cardiac myocytes. Antisense PKCα treatment reduced PKCα mRNA and protein levels and reduced PE induced PKC activity. Inhibition of PKCα was sufficient to inhibit α-SkA gene expression, but had no effect on β-MHC gene expression. Inhibition of PKCα with antisense ODNs significantly inhibited PE induced ANP release, and ET-1 induced release of both ANP and BNP. Antisense PKCα treatment had no effect on agonist induced increase of ANP and BNP mRNA levels or on ET-1 or PE induced protein synthesis or sarcomeric organization. These results suggest that PKCα isozyrne is involved in hypertrophic signaling in cultured cardiac myocytes, but has a minor role in the development of hypertrophic phenotype.

4. Isolated atrial preparate was used as a model to study the role of ERK and p38 MAP kinase in stretch induced cardiac gene expression. p38 MAP kinase, but not ERK, regulated stretch induced transcription factor Elk-1 phosphorylation and DNA binding activity. Atrial stretch also induced an increase in HIF-1α mRNA levels, which was attenuated by inhibition of both ERK and p38 MAPK. Administration of p38 inhibitor also decreased atrial c-fos, β-MHC and BNP gene expression. Studies with cultured rat atrial myocytes demonstrated that ETS binding site within the rat BNP promoter was necessary to confer full BNP promoter activation in response to increased stretch. These findings demonstrate that stretch induced BNP gene expression is regulated by p38 MAP kinase mediated activation of transcription factor Elk-1.
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