CELLULAR PATHWAYS OF ANGIOTENSIN II
SIGNALLING IN THE
HYPERTROPHIC MYOCARDIUM

by

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Date Dec. 10, 1992
ABSTRACT

The general subject of these studies is the cellular action of angiotensin II (ATII) on the hypertrophic myocardium. The significance of this work is both biological and clinical: biological, because ATII has direct effects on the normal heart muscle and the intracellular signalling pathways activated by ATII are not fully understood. Clinical, because ventricular hypertrophy is a strong predictor of mortality in humans and ATII has been implicated in the process of hypertrophic development. The present work was based on the hypothesis that distinct signal transduction pathways are activated by ATII in the hypertrophic heart, as compared to the normal heart, and that these are identifiable in the heart of the Dahl salt-sensitive rat. The aim of the present study was to determine ATII intra-cellular signalling of the hypertrophic cardiac myocyte. Ventricular hypertrophy in the Dahl SS/Jr (Dahl S) rat was dependent upon the time on 6% NaCl diet. This was confirmed to be partly based on differences in viable myocyte size as determined by Fluorescence Activated Cell Sorting analysis. Dahl SR/Jr (Dahl R) rats showed no cardiac hypertrophy after identical treatment. Isolated ventricular myocytes from normal Dahl R rats and hypertrophic Dahl S rats were probed along the adenylyl cyclase-cAMP axis for effects induced by ATII. ATII induced a concentration and time-dependent inhibition of beta receptor stimulated increase in cAMP concentration in Dahl R ventricular myocytes. However, in hypertrophic Dahl S ventricular myocytes ATII induced a significant enhancement of beta receptor stimulated increase in cAMP concentration. This reversal was evident by stimulating adenylyl cyclase directly with forskolin, and was characterized as being dependent upon cAMP generation, not degradation, as well as displaying pertussis toxin sensitivity. The enhancement of stimulation of cAMP was linked to the degree of ventricular hypertrophy. GTP binding protein Gs alpha and Gi alpha subunits were assessed by ADP ribosylation, and immunodetection of Gs alpha. A diminution in the concentrations of these proteins in the hypertrophic Dahl S ventricular myocytes in comparison to Dahl R ventricular myocytes, was observed. Furthermore, the reversal of cAMP inhibition by ATII was mimicked by carbachol, a muscarinic receptor agonist, indicating that the reversal is likely independent of ATII receptor differences between hypertrophic and normal myocytes. This reversal was blocked by the down regulation of protein kinase C. The basis of these effects was further probed by comparing phosphorylation of hypertrophic Dahl S with Dahl R myocyte in response to ATII.
The phosphorylation of a 42 kDa protein was induced by ATII, as well as the phorbol ester, phorbol 12-myristyl 13-acetate (PMA), in the Dahl R and Dahl S cardiac myocyte. Hypertrophic Dahl S myocytes displayed greater phosphorylation of pp42 induced by ATII than Dahl R myocytes, although the converse was observed with PMA stimulation, namely greater PMA sensitivity in Dahl R. Examination of mitogen activated protein kinase (MAP) activation by ATII revealed that ATII regulates activity of p44erk and p42mapk in ventricle. In Dahl R ATII stimulates these MAP kinases, however, in hypertrophic Dahl S ATII inhibits their activity. Both of these effects were mimicked by PMA, and the effects blocked by the protein kinase inhibitor Compound 3. Protein kinase C activity assays verified that ATII signalling follows this route in the ventricle, and indicates possible divergences in this component of the pathway as well. In conclusion, firstly, the Dahl rat model of cardiac hypertrophy provided novel information about signal transduction differences between hypertrophic and normal myocardiocytes. Secondly, potentially important differences in ATII signal transduction have been characterized. These were evident in the adenylyl cyclase activation system, phosphorylation of cellular proteins, and the activations of MAP kinase and protein kinase C.
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<td>ACE</td>
<td>Angiotensin converting enzyme</td>
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<td>ATI</td>
<td>Angiotensin I</td>
</tr>
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<td>ATII</td>
<td>Angiotensin II</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine 3',5' monophosphate</td>
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<td>DAG</td>
<td>1,2 diacylglycerol</td>
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<td>Dahl S</td>
<td>Dahl SS/Jr inbred hypertension sensitive rat</td>
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<tr>
<td>Dahl R</td>
<td>Dahl SR/Jr inbred hypertension resistant rat</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modification of Eagle Medium</td>
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<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
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<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
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<td>G protein</td>
<td>GTP binding protein</td>
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<td>LV</td>
<td>Left ventricle</td>
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<td>LVH</td>
<td>Left ventricular hypertrophy</td>
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<td>MAP</td>
<td>Mitogen activated protein</td>
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<td>PMA</td>
<td>Phorbol 12-myristyl 13-acetate</td>
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<tr>
<td>RAS</td>
<td>Renin-Angiotensin System</td>
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<td>SDS</td>
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Lastly, I wish to thank my family, Nyla and Ntompizani, for their enduring support and patience.
CHAPTER I
INTRODUCTION

1. DEFINITIONS: WHAT IS VENTRICULAR HYPERTROPHY?

"Ventricular hypertrophy" is defined as an increase in myocardial mass. This can be secondary to volume (preload) overload, arterial pressure (afterload) overload, or a combination of these two factors. It can also occur without apparent cause, which is then referred to as being idiopathic.

The pattern of ventricular expansion is dependent upon these factors. On one hand increased preload is associated with high left ventricular volume, which results in left ventricular dilatation. The resulting hypertrophy is referred to as eccentric hypertrophy and occurs in high cardiac output states such as obesity, endurance training, or with regurgitant valvular lesions (such as mitral or aortic regurgitation). Increased afterload, on the other hand, is not associated with left ventricular dilatation, but results in the decrease in the left ventricular radius to posterior wall thickness ratio (Gaash, 1979; Devereux and Reichek, 1980) which is known as concentric hypertrophy. Although these categories are useful as aids in understanding ventricular hypertrophy, they are not mutually exclusive. Clinically, combinations of concentric and eccentric hypertrophy are well known to occur (Messerli, 1983).
2. CLINICAL SIGNIFICANCE OF VENTRICULAR HYPERTROPHY

Left ventricular hypertrophy (LVH) is assessed in human subjects by a number of means including:

- autopsical measurement of weight of the ventricles, which is standardized for total body weight or body surface area (Bove et al., 1966);
- radiographic measurement of heart size, which is adjusted for chest size (Ehstrom, 1948);
- electrocardiographic determinations of QRS deflections, which reflect ventricular mass (Mazzolini 1964); and
- echocardiographic determinations of ventricular mass, which relies on mathematical calculation from measurement of left ventricular (LV) posterior and septal wall thickness.

Electrocardiographic patterns of LVH have long been known as strong predictors of morbidity and mortality independent of blood pressure, age and other risk factors. Echocardiography has confirmed and improved upon electocardiographic studies in showing that LV mass is a potent predictor of cardiovascular morbidity. (Casale et al., 1986; Isles et al., 1987; Levy et al., 1988). In a 5 year follow-up study involving 140 men and echocardiographically-determined LVH, there was a four-fold increase in cardiovascular morbid events (cardiac death, myocardial infarction, stroke, angina pectoris requiring coronary bypass surgery) in the LVH population over those without. Multivariate analysis indicated that LV mass was a stronger independent predictor of cardiovascular morbidity than blood pressure or age (Casale et al., 1986). Other studies have supported and added to these conclusions. Analysis of echocardiographic results of the Framingham study suggests that LV mass is the strongest predictor of cardiovascular morbidity in unselected middle-aged adults (Levy et al., 1988).

These studies suggest that increases in myocardial mass predispose to complications, particularly myocardial ischemia and cardiac arrhythmias.
a) **Myocardial Ischemia**

LV mass and myocardial wall tension are important factors in determining myocardial oxygen consumption. The oxygen requirement of the hypertrophic ventricle is increased. This results in a decrease in coronary reserve (which is the capacity for increased coronary flow in response to increased oxygen demand) by as much as one third (Strauer, 1980). This may be due to an imbalance between muscle mass and the vascular bed as muscle mass increases. Furthermore, the effect is exacerbated by the high arterial pressure and high ventricular wall stress that characterizes hypertension and which often accompanies LVH (Marcus, 1983). This situation is likely reflected in one consequence of ischemia, which is myocardial infarction. Thus, there is a positive relationship between the extent of myocardial infarction associated with sudden death, and LVH (Koyanagi et al., 1982).

b) **Electrophysiological Abnormalities**

Patients with LVH have been documented to be at a greater risk of sudden death than those with normal myocardium (Marcus, 1983). An important mechanism by which LVH predisposes to cardiac morbidity is by inducing ectopic impulses (Messerli et al., 1984) and arrhythmias (Anderson et al., 1983; McLenachan et al., 1987). In some studies, however, no difference was found in the incidence of arrhythmias between patients with essential hypertension and those with normal blood pressure (Messerli et al., 1982). This would suggest that LVH, rather than hypertension, predicts for arrhythmias.

c) **Performance of the Hypertrophic Ventricle**

The course of development of cardiac hypertrophy in response to acute overload has been considered by Meerson (1961) to involve three distinct phases. The utilization of this hypothesis to analyze cardiac hypertrophy, regardless of etiology, is illustrative. During the first of these phases after the onset of acute overload, is a period of "transient breakdown" which lasts several days and is characterized by acute heart failure, dilation of the left
ventricle, pulmonary congestion and low cardiac performance. As the hypertrophy process commences, a period of "stable hyperfunction" occurs in which the increased wall thickness of the ventricles increases cardiac output (leading to relief of pulmonary congestion). The last stage is the "exhaustion" stage during which cell death and fibrosis may lead eventually to heart failure.

Angiographic and echocardiographic techniques have made possible the study of the functional traits of the hypertrophied ventricle. It has become evident that ventricular hypertrophy has both beneficial and detrimental phases. As the myofibrils of the heart muscle develop additional sarcomeres, the muscle as a whole is able to assume greater loads. However, this is a short-lasting benefit since alterations in architecture of the heart, such as increased distance between capillaries (Roberts and Wearn, 1941), hamper the supply of oxygen and nutrients required for energy production. This energy deficit is made worse by the increased number of ATP-utilizing myofibrils present in the hypertrophic heart (Wangler et al., 1982), which increase energy demand by the muscle. So the early stage of hypertrophic development, although producing enhanced contractile performance for a period, eventually degenerates and weakens, leading to drastically impaired contractile performance including slowed relaxation rate (Meerson et al., 1971).
3. BIOLOGICAL ASPECTS OF VENTRICULAR HYPERTROPHY

a) Cardiac Myocyte Development and Cardiac Morphogenesis

Myoblasts are derived from splanchnic mesoderm. In their early, proliferative stage, they possess no identifiable myocyte characteristics. As these cells approach the stage of overt myogenesis, they elongate and synthesize muscle specific proteins. This culminates in formation of visible cross-striations and contractile ability at which point they are considered as developing myocytes (Manasek, 1970). Cytodifferentiation is not synchronized in the heart so myoblasts and developing myocytes initially exist together. Eventually the cells transform to functioning myofibril-containing adult myocytes, unlike skeletal muscle which retains myogenic precursor cells (satellite cells) in the adult stage (Zak, 1974). Unlike skeletal muscle cells, cardiac muscle cells retain mitotic and cytokinetic ability at advanced stages of cytodifferentiation (Manasek, 1968). Whereas skeletal muscle form structural syncytia of multinucleate myofibres, cardiac tissue forms low-resistance junctions between myocytes, which are considered functional syncytia.

At the onset of cardiac morphogenesis in the embryonic chick, the primitive heart tube consists of several layers of myocardial cells surrounding a layer of extracellular matrix and the inner endocardium. At this point there are no fibroblasts, vessels or conductive cells (Manasek, 1970). The tube then forms a loop and non-muscle cells invade the myocardium. Since the non-muscle cell fraction has a greater mitotic rate than the myocytes, they eventually outnumber myocytes (Manasek, 1970). These other cell types are fibroblasts, endothelial cells, nerve cells and phagocytes. Fibroblasts play a role in aiding myocyte glucose metabolism (Schroedl and Hartzell, 1983) and in providing elements of the extracellular matrix for maintaining myocyte morphology (Borg and Caulfield, 1979).

In the embryonic rat heart, beating commences 9-10 days after conception (Goss, 1938). By 15 days the heart responds to stimuli such as catecholamines (Adolph, 1965). In fact, sympathetic innervation may be involved in stimulation of the early stages of myocyte proliferation and differentiation (Bareis and Slotkin, 1978; Claycomb, 1976). So although the
nervous system is a key factor controlling myocyte proliferation, the decline of mitotic ability of these cells is not understood (Bishop, 1983).

Ventricular myocytes still possess mitotic activity at birth and for a few weeks thereafter (Goldstein et al., 1974). In rats, as mitosis declines to 2% at birth, a doubling of ventricular myocyte number occurs by the end of about 4 weeks post-partum (Zak, 1984). After this point mitotic ability is lost completely and heart growth is due to increases in cell size, which increases from about 5 um in length at birth to more than 30 um in the adult (Zak, 1984).

b) The Myocardiocyte of the Hypertrophic Ventricle

i. Morphological Alterations

Since there is limited capacity for myocardial hyperplasia after the first few weeks of life, the response to increased load demand is the enlargement of the myocardial cell as well as hyperplasia of non-myocyte cells under some conditions. The ventricular myocyte increases in length from 49.1 ± 7.1 in normals to 65.6 ± 3.7 um (± SEM) in rats as a result of aortic constriction (Anversa et al., 1980). It should be noted that the length of myocytes is dependent, in part, upon which area of the ventricle they are taken from.

The ability of the hypertrophic heart to achieve a compensatory level of function is due to reorganization of the contractile structures. This was reflected in sarcomere length increases, as well as the number of sarcomeres in series (Laks et al., 1974). Other electron micrographic studies have found that the number of parallel myofibrils also increases in the hypertrophic cell (Page and McAlister, 1973), thus giving a second dimension to the increase in force producing capacity and accounting for the observed increase in cross sectional area of the hypertrophic myocyte. This gives rise eventually to decreased surface area to volume ratio of the hypertrophic myocyte (Kaufmann et al., 1971). Other changes within the contractile elements are widened Z lines, which has been proposed to be due to sarcomerogenesis at this site (Legato, 1970; Schaper et al., 1974).
Similar studies using a number of models, also identified potential bases for the exhaustion seen in the latter stages of hypertrophy: firstly that the mitochondrial volume decreases concomitant with the increase in myofibrillar volume (Anversa et al, 1976; Kamereit and Jacob, 1979), and secondly that the number of capillaries per muscle cell remains constant in the face of increased cell mass (Rakusan, 1971).

ii. Electrophysiologic Abnormalities

Electrophysiological abnormalities include the prolongation of the action potential in the hypertrophic myocardium (Hemwall et al., 1984). This is the most prominent of the electrophysiological alterations which occur and may be due to a delay in the inactivation of open calcium channels (Keung, 1989; Allen et al., 1988).

iii. Biochemical Alterations

There are several isozymes of myosin in the ventricular myocardium, each with a characteristic ATPase activity: V1, a high ATPase activity isozyme consists of two alpha chains; V2, a heterodimer consists of one alpha and one beta chain with lower ATPase activity than V1; and V3, which is made up of two beta chains and has the lowest ATPase activity. These three isozymes can be identified using a series of monoclonal antibodies which specify a number of epitopes (Chizzonite et al., 1983). The ratio of V1/V3 in ventricle correlates not only with ATPase activity, but with the rate of force development and velocity of shortening (Pagani and Julian, 1984; Swynghedauw et al., 1983) and bears an inverse relationship with the energy economy of isometric active force maintenance (Alpert and Mulieri., 1983). An increase in V1 isozyme content in the myocardium occurs in response to hypertrophy due to exercise or volume overload but an increase in V3 content occurs with pressure overload (Litton et al., 1982; Zak, 1984).

Actin and tropomyosin, which are the products of multigene families, also undergo transformation in hypertrophy (Schwartz et al.,1986; Izumo et al., 1988)). These proteins, and perhaps others, revert to isoforms expressed during the fetal stage of development. This has been suggested to be due to the reversion of the myocyte from a state of slow protein
synthesis to the state of rapid protein synthesis seen earlier in ontogeny (Katz, 1990). The full implications of the expression of the neonatal isoforms of contractile elements on contractile function are not yet understood.

Changes in isoforms of other proteins have been reported. These include lactate dehydrogenase (Fox, 1971) creatine kinase (Meerson and Javich, 1982) and sarcolemmal sodium pump (Charlemagne et al., 1986).

iv. **G Protein Alterations**

Guanine triphosphate binding proteins (G proteins) are key components in the signal transduction pathway in myocardial cells, since they are associated with most, if not all, membrane-bound receptors (Fleming et al., 1992). The G proteins play a key role in the regulation of the myocardium since they link receptors to adenylyl cyclase, ion channels and phospholipid hydrolysis. As discussed (vide infra), the generation of cAMP via the activity of adenylyl cyclase controls the contractile capacity of the cardiomyocyte through a number of actions, and also may play a mediating role in macromolecular turnover associated with hypertrophy (Morgan and Baker, 1991). Thus, the activity of both stimulatory G proteins (Gs) and inhibitory G proteins (Gi), by governing adenylyl cyclase activity, are centrally located in the constellation of factors controlling the myocardium.

The G proteins are all heterotrimers consisting of alpha, beta and gamma subunits. The alpha subunit confers the specific functional characteristics of the holoprotein by virtue of structural heterogeneity. The beta and gamma subunits of the different functional classes of G proteins are relatively homogeneous. Characteristics that are specific to the alpha subunit are the GTP binding site and GTPase activity, which is activated by the presence of the beta-gamma complex (Gilman, 1987).

There are at least three types of G proteins which have been identified in cardiac tissue (Robishaw and Foster, 1989): Gs, Gi and Go.

In the heart, Gs acts as a mediator of adenylyl cyclase stimulation by the beta-1 and beta-2 receptors, and also mediates beta regulation of voltage-dependent ion channels, such
as the cardiac Ca\(^{2+}\) channel (Birnbaumer et al., 1990) by a mechanism which operates independent of cAMP changes (Trautwein and Hescheler, 1990). Gs-alpha exists as four isoforms which are encoded by a single gene and generated by alternative splicing of precursor RNA transcripts of the gene. They migrate on SDS gel electrophoresis as two bands of molecular weight 42-45 kDa and 46-52 kDa and differ by 15 amino acids (Bray et al., 1986). In the adult rat ventricle, the major species of Gs are the 45 to 47 kDa isoforms (Murakami and Yasuda, 1986).

Gi acts to mediate the inhibition of adenylyl cyclase activity via muscarinic (Katada et al., 1984), alpha-adrenergic (Brown et al., 1985), adenosine (Neumann et al., 1989), ATII receptors (Allen et al., 1988) and others (Holmer and Homcy, 1991). The mechanism of the inhibitory action on adenylyl cyclase is controversial. Gilman (1984) has presented evidence supporting the hypothesis that the inhibitory receptor causes dissociation of Gi into Gi-alpha and beta/gamma subunits, thereby allowing excess beta/gamma subunits to associate to Gs (by mass action), which inhibits Gs (Gilman, 1984). However, other hypotheses have been put forth based on experiments in which it was shown that in cyc- cells (in which Gs is absent) Gi activation can depress forskolin-stimulated adenylyl cyclase activity (without involvement of Gs) (Hildebrandt et al., 1984).

In addition to the inhibitory action on adenylyl cyclase, muscarinic-linked Gi is also coupled to K\(^{+}\) channel activation in atria (and is thus also named Gk) (Birnbaumer et al., 1990). This action on the heart is thought to be synergistic with adenylyl cyclase inhibition in slowing heart rate, as well as counteracting the arrhythmogenic effects of sympathetic stimulation (Holmer and Homcy, 1991). There are three known isoforms of Gi generated from three different genes of which one isoform Gi-alpha2 predominates in the ventricle (Holmer et al., 1989). The functional correlates of these isoforms are not known. The Gi isoforms present in the adult rat ventricle migrate on SDS polyacrylamide gels at 41-43 kDa (Murakami and Yasuda, 1986).

The function of the third type of G protein in heart, Go, has not been elucidated,
although in the brain, where Go is abundant, it regulates Ca\textsuperscript{2+} channels (Birnbaumer et al., 1989). A fourth, recently identified G protein in cardiac tissue is Gq, a 42 kDa protein linked to activation of phospholipase C (Pappano et al., 1988).

The alpha subunits, Gs and Gi, contain sites of covalent modification by bacterial toxins: Gs is ADP ribosylated by the enzymatic toxin elaborated by Vibrio cholerae, whereas ADP ribosylation of Gi and Go is catalyzed by the toxin elaborated by Bordetella pertussis. ADP ribosylation of Gs-alpha inhibits the GTPase and irreversibly activates adenylyl cyclase and ion channels, whereas ADP ribosylation of Gi-alpha and Go-alpha inhibits the interaction of the subunit with receptor. The cholera toxin catalyzed ADP ribosylation can be used to identify Gs and the pertussis toxin ADP ribosylation can be used to identify Gi by using \([\text{32}} P]\)-labelled ADP ribosylation substrate (Ribeiro-Neto et al., 1985) and electrophoretically analyzing the ribosylated proteins. Endogenous cardiac enzymes that catalyze ADP ribosylation may exist in the myocardium, but these proteins have not been identified (Feldman et al., 1987).

Extracellular signalling of the normal myocardial cell is primarily achieved through the beta-adrenergic and muscarinic cholinergic receptors, regulated by the sympathetic and parasympathetic divisions of the autonomic nervous system. However the contribution of ATII (Baker et al., 1990) and alpha-1 adrenergic (Buxton and Brunton, 1985) receptors may be important in pathological conditions. The muscarinic and alpha-adrenergic receptors are linked through pertussis toxin- and cholera toxin-insensitive G proteins to phospholipase C (Birnbaumer et al., 1990), the activation of which results in the generation of the second messengers inositol trisphosphate (IP\textsubscript{3}) and 1,2-diacylglycerol (DAG) releasing Ca\textsuperscript{2+} from sarcoplasmic reticulum and resulting in the activation of protein kinase C (Berridge, 1987). Thus, G protein alterations in the pathological heart may be an important part of the disease process.

Alterations in G proteins associated with cardiac hypertrophy have been studied in several animal models (Table 1). In the hypertrophic ventricle of hamster, diminished
functional activity of Gs has been reported (Feldman et al., 1990; Kessler et al., 1989) although the mechanism for this change has not been elucidated. In a canine model of pressure overload, hypertrophy Gs-alpha was also shown to be decreased, as assessed by ADP ribosylation studies (Longabough et al., 1988). Other G proteins have also been observed to be altered in hypertrophic cardiomyopathy. Increased pertussis sensitive Gi alpha has been reported in cardiomyopathic Syrian hamster (Sen et al., 1988) and in hypothyroid rats (Levine et al., 1990).

Table 1. Some G Protein Alterations in Heart

<table>
<thead>
<tr>
<th>AUTHOR</th>
<th>SPECIES AND MODEL</th>
<th>G PROTEIN-LINKED FINDINGS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kessler et al., 1989</td>
<td>Syrian Hamster (BIO 14.6) - inherited cardiomyopathy</td>
<td>decrease in Gs activity</td>
</tr>
<tr>
<td>McMahon, 1989</td>
<td>Wistar Rat - maturation (comparing neonate to adult)</td>
<td>decrease in Gs and Gi activity</td>
</tr>
<tr>
<td>Schnabel et al., 1990</td>
<td>Human - failing heart</td>
<td>Gs ADP ribosylation sites - no change</td>
</tr>
<tr>
<td>Feldman et al., 1988</td>
<td>Human - failing heart</td>
<td>Gi activity increase - Gs activity - no change</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gs ADP ribosylation sites - no change</td>
</tr>
<tr>
<td>Levine et al., 1990</td>
<td>Hypothyroid Rats -</td>
<td>Gi-alpha, G-beta mRNA increase</td>
</tr>
<tr>
<td>Longabough et al., 1988</td>
<td>Dogs - Pressure overload hypertrophy</td>
<td>Gs - ADP ribosylation sites- decrease</td>
</tr>
<tr>
<td>Bohm et al., 1992</td>
<td>Rats - Pressure overload hypertrophy</td>
<td>Gi - ADP ribosylation sites-increase</td>
</tr>
</tbody>
</table>

Thus, these studies, taken together, indicate a number of alterations in the G protein complex that occur during the development of cardiac hypertrophy and which are of importance in the adjustment of the response of the myocardium to ligand binding. Factors
that control G protein alterations, such as post-translational modifications to the proteins, have not been completely investigated. Nor is it known whether G protein changes in hypertrophy are a part of the mechanism stimulating hypertrophy, or a response to hypertrophic changes already in progress.

c) **Determinants of Ventricular Hypertrophy**

i. **Hemodynamic Factors**

Since, as discussed above, the myocyte loses mitotic activity shortly after birth, muscle cell enlargement, as well as non-myocyte proliferation, are the only ways in which the heart as a whole can enlarge. It has been a central hypothesis in the study of ventricular hypertrophy that a major factor in ventricular growth is the hemodynamic load placed upon it (Zak, 1986). Experimental models have been developed in order to examine the relationship between hemodynamic load and cardiomyocyte hypertrophy. The two categories of models are those arising out of pressure overload and those involving volume overload models, as shown in Table 2.

**Table 2. Experimental Models of Hemodynamic Effects on Ventricular Hypertrophy**

<table>
<thead>
<tr>
<th>Pressure Overload</th>
<th>Volume Overload</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aortic stenosis - Cutilella et al., 1975</td>
<td>Arteriovenous fistula - Dart and Holloszy, 1969</td>
</tr>
<tr>
<td>Pulmonary artery stenosis - Julian et al., 1981</td>
<td>Aortic insufficiency - Hatt et al., 1970</td>
</tr>
<tr>
<td>Hypertension</td>
<td>Bradycardia - Brockman, 1965</td>
</tr>
<tr>
<td>Renal Ischemia - Goldblatt et al., 1934</td>
<td></td>
</tr>
<tr>
<td>Nephrectomy - Grollman et al., 1944</td>
<td></td>
</tr>
<tr>
<td>Aldosterone - Garwutz et al., 1982</td>
<td></td>
</tr>
<tr>
<td>SHR - Okamoto, 1969</td>
<td></td>
</tr>
<tr>
<td>Dahl rat - Pfeffer et al., 1984</td>
<td></td>
</tr>
</tbody>
</table>

*Spontaneously Hypertensive Rat*
The canine cardiac myocyte, for example, is normally three-fold larger in the adult compared to the neonate, but it enlarges an additional 60% with pressure overload (Zak, 1973), accounting for most of the heart enlargement in pressure overload hypertrophy. The increase in muscle mass with pressure load involves the increase in the number of sarcomeres, both in series and in parallel thereby relieving the load on individual sarcomeres (Legato, 1970; Adomian et al., 1974).

The cellular mechanisms that transform a mechanical stimulus into cellular enlargement are largely unknown. Furthermore, questions have been raised as to whether pressure overload hypertrophy results directly from increased ventricular wall tension or as a replacement response to muscle mass loss since pressure overload is known to cause focal myocardial necrosis (Morgan et al., 1987).

A number of studies on mechanical stimulation of in vitro cardiac tissue have indicated mechanisms by which increases in stretch of tissue can lead to hypertrophy. In particular, the rate protein synthesis has been observed to increase as a result of stretching of quiescent papillary muscle (Peterson and Lesch, 1972) and in response to elevated aortic pressure in hearts arrested by potassium, in which oxygen consumption, intraventricular pressure and energy substrate usage were held constant (Kira et al., 1984).

Increased aortic pressure resulted in a decrease in the rate of protein degradation and increased rates of ribosome formation (Gordon et al., 1986). In contracting cells, feline cardiomyocytes were observed to have larger surface area than quiescent cells after 2 weeks in culture (Cooper et al., 1986). Deformation of the culture substratum also increased uridine incorporation into RNA and phenylalanine into cytoplasmic protein in these cells (Mann et al., 1989). The latter studies, in which cultured cells were placed under stress by deforming the substratum, add considerable support to the link between mechanical stress (ie. load increase) and stimulation of RNA and protein synthesis in the cardiac myocyte.
Cyclic adenosine 3',5' monophosphate as a Second Messenger for Mechanical Stimulation of Ventricular Hypertrophy

The nucleotide cyclic adenosine 3',5' monophosphate (cAMP) is thought to play a role in the control of the hypertrophic process in the heart (Table 3). cAMP is generated in the myocyte by the catalytic action of adenylyl cyclase on ATP to form the cyclized phosphorylated nucleotide. cAMP is then broken down by cytoplasmic phosphodiesterases (Swillens et al., 1988), which means that both the generative or degradative pathways are control points in regulating the level of cAMP in the cell at any given moment. This second messenger is thought to transduce signals from a range of extra-cellular signals. In the myocardium, cAMP availability regulates the activation of cAMP-dependent protein kinase, which catalyzes phosphorylation of troponin I which decreases Ca\(^{2+}\) affinity (Adelstein and Eisenberg, 1980), phosphorylation of myofibrillar C protein (Hartzell and Titus, 1982), phosphorylation and activation of type 1 phosphatase (Ahmad et al., 1989), phosphorylation of phospholamban which regulates Ca\(^{2+}\) ATPase in the sarcoplasmic reticulum (Lindman et al., 1983), and phosphorylation of voltage sensitive Ca\(^{2+}\) channels, which increases Ca\(^{2+}\) across the sarcolemma (Yatani and Brown, 1989). Thus, intracellular cAMP level in the cardiac myocyte is critical in that it is one of the factors governing the contractile capability of the myocardium (Adelstein and Eisenberg, 1980).

Studies focusing on the intracellular signalling of RNA synthesis and protein synthesis which come about as a result of load increases, have provided a partial picture of how mechanical load is translated into a growth response. In guinea pig hearts, an increase in adenylyl cyclase activity follows acute increases in hemodynamic loading (Schreiber et al., 1971). In vitro studies on frog ventricle showed that mechanical stretch could produce a rise in cAMP (Singh, 1982). Other studies demonstrated that increased aortic pressure in tetrodoxin-arrested or beating rat hearts elicited higher rates of protein synthesis and ribosome formation concomitant with rise in cAMP and cAMP-dependent protein kinase activity (Xenophantos et al., 1989; Watson et al., 1989). In these studies, methacholine, a
muscarinic-cholinergic agonist, was found to block the increases of cAMP, as well as cAMP-dependent protein kinase, protein synthesis increases and ribosome formation. The relationship between membrane stretch and cAMP levels has also been observed in non-cardiac cells, namely S49 lymphoma cells (Watson, 1989) and erythrocytes (Morgan et al., 1989), which, when undergoing hypoosmotic swelling, display an increase in intra-cellular cAMP. The transduction of the message of cellular deformation leading to cAMP increase has also been shown to require the actin membrane skeleton since cytochalasin B, which disrupts actin cytoskeleton, eliminated the increase in cAMP produced by hypoosmotic swelling in S49 cells (Watson, 1990).

The mechanism of cAMP regulation of cellular protein synthesis may be through cAMP sensitive protein kinase A which, in turn, has a direct regulatory action on the nuclear binding protein AP-2 (Imagawa et al., 1987), thus leading to faster gene transcription (Roesler et al., 1988).

Table 3 - cAMP Changes in the Heart

<table>
<thead>
<tr>
<th>Experimental Model</th>
<th>Effect on cAMP Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneously Hypertensive Rat (SHR) (Limas and Limas, 1978; Saragoca and Tarazi, 1981)</td>
<td>Lowered cAMP and contractile response to isoproterenol</td>
</tr>
<tr>
<td>SHR (Kumano et al., 1983)</td>
<td>No change in cAMP response to forskolin, NaF Lowered cAMP response to isoproterenol</td>
</tr>
<tr>
<td>Two kidney, one clip rat (Kumano et al., 1983)</td>
<td>Lowered cAMP response to forskolin, NaF, isoproterenol</td>
</tr>
<tr>
<td>Rat - cardiac hypertrophy induced by aortic constriction (Foster et al., 1991)</td>
<td>Lowered cAMP response to forskolin and isoproterenol</td>
</tr>
<tr>
<td>Outbred Dahl rat cardiac hypertrophy (Limas and Limas, 1985)</td>
<td>No change in cAMP response to NaF or isoproterenol</td>
</tr>
<tr>
<td>One kidney, one clip rat (Bohm et al., 1992)</td>
<td>Lowered cAMP response to forskolin and isoproterenol</td>
</tr>
</tbody>
</table>
iii. Ions and Ion Channel Effects

Dietary sodium has been linked to left ventricular hypertrophy independent of changes in ventricular filling pressure (Fields et al., 1991). Neuronal sympathetic activity has been suggested to be a mediator of this effect in the uninephrectomized rat as determined by alpha-1 receptor density and presence of myocardial norepinephrine (Meggs et al., 1988). However, myocardial norepinephrine turnover and tyrosine hydroxylase activity have been demonstrated to remain unaltered in response to high dietary sodium. Thus, sodium may have a direct influence on cellular mechanisms leading to myocardial hypertrophy (Fields et al., 1991).

Another focus of these second messenger studies has been on ion channel changes. The uptake of Na\(^+\) as well as the rate of protein synthesis was found to increase with increasing load imposed on ferret papillary muscle. This effect was blocked by steptomycin and amiloride (which inhibit Na\(^+\) entry) and increased by monensin (which enhances Na\(^+\) entry) (Kent et al., 1989). This apparent link between Na\(^+\) influx and ventricular wall stretch was not, however, borne out in studies on isolated perfused rat heart; in these studies streptomycin did not block the effect of increased aortic pressure in elevating the rate of protein synthesis (Morgan et al., 1989), indicating either important differences between the isolated strip of tissue and the whole organ, or differences between species (or both).

A further indication of a possible role of Na\(^+\) ion comes from a study on fibroblasts. Cellular pH differences between stretched and round 3T3 cells in culture, were eliminated by ethylisopropyl amiloride. Thus, Na\(^+\)-H\(^+\) exchange appeared to be important in setting pH levels, which may be a signal for cellular macromolecule synthesis (Schwartz et al., 1990).

Other intra-cellular mediators of mechanical load on the ventricular cell may include the inositol phosphate-protein kinase C axis (Hardosrf et al., 1989) as well as expression of oncogenes c-fos and c-myc, which were observed to be regulated at the transcriptional level both in vitro (Komuro et al., 1990) and in pressure overload experiments in vivo (Mulvagh
et al., 1987). The role of cellular oncogenes in ventricular hypertrophy will be considered further (vide infra).

iv. Neural Factors

Although hemodynamic elevation can clearly act as a determinant of ventricular hypertrophy, there is also evidence that pressure overload can be dissociated from hypertrophy (Khairallah and Kanabus, 1983; Linz et al., 1989). It has been postulated that other trophic influences are involved, including neural ones.

Alpha-1 Receptor

A number of studies have implicated alpha-1 adrenergic receptor in the control of cardiac muscle growth. Prazosin, an alpha-1 receptor blocker, when used as treatment in patients with cardiac hypertrophy, induced regression of left ventricular mass over 12 months (Leenen et al., 1987).

Stimulation of the alpha-1 adrenergic receptor elicits positive chronotropic and inotropic effects, as well as an increase in phosphatidylinositol turnover (Brown et al., 1985). Levels of cAMP, on other hand, decline, which is due to activation of cAMP phosphodiesterase (Buxton and Brunton, 1985). In vitro studies have employed cultured neonatal rat cardiac myocytes exposed to alpha-1 receptor agonists (Simpson, 1983; Meidell et al., 1986). In this model of mitotic heart cells in culture, protein synthesis and content, myocyte surface area, but not DNA synthesis, were increased with norepinephrine exposure. In vivo studies on rats infused with the alpha-1 receptor agonist, norepinephrine, resulted in increased left ventricular weight to body weight ratios along with an increase in total RNA, and RNA to DNA ratio (Zierhut and Zimmer, 1989). Antagonists of alpha-1 receptor such as prazosin partially blocked the norepinephrine-induced increase in left ventricular weight. Furthermore, verapamil, a calcium channel antagonist, was used to interfere with norepinephrine-induced alterations in contractile function and did not stop ventricular hypertrophy from developing. Thus, norepinephrine stimulated ventricular growth directly and independently of the mechanical stimulation secondary to hemodynamic changes (Zierhut
and Zimmer, 1989).

The intracellular events which transduce stimulation of hypertrophic cardiac myocyte growth through the alpha-1 receptor may involve phospholipase C activation (Henrich and Simpson, 1988) resulting in generation of the two cleavage products: DAG and inositol-1,4,5-triphosphate (IP$_3$) (Abdel-Latif, 1986). DAG is capable of stimulating phospholipid dependent and calcium-dependent protein kinase C, whereas InsP3 has been observed to release sarcoplasmic reticular stores of calcium in skeletal muscle (Abdel-Latif, 1986). The importance of these latter mechanisms in cardiac myocyte hypertrophy has not been fully elucidated. However, it has been demonstrated that the phorbol ester, phorbol 12-myristate 13-acetate (PMA), which stimulates protein kinase C, can mimic alpha-1 adrenoceptor stimulation of cultured cardiac myocyte protein synthesis and growth (Henrich and Simpson, 1988; Fuller and Sugden, 1989). This may lead to phosphorylation of a transcription factor that could bind to the regulatory site of one or more genes, thereby regulating gene transcription. The activities of two such nuclear binding proteins, AP-1 and AP-2, have been shown to be positively regulated by phorbol esters. The activity of AP-2 is also increased by protein kinase A (Lee et al., 1987; Angel et al., 1987). While this is compelling evidence of a possible final step in the mechanism of both phorbol ester and protein kinase A effects of transcription, thus far AP-1 and AP-2 have not been demonstrated in the myocardium.

**Beta Receptor**

The beta receptor is one of the primary components which exerts control over heart rate and cardiac contractility through the adenylyl cyclase - cAMP-dependent protein kinase axis. This makes beta receptor-mediated cardiac hypertrophy more difficult to analyze than alpha-1 receptor mediated hypertrophy *in vivo* and *in vitro* since the induced changes in cardiac work must be controlled. *In vivo* studies have demonstrated that the beta agonist isoproterenol increased myocyte volume as well as heart weight. However, making a direct link between beta receptor stimulation and hypertrophy is not feasible *in vivo* since the beta receptor has concomitant hemodynamic and necrotic effects on the heart (Knufman et al.,
1987). In vitro studies on cultured myocytes also show that chronic exposure to isoproterenol resulted in cellular hypertrophy (Simpson et al., 1982). A separate study demonstrated by means of using non-beating, potassium-depolarized myocytes in culture, that increased contractile activity may be the basis for beta-receptor-mediated hypertrophy (McDermott and Morgan, 1989). However, a number of studies have linked beta-receptor stimulation with hypertrophy through an acute rise in intracellular cAMP (vide supra - section on cAMP changes in hypertrophy).

The depressed adenlylyl cyclase response to beta stimulation may be contributed to, at least in some instances, by alterations in the density of beta receptors. In the Spontaneously Hypertensive Rat (SHR), hypertrophic heart contained diminished beta receptor density compared to Wistar rat hearts (Limas and Limas, 1978; Blumenthal et al., 1982; Upsher and Khairallah, 1985). However, beta receptor control is dependent upon the model studied since Renal Hypertensive Rat cardiac hypertrophy displayed an increase in beta receptor density, as compared with a decrease in the beta receptor density in the SHR in the same study (Upsher and Khairallah, 1985). A similar study in Dahl rats indicated that the hypertrophic heart of the outbred Dahl S rat contained elevated beta receptor density (Limas and Limas, 1985).

v. Endocrine Factors

Thyroid Hormone

Thyroid hormone has been demonstrated to directly alter the heart through a variety of mechanisms: indirectly, by stimulating the adrenergic nervous system, and directly, by increasing protein synthesis. In vivo studies showed that administration of thyroxin to mice resulted in cardiac hypertrophy, independent of changes in circulation (Cohen et al., 1966). Blockade of the beta receptors with propanolol, while diminishing acceleration of heart rate by thyroid hormone, had no effect on the development of cardiac hypertrophy (Bedoto et al., 1989). T4-treated rats have been shown to exhibit cardiac hypertrophy concomitant with accelerated protein synthesis, as well as accelerated protein degradation due to
increased cardiac rRNA and mRNA content (Parmacek et al., 1986). However, this conclusion has been contradicted in other work also using propranalol in which T4-induced hypertrophy was blocked, indicating an indirect, rather than a direct effect (Klein I, 1988).

*In vitro* studies indicate that thyroid hormone is one of the factors which is capable of shifting myosin isoform content from V3 to the fetal V1 form (Nag and Cheng, 1984). In cultures of embryonic chick cardiac myocytes, protein synthesis and cell growth were stimulated by T3 treatment (Carter et al., 1985) supporting the concept of a direct action of thyroid hormone on cardiac cell hypertrophy.

The mechanism of thyroid hormone action may involve binding of the receptor-ligand complex to specific DNA sequences (Gustafson et al., 1987). However, cardiac cAMP levels have also been observed to rise and remain elevated prior to the induction of acceleration of protein synthesis by thyroid hormone (Zimmer and Pfeffer, 1986).

vi. Proto-oncogenes

Oncogenes were first identified as exogenous DNA sequences associated with neoplasia (Gordon, 1985). Subsequently, endogenous genes were discovered which contained a high degree of sequence homology to transforming oncogenes. These have been termed proto-oncogenes. The proteins encoded by these genes can be classified according to a handful of functions in the cell: cytoplasmic protein kinases, membrane GTP binding proteins, growth factors, growth factor receptors and DNA binding proteins. It has become evident that some proto-oncogenes play key roles in the regulation of normal cell growth and division (Weinberg, 1985). The induction of protein synthesis leading to myocyte growth in hypertrophy may involve the induction of proto-oncogene expression as one of the intermediate steps (Parker and Schneider, 1991).

The presence of c-sis mRNA, which corresponds to Platelet-Derived Growth Factor (PDGF) beta chain, in rat myocardium, suggests that it may play a role in myocardial growth (Claycomb and Lanson, 1987). Sis may be induced in the pressure overloaded rat heart (Re, 1987). Other growth factor receptors which have been detected in the
myocardium include erb-A, a member of the thyroid/steroid receptor family, in neonatal and adult rat myocytes (Claycomb and Lanson, 1987). This is of potential importance in the understanding of the thyroid hormone actions discussed previously. While the insulin receptor related- c-ros, c-met and c-trk have not been studied in heart, insulin receptors have been found on myocardial cells (Clark et al., 1988). Similarly erb-B mRNA, which encodes the Epidermal Growth Factor (EGF) receptor, has not been detected in rat myocardium. However, the acute chronotropic response to EGF observed in chick cardiac myocyte aggregates may indicate the presence of the receptor, at least in avian heart (Rabkin et al., 1987).

Cytoplasmic signal-transducing proto-oncogenes have also been studied in the myocardium. C-raf, which encodes a serine/threonine protein kinase is expressed in neonatal and adult myocardium (Claycomb and Lanson, 1987). Tyrosine kinase encoding proto-oncogenes c-src, c-abl and c-fps\fms have been detected only in neonatal heart (Schneider and Parker, 1990; Claycomb and Lanson, 1987).

Proto-oncogenes encoding nuclear binding proteins and found to express mRNA in the myocardium include c-ski, c-myc (Jackson et al., 1990; Claycomb and Lanson, 1987) and c-fos (Komuro et al., 1988). Proto-oncogenes c-myc, c-fos and c-Ha-ras may be involved in the mediation of pressure overload-induced hypertrophy (Komuro et al., 1988). C-fos expression is also increases with age of the myocardium (Komura et al., 1988) and by contractility of the rat heart (Bauters et al., 1988). Both c-myc and c-fos expression are rapidly (within 30 min) induced by the phorbol ester, PMA (Claycomb, 1988; Starksen et al., 1986).

Other signals which are thought to link pressure increase with proto-oncogene activation include the calcium ion, which has been well documented to be a key signal in controlling cell growth in other tissues (Marban and Koretsune, 1990).

Thus, several proto-oncogenes appear to be elements in the mediation of cardiac growth and hypertrophy.
4. ANGIOTENSIN AND THE HEART

Although it falls into the category of endocrine factors linked to cardiac hypertrophy, angiotensin is one of the principle elements of this dissertation, therefore it is discussed separately in this section.

a) Sources of Angiotensin: Renin-Angiotensin Systems

The classically held view of the renin-angiotensin system (RAS) revolves around the juxtaglomerular cells of the kidney, where renin, an aspartyl protease, is released into the blood in response to loss of pressure in the afferent arteriole. Renin release, which is also stimulated by the baroreceptor reflex, sodium concentrations at the macula densa, and via sympathetic stimulation of the juxtaglomerular cells, enzymatically converts the hepatically-synthesized angiotensinogen, a 55 kDa glycoprotein of the alpha2 globulin class, into the decapeptide angiotensin I (ATI). Conversion of ATI into angiotensin II (ATII), an octapeptide, occurs during passage through the pulmonary circulation, where it encounters the dipeptidyl carboxypeptidase, angiotensin converting enzyme (ACE). ATII is one of the most potent vasoconstrictors known. It also stimulates the release of Anti-diuretic Hormone (ADH) from the posterior pituitary, and aldosterone from the adrenal cortex. All of these actions serve to maintain perfusion pressure (Peach, 1977).

However, evidence has mounted over the last two decades which indicate that RAS is rather more diverse than described above. Two general foci of evidence add significantly to the classically held view of RAS: firstly that RAS is present as an autocrine or paracrine system in a number of tissues and that this accounts for ATII in these tissues. Secondly, ATII and to a lesser extent ATI, have broad and important biological actions other than vasoconstriction and stimulation of hormone release. The widened concept of this system has implications in physiology and pathology.
Figure 1 The Renin Angiotensin System
Scheme indicating the movement of renin and angiotensinogen, angiotensin I (ATI) and angiotensin II (ATII) between tissue and blood and the conversion to ATII.

i. Circulating RAS

Whereas the classically held notion is that ATI is converted to ATII and then conveyed to the peripheral tissues, evidence suggests that the major site of production of ATI and ATII occurs at peripheral tissues in the following way: plasma renin (from the kidneys) acts on plasma angiotensinogen (from the liver) at the peripheral tissue to generate ATI which is then converted to ATII by tissue converting enzyme (Campbell, 1985). Thus, in one revised model, circulating RAS does not result in the systemic delivery of ATII to tissues, but in the delivery of renin and angiotensinogen to tissues. In this model, plasma angiotensin is thought to be the result of spillover from the site of production at the tissues (Campbell, 1987). Plasma angiotensin levels are known to drop following nephrectomy, supporting the importance of kidney renin in angiotensin production (Catt et al., 1967).

One of the important aspects of this decentralized mechanism of plasma angiotensin production may be in the diversity of control mechanisms that are brought to bear on ATII generation.
ii. Tissue RAS

The results of several studies have led to speculation that a complete RAS exists within specific tissues, or even within cells. These studies have employed a host of inhibitors of the RAS, such as the ATII receptor antagonist Saralasin, enzyme inhibitors of ACE, as well as immunological detection of RAS components. However, immunological detection of elements of the RAS system is insufficient to draw conclusions about a local autocrine system in operation since spillover and accumulation from circulating RAS can confound the results. This methodological difficulty has been overcome in more recent work in which mRNA for renin, angiotensinogen and angiotensin converting enzyme (ACE) has been detected in the tissues. This has gone a long way to supporting the contention of the existence of tissue RAS. The gene for ACE, recently cloned (Soubrier et al., 1988), has been found to have tissue-specific transcripts in endothelium and testis (Lattion, 1989).

The existence of local angiotensin generating systems which operate independently of circulating RAS has been suggested by studies in brain, kidney, uterus, adrenal, testis, arterial wall and heart. Messenger RNA encoding angiotensinogen, which is the only known precursor of ATII, has been detected in kidney, brain, spinal cord, aorta, mesentery, adrenal, atria, lung, stomach, colon, spleen and ovary (Campbell and Habener, 1986).

Renin mRNA has been detected in adrenals, testes, brain and heart (Dzau et al., 1987). A number of enzymes are capable of cleaving angiotensinogen to generate ATI, including tonin (Boucher et al., 1977), cathepsin (Tonnesen et al., 1982), trypsin (Arakawa, 1980) and kallikrein (Maruta and Arakawa, 1983). So it is quite possible that in vivo, renin is not required for the generation of ATI and ATII at the local tissue level.

However, comparisons of angiotensinogen and renin from the same tissue show that angiotensinogen mRNA is far more abundant, suggesting that if renin is the primary enzyme active in converting angiotensinogen, then renin would be the rate limiting step in these local systems (Dzau et al., 1987).
iii. Cardiac RAS

There are several lines of evidence supporting the existence of an operational RAS in the heart. Renin activity has been found in dog heart (Hayduk et al., 1970), as well as isolated rat cardiomyocytes (Re et al., 1983). In these studies, depletion of dietary sodium increased renin activity in the tissue. Isolated rats hearts have been demonstrated to be capable of converting ATI to ATII, as detected by high performance liquid chromatography (Xiang et al., 1985), thus indicating the presence of renin or renin-like activity. Perfusion with ATI leads to appearance of ATII, suggesting the presence of ACE (Lindpainter et al., 1988). The inhibition of this system by pretreatment with ACE inhibitors has also been demonstrated in the isolated rat heart, reducing the level of ATII when perfused with ATI (Linz et al., 1986). More recent work in this area is the radioimmune detection of the spontaneous release of angiotensinogen from isolated rat hearts (Lindpaintner et al., 1990). This study indicated that intracardiac angiotensinogen is present in the heart and is converted to ATI in the presence of renin in the perfusate and subsequently to ATII, and also indicated that exogenous angiotensinogen in the perfusate can later be released and converted. Anti-renin antibodies have also been employed to detect renin in isolated ventricular myocytes suggesting this as a site of renin synthesis, as opposed to other cell types with the heart (Dzau et al., 1987).

In support of this, renin gene expression has been detected in atria and ventricles of rats and mice (Dzau et al., 1987; Suzuki et al., 1988). Expression of renin mRNA is higher in ventricles than in atria (Suzuki et al., 1988) and compares as approximately 2% of renin mRNA concentrations found in kidney (Dzau et al., 1987). Cardiac renin mRNA is subject to regulation, such as elevation in response to sodium depletion (Dzau et al., 1987).

ACE is most abundant in the lung, but it has also been demonstrated in cardiac tissue in the rat (Cushman and Cheung, 1971; Fabris et al., 1989), cat (Cross et al., 1981), and human (Sakharov et al., 1987). Recently, ACE has also been localized in the heart using in vitro autoradiography by using a radioiodinated tyrosyl derivative of lisinopril, an ACE
inhibitor, which specifically binds the ACE protein. This method revealed high density of ACE at valve leaflets (aortic, pulmonary, mitral and tricuspid) and coronary bed with lower density in the endocardium (Yamada et al., 1991). ATII formation in the rat heart is likely reliant upon ACE to a large extent, however new studies have indicated that in human and hamster hearts an ACE inhibitor-insensitive, soybean-trypsin inhibitor-sensitive enzyme actively converts ATI to ATII (Urata et al., 1990). Other proteases may be active in the heart as well.

ATII receptors have been characterized from cardiac tissue from chick (Peach, 1981), rat (Rogers et al., 1986), as well as rabbit (Wright et al., 1983; Baker et al., 1984) calf (Rogers, 1984) and human (Urata et al., 1989). Most of these studies found low-affinity, low-binding capacity, as well as high affinity, high binding capacity components. The ATII binding sites are both saturable, specific and reversible, fulfilling key requirements of a hormone receptor. The difference in affinities between the two receptors is from 10- to 100-fold, with a 3- to 20-fold difference in binding capacities. The receptor density on the neonatal rat myocyte was estimated at 45,000 per cell (Rogers et al., 1986). Autoradiographic localization of receptors shows them on the myocardium, coronary vessels and sympathetic nerves of the heart. Similar receptor density was observed between left and right ventricles (Urata et al., 1989). The bovine ATII receptor has been identified as a 116 kDa protein by affinity cross-linking studies (Rogers, 1984) which is suggested to be a dimer whose subunits aggregate upon ligand binding (Peach, 1981). Receptor sites were shown to decrease between day 1 and day 10 after birth in rats (Urata, 1989). This regulation appears to differ between species since ATII receptor sites on chick hearts increase in number during development (Peach, 1981).

b) Direct Physiological Effects of Angiotensin on the Heart

ATII acts on the heart indirectly, through its potent pressor and trophic activities on the vasculature (Griendling and Alexander, 1991) and through direct effects. In this section
only the direct effects of ATII on the heart will be considered.

One of the first observed direct effects of ATII on the heart was a positive inotropic action in several species including cat (Koch-Weser, 1964), chick, rabbit (Freer et al., 1976) and others (Peach, 1977 for review). A number of methods were used to demonstrate that the effect was due to ATII and not through modulation of sympathetic nervous function. These included using beta blockade (Peach, 1981), cardiac denervation (Rioux et al., 1975) and isolated cardiac myocytes (Allen et al., 1988; Dosemici et al., 1988; Neyses and Vetter, 1989). Thus, after experimentally eliminating sympathetic effects, ATII has a dose-dependent, positive inotropic action on the myocardium. In neonatal rat heart cultures the inotropic action is reportedly negative rather than positive, thus making this system an exception (Allen et al., 1988), especially in light of reports that the adult rat myocyte was found non-responsive to ATII (Peach, 1977) and others in which ATII had a positive inotropic effect on the adult rat ventricular myocyte (Neyse and Vetter, 1989; Doggrell, 1989; Nakamura et al., 1989). The mechanism of action of the inotropic effects in the neonatal rat has been associated with concomitant increases in the slow inward calcium current, as has the positive inotropic effect seen in rabbit and chick (Freer et al., 1976). This has been associated with concomitant cadmium-sensitive "L-type" calcium channel activity and with formation of inositol monophosphate and inositol bisphosphate in neonatal rat heart cultures (Allen et al., 1988).

Recently, human trabecular strips were found to be responsive to ATII stimulation in vitro although a wide heterogeneity was reported, and a greater response was elicited in functionally normal hearts compared to failing hearts (Moravec et al., 1990). In the neonatal rat the negative inotropic effect and calcium channel activity increase were mimicked by the phorbol ester, PMA, which is known to activate protein kinase C (Dosemici et al., 1988). This suggests protein kinase C may mediate the action of ATII in the neonatal rat.

ATII acutely alters transport of other ions besides calcium in the myocardium. Phosphate efflux is stimulated and influx inhibited in isolated rat ventricular cells, actions
which are also mimicked by the protein kinase C stimulant, PMA (Sunga and Rabkin, 1991). The frequency of opening and rates of activation and inactivation of sodium channel was increased in patch-clamp experiments on neonatal rat ventricular myocytes, and this too was mimicked by PMA (Moorman et al., 1989). Both of these are possible candidates as components in the mechanism of contractile changes described above.

c) Angiotensin, Cell Growth and Cardiac Hypertrophy

Cardiac hypertrophy is based mainly on increase in myocyte size, as opposed to myocyte proliferation (vide supra). Part of the process of growth of myocytes involves increased macromolecular turnover. Angiotensin is emerging as a potential player in the regulation of this process.

The first kind of evidence is inferential. ACE inhibitors, agents which block the conversion of ATI to ATII, are well-documented to prevent and even reverse hypertrophy in rats (Sen et al., 1980; Kromer and Riegger, 1988) and humans (Dunn et al., 1984). However, the effects of ACE inhibitors on the extra-cardiac vascular system and the resulting hemodynamic repercussions for the heart make it difficult to draw conclusions on the basis of this work alone. However, a different approach, the treatment of aortically banded rats with sub-pressor doses (doses too low to raise blood pressure) of the ATII analogue (Sar\textsuperscript{1}Ile\textsuperscript{8}) has also been shown to result in hypertrophy (Khairallah and Kanabus, 1983). Likewise, doses of ACE inhibitors too low to decrease blood pressure were recently shown to result in prevention and regression of hypertrophy in the aortic-banded rat (Linz et al., 1989). This work is strengthened by the demonstration that ATII increases RNA content and protein synthesis in chick myocardiocyte cultures (Baker and Aceto, 1990; Aceto and Baker, 1990) and DNA turnover, RNA turnover and protein synthesis in isolated rat ventricles (Khairallah et al., 1972).

ATII may act directly at the nucleus, since it has been localized autoradiographically to the nucleus of rat cardiomyocytes (Robertson and Khairallah, 1971) and associates with
high affinity chromatin receptors (Re et al., 1983). ATII induces expression of the nuclear proto-oncogene, c-fos (Hoh et al., 1990), an action which takes place through protein kinase C and modulation of calcium flux in aortic smooth muscle (Taubman et al., 1989). Furthermore, cardiac tissue responds to experimental pressure overload with hypertrophy and c-fos and c-myc expression (Izumo et al., 1988). Another factor associated with cell growth, early growth response gene 1 (Egr-1), is also activated by ATII in rabbit cardiomyocytes, and is also associated with protein kinase C activation (Neyes et al., 1989).

The impact of ATII on the hypertrophic myocardium may be accentuated by the presence of an endogenous cardiac renin-angiotensin system which is amplified in pressure-overload hypertrophy (Baker et al., 1990). The hypertrophic heart of the Spontaneously Hypertensive Rat displays increased renin expression (Lindpaintner et al., 1987), while aortic banded rats display accelerated angiotensinogen gene expression (Baker et al., 1990) and increased ACE enzymatic activity (Lorell et al., 1989).

Thus ATII, whether generated within the heart or not, has a number of actions on the heart, some of which may be central to the control of growth, and others which are certainly important in cardiac contractility. In the hypertrophic heart ATII may be present in higher concentrations than in the normal heart, making analysis of ATII actions on this clinically important condition a compelling area of study.
6. CHOICE OF THE INBRED DAHL RAT MODEL OF CARDIAC HYPERTROPHY

Meneely and Ball, upon review of work carried out in the 1950s, showed that the blood pressure in a large population of Sprague-Dawley rats varied linearly with the amount of the NaCl in the diet (between 0-10% NaCl diet)(Meneely and Ball, 1958).

Lewis K. Dahl took advantage of the variability of the hypertensive response to high NaCl by inbreeding Sprague-Dawley rats for sensitivity or resistance (denoted S or R respectively) to the hypertensive effect of high sodium diet. The basic trait of S rats which Dahl selected for, was that upon several weeks of high NaCl diet, high blood pressure developed, whereas R rats, given the same diet, maintained a constant blood pressure. The strains were selectively inbred for several generations before being outbred. The hypertensive traits were then maintained by selection and the avoidance of inbreeding. These two strains were then used for the study of various aspects of hypertension. Subsequently, in the 1970 and 1980s, the strains were inbred by Rapp (Rapp, 1987), so fixing the genetic traits of each strain. They have by now been inbred for more than 80 generations.

Characteristics of the Dahl R and S Strains

Dahl S rats on a 8% NaCl diet from 21-23 days of age develop hypertension and die by 16 weeks of this diet. Identically treated Dahl R rats retain normal blood pressure and survived to 48 weeks on this diet (Knudsen et al., 1970). The Dahl S rat exhibits a markedly higher pressor response to bolus infusion of ATII, as well as containing less tissue and plasma ATII. The pressor difference is more pronounced with high NaCl diet feeding (Iwai et al., 1970). When other pressor substances such as ADH, or norepinephrine are administered intravenously Dahl S rats experience a greater increment in blood pressure than Dahl R rats (Dahl et al., 1964). This occurs when the animals are on low NaCl diet (0.4%) in which there was no significance difference in blood pressure between the strains. Since this latter effect is abolished by ganglionic blockade, which also eliminates the
baroreceptor reflex, the suggestion has been made that this difference in response to pressors is partially due to altered baroreceptor reflex in the Dahl S rat (Gordon et al., 1981).

The lowered tissue and plasma ATII levels may be partly dependent upon a polymorphism in the renin gene which co-segregates with blood pressure differences between Dahl R and Dahl S (Rapp et al., 1989). Cardiac differences in ATII response are suggested also by recent work in which the non-peptide ATII receptor agonist DuP753 was demonstrated to significantly lower mortality in Dahl S rats on high salt, without significantly lowering blood pressure (von Lutterotti et al., 1991).

Pfeffer et al. (1984) examined the cardiac performance of rats in the Dahl strains, tracing the progress of ventricular hypertrophy and the cardiac performance of hypertrophic hearts in Dahl S compared to normal Dahl R hearts. They observed that cardiac output was preserved (similar to Dahl R hearts) despite increased arterial pressure (preload), and noted that the degree of hypertrophy was dependent on the amount of NaCl in the diet, as well as the time on high NaCl diet (Pfeffer et al., 1984)

Thus the choice of the Dahl model of hypertrophy for the study of the actions of ATII on the hypertrophic heart is based on the following considerations:

i. simple dietary control of hypertrophy, through NaCl supplemented diet, which allows different degrees of ventricular hypertrophy to be studied (with different concentrations of NaCl in the diet, and different lengths time on high NaCl diet (Pfeffer et al., 1984);

ii. amplified response to ATII in the Dahl S rat;

iii. genetic homogeneity in the inbred strains;

iv. possibility of having two types of controls: the non-hypertrophic (low NaCl) Dahl S rat, and the high NaCl-fed sister strain, the Dahl R rat; and

v. the ease of handling of these animals and of isolating viable myocytes from them are added bonuses.
7. HYPOTHESES AND OBJECTIVES

There is a considerable amount known about the relationship between ATII and the myocardium, both in that inhibitors of ATII production, such as ACE inhibitors (Kromer and Riegger, 1988), have direct effects on the myocardium, and in that ATII has various effects on the myocardiocyte (Schelling et al., 1991; Allen et al., 1988; Neyses and Vetter, 1989; Aceto and Baker, 1990). However, ATII effects on the hypertrophic myocardium have not been examined fully, even though this is of potential importance, since ATII may be more active in hypertrophic ventricle than normal ventricle (Baker et al., 1990). The hypotheses and objectives of this project developed in the context of using the Dahl rat strains in a novel way: as a model of the effects of ATII on the hypertrophic ventricle.

The principle hypotheses were that

1. ATII has distinct effects on the hypertrophic Dahl S rat ventricle.
2. Divergences in ATII intracellular signalling occur in the hypertrophic Dahl S rat ventricle compared to the Dahl R ventricle.

The specific objectives were to

1. Establish the Dahl rat as a model for studying the effects of ATII on cardiac hypertrophy.
2. Define the effects of ATII on the hypertrophic Dahl S and normal Dahl R myocardium by comparing ATII effects along the beta receptor-adenylyl cyclase-cAMP signal transduction axis in the normal and hypertrophic myocardium.
3. Identify differences in intracellular signalling in the hypertrophic Dahl S rat ventricle compared to the normal Dahl R ventricle by examining the effects of ATII on protein phosphorylation.
CHAPTER II
MATERIALS AND METHODS

1. Animal Treatment

Inbred Dahl SR/Jr and SS/Jr and outbred male Dahl DR and DS rats, from Harlan Sprague Dawley (Indianapolis, USA), were placed on a diet of powdered Purina Lab Chow supplemented with an additional 6% NaCl from weaning (21 days of age). Animals were permitted to drink tap water ad libitum. The normal diet was not NaCl free, but contained 1% NaCl, therefore the high NaCl diet contained a total of 7% NaCl. Hypertension-sensitive (S) and hypertension resistant (R) rats were paired according to date of birth, dietary regime and sex such that each S rat had an identically treated, age-matched, gender-matched counterpart.

2. Isolation of Adult Rat Cardiac Myocytes

Viable adult cardiac myocytes were isolated from 150-300 g rats by the method of Kryski and Severson (1985), as modified by Sunga and Rabkin (1991). Rats were anaesthetized with 3% halothane in air and killed by cervical dislocation. Hearts were rapidly excised, rinsed in oxygenated, 35°C Joklik's Modified Minimal Essential Medium containing 1.2 mM MgSO₄, 23.8 mM NaHCO₃ and 0.5 mM L-carnitine (solution A) and perfused through the aortas with oxygenated, 35°C solution A at 7 ml/minute for 5 min. The pumped solution was changed to solution B (solution A plus 238 mg/ml type II collagenase 1 mg/ml, fatty acid free bovine serum albumin and 25 μM CaCl₂) and perfusion continued for 20 min during which time the perfusate was collected, warmed, oxygenated and recycled. After being trimmed of major vessels and atria, ventricles were placed in solution C (solution A plus 1% bovine serum albumin plus 1.5 mM CaCl₂) and quickly blotted and weighed. The ventricles were minced, shaken for 10 min and the supernatant discarded. Subsequently, the perfusate of solution B was added for a further 15 min of shaking.
Isolated myocytes were harvested, diluted 10 X in solution C and centrifuged at 45 G for 1 min. The isolated cells were washed twice in solution C before being resuspended in Dulbecco's Modification of Eagle Medium (DMEM) and stored under 95% O₂ and 5% CO₂. Experiments were carried out within 6 hr of harvesting myocytes. Viability of cells was usually between 75 and 85% as determined by trypan blue exclusion. Isolated cells were characteristically rod-shaped and quiescent. The yield of viable cells was 15 to 20 million per heart.

3. Fluorescence Activated Cell Sorting Analysis of Ventricular Myocyte Size

Isolated, dispersed cells from hypertrophic Dahl S and age and diet matched Dahl R hearts were diluted in 37°C oxygenated DMEM to a final concentration of 10⁵ cells/ml and analyzed for cell size using a Coulter Epics C Clinical Flow Cytometer with a Multi-parameter Sensor System and argon laser (Coulter Inc., Hialeah, FLA). Forward right angle light scatter was determined for a window which allowed relative size comparison and excluded cell debris. The distribution of cell size was analyzed into 256 channels. A window of channel 120 to channel 250 was examined. Dahl R and Dahl S myocyte populations with 74% viability as previously assessed by trypan blue exclusion, were analyzed in identical fashion, within 5 min of each other.

4. Platelet Preparation

Blood from Dahl rats was made to 25 mM Na citrate before being centrifuged at 150 X g for 10 min and the platelet-containing plasma removed. Platelets were examined by phase contrast microscopy and counted using a hemacytometer before centrifugation for 10 minutes at 1500 X g. Platelets were resuspended in phosphate-free DMEM containing 250 uCi/ml carrier-free [³²P] orthophosphate for 60 min. Samples were then centrifuged at 12,000 x g, the labelling medium aspirated and the pellet dissolved in 200 ul of isoelectric focusing buffer (9.5 M urea, 2% Triton X-100, 5% mercaptoethanol and 2 mls of ampholytes) for
later determination of protein content and analysis by two-dimensional gel electrophoresis.

5. Isolated Heart Preparation

Male SR/Jr and SS/Jr rats (150-250 g) were used for all experiments. Animals were anaesthetized with 3% halothane in air and killed by cervical dislocation. Contractile function was assessed as described by Rabkin (1983). Hearts were rapidly excised and placed immediately in cold Kreb-Henseleit solution containing (mM) NaCl (117.4), KCl (4.7), CaCl₂ (3.0), MgSO₄ (1.2), KH₂PO₄ (1.2), EDTA (0.5), NaHCO₃ and glucose (11.0), pH 7.4. The heart was trimmed of pericardial tissue and the aorta tied to a cannula and perfused retrogradely with Krebs-Henseleit that was being continuously gassed with 95% O₂/ 5% CO₂ and maintained at 37°C. Perfusion was maintained at a constant flow rate of 6 ml/min by means of a Masterflex constant flow pump (Cole-Parmer Instruments, Chicago, USA). Through an incision in the left atrium a fluid-filled latex balloon was inserted into the left ventricle and tied with silk suture. The balloon was attached via PE 50 polypropylene tubing to a P-23 Gould pressure transducer and pressures continuously recorded on a Gould 2400 recorder. Silver wire was attached beneath the epicardium to pace the heart at 250 beat/min using a Pulsar 6i (Frederick Haer and Co.) stimulator. Perfused hearts were allowed to equilibrate in order to establish a baseline. Forskolin or ATII were introduced into the perfusate by Sage syringe pumps (Orion Research Corp., Cambridge, Mass.). ATII was given prior to addition of forskolin.

6. Biochemical Techniques

a) Sarcolemmal Isolation

Sarcolemmal membranes were isolated according to the procedure of Anand-Srivastava (1989) with the following modifications for isolated myocyte preparations. After isolation of viable myocytes, samples of 1 x 10⁶ cells were suspended in 1 ml of 10 mM Tris-HCl, 1 mM EDTA, pH 7.4 and homogenized with 10 strokes in a glass-teflon homogenizer.
Samples were centrifuged at 1000 x g for 10 min and the pellet suspended in 1 ml of 10 mM Tris-HCl buffer, pH 7.4, inverted in for 30 min at 4°C and centrifuged for 10 min at 1000 x g. This cycle was repeated two times, suspending in 1 ml of 10 mM Tris-HCl pH 8.0, then the same buffer at pH 7.4. The pellet was then inverted in Tris-HCl, 0.4 M LiBr for 45 min and centrifuged at 1,000 x g for 10 min. The pellet was suspended in 10 mM Tris-HCl pH 7.4 and finally suspended in 100 ul of 10 mM Tris-HCl, 1 mM dithiothreitol, 1 mM EDTA pH 7.4, aliquoted and frozen at -80°C for in vitro ADP ribosylation reactions.

b) $^{32}$Porthophosphate Incorporation

Isolated myocytes were washed with Pi-free DMEM twice and incubated in Pi-free DMEM for 30 min to reduce intra-cellular phosphate content. Cells were labelled with 250 uCi/ml carrier-free $^{32}$Porthophosphate in DMEM for 2 h. One uM carbachol or ATII were added to samples of 0.5 ml containing 0.5 X $10^6$ cells for 10 min after which 10 uM forskolin was added at final portion of the labelling period. The reactions were stopped by centrifugation (13,500 x g for 5 sec), aspiration of the labelling medium and addition of SDS gel electrophoresis buffer to the cell pellet.

c) $^{35}$S methionine Incorporation

Samples of 1 x $10^6$ isolated myocytes were rinsed in methionine-free DMEM twice then incubated in this medium for 1 h. Samples were pulsed with 60 uCi/ml $^{35}$S methionine in methionine-free DMEM for 2 h after which the pulse medium was replaced with fresh DMEM. Myocytes were harvested by a brief centrifugation up to 13,500 x g for 5 sec after which cell pellets were dissolved in immunoprecipitation buffer.

d) Pertussis Toxin Treatment of Isolated Ventricular Myocytes

Isolated myocytes were treated with pertussis toxin by addition of the toxin to isolation medium to a final concentration of 50 ng/ml. Myocytes were maintained at 37°C in the presence of 5% CO$_2$. Viability of cells was not affected under these conditions, as determined by spontaneous beating and trypan blue exclusion.
e) **Immunoprecipitation**

For immunoprecipitation, cell pellets were lysed in a buffer containing 10 mM Tris, 1% Triton X-100, 5 mM EDTA, 50 mM NaCl, 1 mM phenylmethylsulphonylfluoride, 1 mg/ml aprotinin, pH 7.6 at 4°C. The lysate was rocked overnight with rabbit anti-Gi antibody-linked Protein G agarose beads (Genex Inc., Gaithersburg, MD) at 4°C. The slurry was centrifuged at 13,500 x g for 10 min and washed twice with 1.0 ml of 0.01 M sodium phosphate pH 7.4, 0.15 M NaCl, 0.05% sodium azide. SDS gel electrophoresis buffer was added and the mixture heated to 90°C for 10 min. The eluate was centrifuged to remove the beads and analyzed on 10.5% polyacrylamide SDS gels.

f) **Protein Determination**

Protein content was assessed using the method of Bradford for most samples (Bradford, 1976). Bradford reagent was purchased from Bio-Rad Canada Ltd (Mississauga, Ont.). For samples dissolved in urea-containing buffers for isoelectric focusing, the method of Ramagli and Rodriguez (1985), which is a modification of Bradford’s technique, was employed. Bovine serum albumin was used as a standard.

g) **In vitro ADP Ribosylation**

**In vitro** ADP ribosylation was carried out according to the procedure of Ribeiro-Neto et al. (1985) with modifications. Cholera or pertussis toxin was activated by incubation in 25 mM dithiothreitol, 25 mM Tris-HCl, 0.075 % bovine serum albumin at pH 7.5 for 30 min at 35°C. Cholera toxin catalyzed ADP-ribosylation was carried out by preparing a reaction mixture containing the following 10 uM NAD⁺, 300 mM KH₂PO₄, 50 nM ADP-ribose, 10 mM thymidine, 1 mM ATP, 0.1 mM GTP, 1 mM ethylenediamine tetraacetic acid, 10 mM MgCl₂, 25 mM Tris-HCl, 0.02 mg/ml DNase 1, 125 ug/ml bovine serum albumin, 1.66 mM dithiothreitol, 100 ug/ml cholera toxin, or 10 ug/ml pertussis toxin. Membrane protein, 5 ug/ml, was added. Reactions were started with the addition of [³²P]NADP. The reactions were carried out at 32°C for 45 min and were stopped by addition of 1.0 ml of ice cold 20% trichloroacetic acid. After 20 min, the mixtures were centrifuged for 20 min at 2,100 x g at
4°C and the supernatant removed. Pellets were washed in 1.5 ml diethyl ether, centrifuged as before and the reactions tubes warmed to room temperature and exposed to air until the ether evaporated. Membrane proteins were then assayed for protein content and prepared for sodium dodecyl sulphate 10.5% electrophoretic analysis.

h) SDS Polyacrylamide Gel Electrophoresis

SDS PAGE of extracts was performed on 10.5% separating gels according to the method of Laemmli (1970). Samples were dissolved in SDS gel electrophoresis buffer (125 mM Tris-HCl (pH 6.8) 4% SDS, 0.01% bromophenol blue, 10% mercaptoethanol, 20% glycerol), boiled for 5 min, and equal amounts of protein loaded onto 4% stacking gels overlying 10.5% polyacrylamide separating gels. Following electrophoresis, gels were stained with Coomassie Brilliant Blue R250, destained, and dried onto paper.

i) Two dimensional electrophoresis

Two dimensional electrophoresis was performed according to the method of O'Farrell (1975). Samples for isoelectric focusing gels were dissolved in 9.5 M urea, 2% Triton X-100, 5% mercapto-ethanol, 1.6% pH 5/7 ampholytes, 0.4% 3/10 ampholytes and frozen at -80°C overnight. Samples were assayed for protein content by a modification of the Bradford method (Ramagli and Rodriguez, 1985) and 30 μg of protein were loaded onto tube gels. Isoelectric focusing tube gels were electrophoresed at constant voltage of 750 V for 4.5 h after which time the current stabilized to 0.02 mA/gel. Tubes were frozen at -80°C overnight. Second dimension 10.5% polyacrylamide slab gels were electrophoresed as described (vide supra).

j) Quantitation of Radio-labelled Proteins

[32P]phosphate labelled protein gels were mounted, dried and autoradiographed on Kodak X-AR5 X-ray film using intensifier screens at -80°C. [35S]methionine labelled protein gels were soaked in Amplify (Amersham Canada Ltd., Oakville, Ont.) for 10 min before being dried and exposed to Kodak X-AR5 or RP-1 film for 5 days. Densitometric analysis was performed on a Bio-Rad Video Densitometer (Bio-Rad Canada Ltd, Mississauga, Ont.)
to quantitate SDS polyacylamide gel autoradiographs. Internal comparisons were made with extraneous bands to ensure consistency of densitometric determinations.

k) **Immunoblotting**

Following electrophoresis, the separating gel was soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) and then placed together with a nitrocellulose membrane in a sandwich. Proteins were transferred for 3 h at 75 V. Subsequently, the nitrocellulose membrane was blocked with TBS (Tris-buffered saline) containing 5% skim milk powder for 2 h at 20°C. The membrane was washed twice with TBS containing 0.05% Tween 20 (TTBS) for 5 min before incubation with the primary antibody overnight at 20°C. The membrane was then washed twice with TTBS and incubated with the secondary antibody (goat anti-rabbit IgG coupled to alkaline phosphatase in 0.05% Blotto) for 2 h at 20°C. The membrane was rinsed with two washes of TTBS followed by one wash with TBS, and then incubated with 5-bromo-4-chloro-3-indoly phosphate/nitro blue tetrazolium colour development solution (a mixture of 3% nitro blue tetrazolium in 1 ml of 70% dimethylformamide and 1.5% 5-bromo-4-chloro-3-indoly phosphate in 1 ml of 100% dimethylformamide subsequently added to 100 ml of 0.1 M NaHCO₃, 10 mM MgCl₂, pH 9.8). The colour was developed in 1-24 h at 20°C in the dark, and the reaction was stopped by rinsing the membrane in water.

l) **cAMP Determination**

Samples containing 1 million viable isolated myocytes were centrifuged up to 10,000 x g for 5 sec and the medium aspirated. Deproteination of the sample was achieved by ethanol precipitation and the pellet suspended in 1 ml ice cold 0.1 N HCl in 95% ethanol. The samples were then vortexed until the pellet was thoroughly dissolved then centrifuged for 5 min at 10,000 x g. The supernatant was saved for cAMP determination. The pellet was washed again in 1 ml of 35% ethanol, vortexed and centrifuged as before and the supernatant combined with the acid supernatant from the previous step. Supernatants were evaporated to dryness at 55°C under a stream of N₂ in preparation for cAMP determination.
The pellets were retained for protein determination.

The determination of cAMP was based on the method of Gilman (1970) which relies on competitive binding between unlabelled cAMP and a fixed quantity of $[^3H]$ labelled cAMP to a protein with high binding specificity for cAMP. The materials were supplied freeze dried purchased from Amersham Canada. The assay protocol was, briefly, 50 ul of 0.5 M Tris, 4mM ethylenediamine tetraacetic acid (EDTA) were added to each 50 ul of each sample on ice. Fifty ul of $[^3H]$cAMP were added, followed by the addition of 100 ul of binding protein. The samples were vortexed for 5 sec, placed in an ice bath and allowed to incubate for 2 h. At the end of the 2 h period 100 ul of charcoal suspension were added and the samples vortexed for 10 sec and placed in a refrigerated microcentrifuge (Heraeus, W.Germany) and centrifuged at 12,000 x g for 2 min at 4°C. Samples of 200 ul of supernatant were removed for scintillation counting.

m) Preparation of Ventricular Cytosolic Extract

After the period of stimulation of tissue pieces, DMEM was aspirated from ventricular pieces. The pieces were rinsed with ice cold homogenization buffer (125 mM beta-glycerol phosphate, 12 mM EDTA, 5 mM EGTA, 2 mM sodium vanadate, 1 mM diothiothreitol, 1mM phenyl methyl suphonyl fluoride, pH 7.2) once, then placed in 1 ml of homogenization buffer. Samples were homogenized using a microtip equipped Polytron (Brinkman Instruments) at setting 7 for 3 bursts of 10 sec/burst, in an ice bath. The homogenates were centrifuged at 200,000 x g for 15 min on a Beckman TL-100 centrifuge and the supernatant retained for protein determination.

Protein content of the supernatant was determined using the Bradford assay using bovine serum albumin as a standard. Samples were either stored at -70°C or chromatographed immediately on a MonoQ column.

n) MonoQ Column Chromatography

Anion exchange chromotrography was used to fractionate protein from the extracts of ventricular myocytes. Cytosolic protein (5.0 mg) of myocyte extracts were loaded onto a
MonoQ column (Pharmacia)(1 ml bed volume) in buffer B (5 mM Mops (pH 7.2), 5 mM EGTA, 1 mM sodium orthovanadate and 1 mM dithiothreitol) at a flow rate of 1 ml/min in a Pharmacia FPLC system. The column was developed with a 15 ml linear 0-0.8 M NaCl gradient in buffer B at the same flow rate. Forty fractions of 0.25 ml volume were collected for myelin basic protein kinase assays.

o) Myelin Basic Protein Kinase Activity Assay

MBP Kinase assays contained in a final volume of 25 ul the following: 1 mg MBP/ml; 50 uM [gamma\textsuperscript{32}P]ATP (~1000 cpm/pmol); 0.5 uM cAMP dependent protein kinase inhibitor peptide and assay dilution buffer (30 mM beta-glycerol phosphate, 20 mM Mops, pH 7.2, 20 mM MgCl\textsubscript{2}, 5 mM EGTA, 1 mM DTT, and 0.5 mM sodium vanadate). All reaction pre-incubations were at 0\textdegree C. The kinase reactions commenced upon the addition of [gamma\textsuperscript{32}P]ATP and was of 5 min duration at 30\textdegree C. The reaction rate was non-linear in excess of 15 min duration or when the protein concentration was more than 0.3 mg/ml. The assay reactions were terminated by spotting 20 ul aliquots onto a 1.5 cm\textsuperscript{2} piece of Whatman P81 phosphocellulose paper. The papers were washed by 10 changes of 1% phosphoric acid and overnight washing before being transferred into 6 ml plastic scintillation vials containing 3 ml of scintillation fluid and counted on a Packard Tri-Carb 2200 scintillation counter.

p) Protein Kinase C Activity Assay

Fractions from MonoQ column chromatography were assayed for kinase activity in a 10 min reaction at 30\textdegree C in a 25 ul volume. The incubation included the following components: 1 mg/ml histone H1 (Type IIIS), 50 uM [gamma\textsuperscript{32}P]ATP (1500 cpm/pmol), 25 mM beta-glycerophosphate, 10 mM Mops pH7.2, 15 mM MgCl\textsubscript{2}, 2 mM EGTA, 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol and 500 nM protein kinase inhibitor peptide. Where indicated as containing lipid, the incubations also included 4.5 mM CaCl\textsubscript{2}, 60 ug/ml phosphatidylinositol serine and 6 ug/ml diolein. The reactions were terminated by spotting 20 ul samples onto 1.5 cm\textsuperscript{2} pieces of Whatman P81 phosphocellulose paper and 30 sec later washing 10 times with 1% (v/v) phosphoric acid. The wet papers were transferred
into 6 ml plastic scintillation vials containing 3 ml of scintillation fluid and counted on a Packard Tri-Carb 2200 scintillation counter.

7. Methods of Statistical Analysis

The t-test was used to determine the significance of difference between two means. Analysis of variance was used to examine for group comparisons. Linear regression analysis was used and analysis of variance used to assess the underlying relationship. The 5% level was considered significant.

8. Materials

a) Animals

Dahl rats were purchased from Harlan-Sprague Dawley (Indianapolis, Indiana) and housed in controlled conditions in the animal unit at the University Hospital (Shaughnessy) Research Centre.

b) Radiochemicals

\[^{35}\text{S}]\text{methionine was from New England Nuclear, Canada (Toronto, Canada). Carri}\]er-free \[^{32}\text{P}]\text{orthophosphate, }[^{32}\text{P}]\text{NADP, }[^{14}\text{C}]\text{labelled high molecular weight protein markers and }[^{3}\text{H}]\text{cAMP were from Amersham Canada (Oakville, Ontario). Gamma }[^{32}\text{P}]\text{ATP was from ICN (St. Laurent, Quebec).}

c) Biochemicals

Collagenase I was and DNAase I was from Worthington Biochemicals (Freehold, New Jersey). Forskolin, angiotensin II and PMA, Saralasin and ionomycin were from Peninsula Laboratories (Belmont, CA, USA). Pertussis toxin and cholera toxin were from List Biological Laboratories (Campbell, CA). The synthetic peptide TTYADFIASGRTGRNRNAIHD (Protein Kinase Inhibitor Peptide, a cAMP-dependent protein kinase inhibitor peptide), was produced in the lab of Dr. E. Krebs. Isoproterenol, A23187 and carbamyl choline, phosphatidylserine, aprotinin, phenylmethylsulfonyl fluoride, diolein, ATP, Histone H1, and
other chemicals were from Sigma Chemicals (St.Louis, Missouri).

d) Antibodies

Anti-muscle actin (clone C4) and anti-actin (clone B4) monoclonal antibodies were obtained from ICN Biomedicals (St.Laurent, Quebec). Rabbit anti-Gs (clone RM-1) and anti-Gi antibodies were from New England Nuclear. Antibodies against phospho-tyrosine (PY-20) were purchased from ICN and Amersham Canada. Antibodies against MAP kinases were developed in the laboratory of Dr. S.L. Pelech (University of British Columbia).
CHAPTER III

A NOVEL ACTIVITY OF ATII IN THE ADENYLYL CYCLASE AXIS OF HYPERTROPHIC MYOCARDIOCYTES

1. ATII ELEVATION OF ISOPROTERENOL-INDUCED INCREASES IN cAMP IN THE HYPERTROPHIC DAHL VENTRICULAR MYOCYTE: A REVERSAL
   a) Summary

   Isolated ventricular myocytes from hypertrophic Dahl S ventricles and Dahl R ventricles were treated with 1 μM ATII prior to stimulation with isoproterenol and measurement of cAMP concentration. Accumulation of cAMP in hypertrophic ventricular myocytes from Dahl S rats was less responsive to isoproterenol stimulation than Dahl R ventricular myocytes. Moreover, ATII inhibited isoproterenol-induced cAMP accumulation in Dahl R but increased isoproterenol-induced cAMP accumulation in hypertrophic Dahl S myocytes. The enhancement of isoproterenol stimulation of cAMP accumulation by ATII was positively related to the degree of cardiac hypertrophy. ATII stimulation alone did not alter cAMP concentrations. The reversal of the inhibitory action of ATII points to an alteration in the signal transduction of beta-receptor stimulation in the hypertrophic heart and is of potential importance in defining the role of ATII in cardiac hypertrophy.
b) Introduction

The beta-adrenergic agonist, isoproterenol, is capable of causing cardiac hypertrophy in rats, an effect which likely occurs through beta-receptor mediated elevation in cAMP (Zimmer, 1984). This is supported by studies directly implicating increases in cAMP in the process leading to cardiac hypertrophy (Xenophantos et al., 1989). Increasing the load on ventricular muscle elicits rapid increases in cardiac cAMP content concommitant with increased rates of protein synthesis and ribosome formation (Singh, 1982). Pre-treatment of hearts with the muscarinic agonist, methacholine, blocks pressure-induced increases in cAMP and blocks increases in protein synthesis (Haneda et al., 1990). Thus, cAMP-linked protein synthesis in the cardiac cell is subject to both positive and negative control and may be one of the underlying mechanisms by which the myocyte undergoes hypertrophy (for review see Morgan and Baker, 1991).

The signal transduction of ATII stimulation of cardiac myocytes involves increase in calcium current, increase in cytosolic inositol mono- and bi-phosphate accumulation (Allen et al., 1988) and activation of protein kinase C (Dosemici et al., 1988; Urabe et al., 1990). ATII is also functionally linked to the adenylyl cyclase-cAMP axis through an inhibitory mechanism (Allen et al., 1988; Anand-Srivastava, 1989). In normal rat ventricular myocytes, the beta receptor stimulates adenylyl cyclase activity through the stimulatory guanyl nucleotide binding protein (Gs) (Gilman, 1984), whereas stimulation of ATII and muscarinic receptors inhibit adenylyl cyclase through a pertussis toxin sensitive G protein (Gi) (Allen et al., 1988; Anand-Srivastava, 1989; Buxton and Brunton, 1985). In turn, the G protein complex may be subject to both developmental regulation (Allen et al., 1988) as well as down-regulation by chronic exposure to catecholamines (Reithmann et al., 1989) and pertussis toxin (Watkins et al., 1989).

In this section, ATII effects on cAMP generation in the non-hypertrophic Dahl R, non-hypertrophic Dahl S and hypertrophic Dahl S ventricular myocyte were studied.
c) Results

i. Cardiac Hypertrophy in the Dahl S Rat

Dahl S rats fed high salt diet developed ventricular hypertrophy as determined by increased ventricular weight relative to total body weight (Figure 2). The ventricular weight to body weight ratio of Dahl S rats fed high NaCl diets was 7.51 ± 0.428 g/kg (means ± S.E.M.), which was significantly (P < 0.05, n=28) greater than identically treated Dahl R rats which displayed a ratio of 4.82 ± 0.52 g/kg. Furthermore, there was a significant (P <0.05) relationship (r=0.754) between duration of high NaCl diet and the degree of ventricular hypertrophy in Dahl S rats. Age and sex matched inbred Dahl R rats fed the identical diet maintained a constant ventricular weight to body weight ratio with no signs of ventricular hypertrophy.

ii. Increased Cell Size in Hypertrophic Dahl S Ventricular Myocytes

Isolated ventricular myocytes were between 75 and 85% viable as determined by trypan blue exclusion. Ventricular myocytes were characteristically rod-shaped and quiescent (Figure 3).

Isolated ventricular myocytes were subjected to Fluorescence Activated Cell Sorting (FACS) analysis and compared. The amount of forward light scatter is directly proportional to the cell size. The forward light scatter was analyzed into 256 channels so that a higher channel number reflects larger cell size.

Ventricular myocytes from Dahl S hypertrophic hearts were larger than myocytes from normal Dahl R hearts (Figure 4). Dahl S myocytes from a heart with a ventricle/body weight of 7.8 g/kg displayed a relative cell size which placed them in a mean peak channel of 43.42 ± 5.95 (± S.D.). This compared to Dahl R myocytes from a heart with a ventricle/body weight of 4.88 g/kg which displayed a relative cell size which placed them in a lower mean peak channel of 25.49 ± 2.09 (± S.D.). The number of cells counted within the window was greater than $10^4$. 
Figure 2. Development of ventricular hypertrophy in the inbred Dahl SR/Jr and SS/Jr rat.

Rats were fed powdered chow supplemented with 6% NaCl beginning in their third week of life. SR rats (open triangles) maintained a constant ventricle to body weight ratio throughout the course of high salt feeding. SS rats (closed circles) displayed an increased ventricular to body weight eventually leading to early death. The regression line ($r = 0.754$) relationship between the ventricle/body weight index and the time on 6% NaCl diet is displayed.
Figure 3. Phase contrast micrograph of isolated ventricular myocytes from a hypertrophic Dahl S rat heart.

Myocytes from a hypertrophic heart from a Dahl S rat were isolated as described in Materials and Methods. Cells were dispersed on slide unfixed and photographed at 630X magnification using phase contrast optics.
Figure 4. FACS analysis of populations of myocytes from hypertrophic Dahl S hearts and normal Dahl R hearts.

Myocytes were isolated from Dahl S hearts and Dahl R hearts and subjected to cell size analysis. The distribution of cell size was analyzed into 256 channels. A higher channel number indicates larger cell size. A window of channel 120 to channel 250 was examined. Dahl S ventricular myocytes (upper graph) display a peak cell count which falls into channel 40 with a channel mean of 43.42 ± 5.95 (S.D.). Dahl R ventricular myocytes (lower graph) display a peak cell count size which falls into channel 24 and a channel mean of 25.49 ± 2.09 (S.D.). The ordinate lacks a numerical because scaling by the analyzer is adjusted with the count, however the area under the curves represent 10,578 Dahl S cells counted, and 22,767 Dahl R cells counted within the channel 120 - channel 250 window.
iii. Response of Dahl R and Dahl S Ventricular Myocyte cAMP to Isoproterenol Concentrations

Isoproterenol produced a significant (P<0.05), concentration-dependent increase, compared to untreated controls, in cAMP in intact ventricular myocytes from Dahl R rats (Figure 5). In contrast, the response of hypertrophic Dahl S myocytes was significantly (P<0.05) less than the response of ventricular myocytes from age and diet-matched Dahl R rats.

iv. Difference in cAMP Response to Isoproterenol in Populations of Dahl R and Hypertrophic Dahl S Hearts

cAMP accumulation after isoproterenol stimulation was significantly (P < 0.05) lower in hypertrophic myocytes from Dahl S myocytes with a mean of 27.67 ± 3.8 pmol/cAMP/mg protein/min compared to myocytes from non-hypertrophic hearts from Dahl R rats, which displayed a mean cAMP accumulation of 48.29 ± 5.5 pmol cAMP/mg protein/min (Figure 6).

v. Isoproterenol-induced Changes in cAMP levels After Pre-treatment with ATII

Pre-treatment of myocytes with ATII (1 uM) for 5 min led to a significantly (P<0.05) lower cAMP response of R myocytes at all concentrations of isoproterenol compared to R myocytes that were not exposed to ATII (Figure 7a). Myocytes from Dahl S rat hearts without hypertrophy displayed no ATII-induced depression of cAMP accumulation in response to isoproterenol (Figure 7b). In contrast, hypertrophic S ventricular myocytes respond to ATII pre-incubation with an significantly (P<0.05) elevated stimulation of cAMP levels (Figure 7c).

vi. Effect of ATII Alone on cAMP Levels

ATII alone did not significantly alter basal levels of cAMP in Dahl R or hypertrophic Dahl S or myocytes while in cells from the same pair of hearts, isoproterenol caused an approximately 2-fold increase in hypertrophic Dahl S and a 4-fold increase in Dahl R (Figure 8).
Figure 5. Hypertrophic Dahl S myocytes display diminished cAMP increases in response to isoproterenol stimulation.
Viable ventricular myocytes from Dahl R with ventricle to body weight index of 4.9 g/kg (open circles) or hypertrophic Dahl S with ventricle to body weight index of 7.4 g/kg (solid circles) were stimulated with the indicated concentrations of isoproterenol for 1 min. cAMP and protein levels were determined as described in Materials and Methods. Each point represents the mean ± SEM. Experiments were performed seven times in triplicate with similar results.
Figure 6. Relationship between cAMP response to isoproterenol and ventricle to body weight ratio in Dahl R and Dahl S rats.

The cAMP accumulation in response to 1 min, 1 uM isoproterenol stimulation was determined in a group of Dahl S (open circles) and Dahl R (closed circles) rats as described in materials and methods. Each point represents the mean ± SEM for a single ventricle. Each sample was assayed in triplicate.
Figure 7. **Effect of ATII on isoproterenol induced cAMP in Dahl R and S myocytes.** Ventricular myocytes from a Dahl R rat with ventricle to body weight index of 4.9 g/kg (panel a), and Dahl S rat with ventricle to body weight ratio of 5.2 g/kg (panel b) and a hypertrophic Dahl S ventricle with ventricle to body weight of 8.1 g/kg (panel c), were treated with ATII (solid circles) or no ATII (open circles) for 5 min prior to 1 min stimulation with 1 uM isoproterenol. Each point represents the mean ± SEM. Experiments were performed in three times in triplicate.
isoproterenol concentration (log M)

(a) cAMP (pmol/mg protein)

(b) cAMP (pmol/mg protein)

(c) cAMP (pmol/mg protein)
Figure 8. **ATII alone had no effect on cAMP levels in Dahl R or S myocytes.** Ventricular myocytes from Dahl R or hypertrophic Dahl S rats were treated with 1 μM isoproterenol for 1 min, with 1 μM ATII for 5 min followed by 1 μM isoproterenol for 1 min or with 1 μM ATII for 5 min. Each point represents the mean ± SEM. Experiments were done three times in triplicate.
vii. Relationship of ATII Elevation of cAMP Stimulation to Cardiac Hypertrophy

The stimulation of cAMP accumulation by ATII in isoproterenol-stimulated hypertrophic myocytes was directly related to the degree of ventricular hypertrophy in Dahl S rats (Figure 9 - upper). Hearts from Dahl R rats, which had a smaller range of heart to body weight index, displayed consistent ATII inhibition of isoproterenol-stimulated cAMP accumulation (Figure 9 - lower). Basal levels of cAMP were similar, but not consistent between individual hearts. Therefore cAMP levels after isoproterenol stimulation were standardized by dividing by basal cAMP levels.

d) Discussion

Cardiac hypertrophy was indicated by an increase in the ventricular to body weight index. The assumption that ventricular weight to body weight index is indicative of ventricular myocyte size has been validated in studies in which various techniques have shown that the remodeled, enlarged heart consists of myocyte hypertrophy (Smith et al., 1988; Zak, 1974). Furthermore, it is known that ventricular cardiomyocytes cease to divide during the first few weeks after birth (Zak, 1984) and respond to increased demands upon the heart by increasing myocyte volume. However, the increased myocyte size of the hypertrophic Dahl S heart, as compared to the non-hypertrophic Dahl R heart, was confirmed by FACS analysis.

These experiments demonstrate that hypertrophic ventricular myocytes isolated from Dahl S rats accumulate significantly less cAMP in response to isoproterenol stimulation compared to control myocytes from age and sex matched Dahl R rats. Secondly, ATII causes a distinct enhancement of isoproterenol-stimulated increase in cAMP concentration in hypertrophic myocytes. In contrast, ATII inhibits isoproterenol-induced cAMP accumulation in R myocytes or caused no significant change in non-hypertrophic S myocytes. ATII alone had no effect on cAMP in either R or hypertrophic S myocytes.

Diminished response to isoproterenol has been previously noted in other models of cardiac hypertrophy after aortic constriction (Foster et al., 1991), renal vascular hypertension
Figure 9. ATII enhancement of isoproterenol stimulation of cAMP was a function of cardiac hypertrophy.
Concentration of cAMP after stimulation for 1 min was divided by the basal concentration of cAMP in order to standardize basal cAMP levels between individual hearts. Effect of ATII was inhibitory in Dahl R myocytes (lower panel) and stimulatory in Dahl S myocytes (upper panel). Each point represents the mean ± SEM. Each point represents myocytes from one heart assayed in triplicate.
in rat (Ayobe and Tarazi, 1983) and in the spontaneously hypertensive rat (Saragoca and Tarazi, 1981). In each of these studies, contractile function of the ventricle was found to be lowered in the hypertrophic heart. The present study shows that diminished cAMP signalling occurs in hypertrophy in the Dahl salt sensitive model of hypertension. In addition this study was carried out using isolated ventricular myocytes, which closely links alterations in signal transduction via cAMP, to direct effects on hypertrophic myocytes rather than on other components of the heart.

Diminished adenylyl cyclase activation in response to beta-receptor stimulation has also been observed in experimental models of cardiac hypertrophy including the renal hypertensive rat (Anand-Srivastava et al., 1983) and in the Spontaneously Hypertensive Rat (Upsher and Khairallah, 1985). The mechanism of diminution of cAMP response was attributed to reduced beta receptor density (Upsher and Khairallah, 1985; Limas and Limas, 1978) but may also be due to alterations in stimulatory G proteins (Feldman et al.1990). At present it is not clear whether beta-receptor down-regulation or G protein alterations are involved in the cellular mechanism that gives rise to the observed differences in the response to isoproterenol between R and hypertrophic S myocytes.

The most important aspect of the present study is that ATII enhanced isoproterenol-stimulated cAMP in hypertrophic Dahl S ventricular myocytes. The inhibitory action of ATII in Dahl R ventricular myocytes is consistent with reports in which ATII was found to inhibit adenylyl cyclase in ventricular sarcolemmal membranes through a pertussis toxin-sensitive inhibitory G protein (Allen et al., 1991; Anand-Srivastava, 1989). To my knowledge, this is the first data to demonstrate the reversal in functional capacity of adenylyl cyclase in the heart in response to stimulation by ATII. Since this is an ATII mediated effect, it likely involves a beta receptor-independent mechanism. The enhancement by ATII of cAMP accumulation of isoproterenol-stimulated cells in Dahl S myocytes was clearly a function of cardiac hypertrophy, but it is not known from these experiments whether the observed ATII effect on cAMP was linked causally to the hypertrophic process. However, ATII has been associated with cell growth (Schelling et al., 1991). Studies in which [Sar¹]angiotensin II was
observed to stimulate protein synthesis in growing chick cardiac cell cultures have demonstrated that ATII is a potent cardiac cell growth factor (Aceto and Baker, 1990). Other direct evidence for ATII involvement in the hypertrophic process has come from in vivo studies in rats in which it was demonstrated that the ATII antagonist [Sar\textsuperscript{1} Ile\textsubscript{8}] angiotensin was able to produce cardiac hypertrophy at sub-pressor doses (Khairallah and Kanabus, 1983). Furthermore, angiotensin converting enzyme inhibitors can prevent or cause regression of the hypertrophic process independent of hemodynamic changes (Kromer and Riegger, 1988).

In conclusion, these results demonstrate that in hypertrophic ventricular myocytes, ATII enhances isoproterenol-induced increase in cAMP concentration. Since both ATII and cAMP have been implicated in cardiac growth, this work provides support and a possible, hitherto unreported, association between them. This suggests a mechanism by which ATII, by enhancing intracellular cAMP accumulation, may play a role in the control of ventricular hypertrophy.
2. STUDIES INTO THE MECHANISM OF ANGIOTENSIN II STIMULATION OF
cAMP IN THE HYPERTROPHIC DAHL VENTRICULAR MYOCYTE

a) Summary

The purpose of this study was to explore the action of ATII on the modulation of cAMP
generation in the hypertrophic myocardium. Ventricular myocytes were isolated from inbred Dahl
S and Dahl R rats which had been on a diet supplemented with 6% NaCl. In the hypertrophic
Dahl S rat, inhibition of cAMP generation by ATII is reversed such that forskolin-stimulated
cAMP generation is increased 2.5-fold after 1 uM ATII incubation. Thus, the mechanism of
ATII enhancement of cAMP generation in the hypertrophic heart was investigated. The
phosphodiesterase inhibitor, isobutyl-1-methyl xanthine, while increasing intracellular cAMP
generally, did not abolish the enhancement process by ATII in the hypertrophic myocyte. The
presence of 1 uM carbamyl choline induced a similar enhancement of cAMP accumulation in
hypertrophic myocytes as did ATII. Pertussis toxin, while abolishing both inhibition or
enhancement after 4 hr, abolished the enhancement but not the inhibition of adenylyl cyclase
after 1 h. The hypertrophic Dahl S ventricular myocyte contained 67% less Gs alpha and 40%
less Gi alpha than Dahl R ventricular myocytes as determined by in vitro ADP ribosylation in
the presence of cholera toxin and pertussis toxin, respectively. De novo synthesis of Gi alpha,
as measured by [35S]methionine incorporation, was lower by 34% in hypertrophic myocytes. The
reversal of control of cAMP generation was reflected in contractile performance of isolated
hearts as ATII enhanced the action of forskolin perfusion to increase the left ventricular
developed pressure in the hypertrophic heart but decreased it in the control heart. The phorbol
ester PMA was used to abolish ATII-induced inhibition of cAMP generation in the Dahl R and
stimulation of cAMP in the hypertrophic Dahl S myocytes, implicating protein kinase C in the
ATII mediated cAMP regulation. Furthermore, the inhibition and enhancement were mimicked
by exogenous phosphatidylcholine-specific phospholipase C, suggesting a possible role of
phosphatidylcholine hydrolysis in the signalling pathway.
In conclusion, these data indicate that reversal of control of cAMP accumulation in the hypertrophic Dahl S heart is due to changes in the generative control of cAMP which is based in part on alterations in the G protein complex but also on other probable divergences in ATII signalling through protein kinase C.
b) Introduction

The accompanying study on the beta receptor-adenylyl cyclase axis demonstrated that ATII significantly enhances beta-receptor stimulation of the second messenger cAMP in cardiac hypertrophy, as distinct from the inhibitory influence observed in normal myocytes. Furthermore, this reversal was shown to be dependent upon the degree of cardiac hypertrophy (Sunga and Rabkin, 1991). The enhancement of cAMP generation by ATII in the hypertrophic Dahl S should be of importance in the hypertrophic process of the cardiac myocyte since cAMP content and protein kinase A activity have been linked to increased rates of ribosome formation and protein synthesis in the rat ventricle (Xenophantos et al., 1989; Haneda et al., 1990; Watson et al., 1989). Adenylyl cyclase activity has also been found to be involved in the regulation of growth and proliferation of vascular smooth muscle in culture (Boynton and Whitfield, 1983; Franks et al., 1984). In the present studies the cellular mechanism and functional consequences of ATII-induced cAMP accumulation in the hypertrophic Dahl S rat myocardium were explored.

c) Results

i. Forskolin Stimulation of Dahl R and Hypertrophic Dahl S Ventricular Myocytes

Ventricular myocytes were stimulated with forskolin, which directly activates adenylyl cyclase (Seamons et al, 1981; Seamons and Daly, 1981). Forskolin produced a concentration-dependent increase in cAMP (Figure 10). The response of hypertrophic Dahl S myocytes (Figure 10a) was significantly (P<0.05) less than that of Dahl R myocytes, which indicated lower responsiveness of the adenylyl cyclase system in myocytes from the hypertrophic heart.

Treatment of Dahl R myocytes with 1 uM ATII for 5 min caused a significant (P<0.05) lowering of the response of cAMP concentration to forskolin compared to controls stimulated with forskolin. This ATII effect was evident at forskolin concentrations of $10^5$ M and higher (Figure 10b). In contrast, ATII treated hypertrophic S cardiac myocytes display a significant (P<0.05) elevation of cAMP concentrations at concentrations of
Figure 10. Forskolin stimulation of cAMP accumulation in Dahl SR and hypertrophic Dahl SS ventricular myocytes.

Ventricular myocytes from hypertrophic Dahl S (7.51 ± 1.97 g/kg mean ventricular/body weight ± S.D.) (panel a) or Dahl R (4.82 ± 0.52 g/kg) (panel b) were exposed to different concentrations of forskolin for 2 min after pre-incubation with 1 uM ATII (closed circles) or without ATII (open circles) for 5 min. Cellular cAMP concentration and protein content was determined as described in Materials and Methods section. Points are means ± SEM. Experiment was performed using three matched pairs of Dahl R and Dahl S rats assayed in triplicate.
Figure 11. Time course of ATII inhibition or enhancement of forskolin stimulation of cAMP generation.

Ventricular myocytes from hypertrophic hearts of Dahl S rats (panel a) or normal hearts of Dahl R rats (panel b) were exposed to 1 uM ATII for different times, followed by 10 uM forskolin for 2 min. Cellular cAMP and protein content was determined as described in Materials and Methods section. Points are means ± SEM. Experiments were repeated in duplicate on three pairs of matched animals.
forskolin of $10^{-5}$ M or higher (Figure 10a). The 2 min time point for forskolin treatment was based upon preliminary studies in which a rapid cAMP accumulation was observed. The 5 min time period for ATII incubation was based upon studies of the time course of the ATII-induced increase in cAMP in which hypertrophic Dahl S myocytes showed a peak effect at 5 min (Figure 11a). In contrast, the time course of inhibition in Dahl R myocytes displayed a maximal lowering of cAMP stimulation at 30 min of incubation with ATII (Figure 11b).

ii. **Attenuation of ATII Effect on cAMP accumulation with the ATII receptor antagonist Saralasin**

Saralasin (Sar\(^1\)Ala\(^8\) angiotensin II), an antagonist of the ATII receptor, was used to determine the specificity of ATII action on cAMP accumulation. Saralasin was found to be effective in attenuating both the inhibition of cAMP accumulation in Dahl R myocytes and the enhancement of cAMP accumulation in Dahl S myocytes. All samples were treated with 10 uM forskolin, for 2 min. ATII increased forskolin stimulation of cAMP in the S myocytes by 78 ± 9% (S.E.M.) but in the presence of the 10 uM saralasin and 1 uM ATII the increase in forskolin was 28 ± 10% (S.E.M.) (Figure 12). ATII decreased cAMP accumulation in the Dahl R myocytes by 30 ± 8% but with the addition of saralasin this decrease was 16 ± 5%. Saralasin alone had no significant effect on either the increase or decrease of cAMP accumulation.

iii. **ADP-ribosylation of Gs alpha**

The presence of stimulatory subunit (Gs) of the G protein complex from normal and hypertrophic hearts was probed for by using in vitro \([^{32}\text{P}]\)ADP-ribosylation of Gs in the presence of cholera toxin. A Mr = 45,000 protein which was ADP-ribosylated in the presence of cholera toxin displayed varying \([^{32}\text{P}]\) content, as assessed by video densitometry, in relation to the concentration of cholera toxin in the incubation mixture (Figure 13). A Mr = 36,000 band was unresponsive to cholera toxin concentration and was therefore used as an internal reference. Hypertrophic Dahl S cardiac membranes displayed a lower response to cholera toxin at 25 ug/ml of cholera toxin than did Dahl R cardiac membranes. At 25 ug/ml cholera toxin, the hypertrophic Dahl S membranes contained 67% less ADP.
Figure 12. Attenuation of ATII-induced stimulation of cAMP by Saralasin. Ventricular myocytes from hypertrophic Dahl S and normal Dahl R rat hearts were exposed to 1 uM ATII for 5 min or to 10 uM Saralasin for 10 min with 1 uM ATII added for the last 5 min. All samples were stimulated with 1 uM forskolin for 2 min. Points represent the mean ± 1/2 the range of means. Experiments were done two times in triplicate.
Figure 13a. **In vitro ADP-ribosylation of Gs in hypertrophic ventricular myocytes.**
Sarcolemmal membranes from Dahl R (5.4 g/kg ventricular/body weight) and hypertrophic Dahl S ventricular myocytes (8.1 g/kg ventricular/body weight) were incubated in different concentrations of cholera toxin in ADP-ribosylation mixture as described in Materials and Methods. 5 ug of ADP-ribosylated protein was electrophoresed per lane in 10.5 % SDS gels and autoradiographed for 7 days. Lane 1-6: Dahl R. Lane 7-12: Dahl S. Lanes contained the following concentrations of cholera toxin: 1 and 7 - none, 2 and 8 - 25 ug/ml, 3 and 9 - 5 ug/ml, 4 and 10 - 1 ug/ml, 5 and 11 - 0.2 ug/ml, 6 and 12 - 0.1 ug/ml. Experiments were repeated on three pairs of animals of which this data is representative.

Figure 13b. **Densitometric analysis of ADP-ribosylation concentration response curves.**
R45 and R36 designate, respectively, the 45 kDa and 36 kDa cholera toxin substrate from Dahl R ventricular myocytes. S45 and S36 designate, respectively, the 45 kDa and 36 kDa cholera toxin substrate from hypertrophic Dahl S ventricular myocytes.
Cholera Toxin Concentration (ug/ml)

O.D. (arbitrary units)

Cholera Toxin Concentration (ug/ml)
riboylated Mr = 45,000 substrate than Dahl R membranes (Figure 13b).

iv. **Determination of de novo Synthesis of Gs alpha in Dahl R and Dahl S Cardiomyocytes**

An assessment of the relative synthesis of Gs alpha was carried out by pre-labelling Dahl R and hypertrophic Dahl S myocytes with $[^{35}S]$methionine and detecting immunoprecipitated protein by SDS polyacrylamide electrophoresis and autoradiography. A distinct 45 kDa band was present in radiographs of both Dahl R (Figure 14 lane 1-3) and hypertrophic Dahl S (Figure 14 lane 7-9). The 45 kDa band was apparent in immunoprecipitates obtained using a polyclonal antibody against rat brain Gs alpha subunit (RM-1 antibody) but not in immunoprecipitates obtained using a polyclonal antibody against rat brain G beta subunit (MS-1 antibody) (Figure 14 lane 4-6 and 10-12). This indicated that the RM-1 immunoprecipitate was indeed cardiac Gs-alpha subunit. Results of densitometric analysis of the 45 kDa radiographic band indicated that immunoprecipitate from the hypertrophic Dahl S myocytes contained $14\pm4\%$ less $[^{35}S]$methionine than Dahl R myocytes.

v. **Effect of Phosphodiesterase Inhibition on Angiotensin II Enhancement of cAMP Accumulation**

To assess the contribution of phosphodiesterase to the ATII-induced elevation of cAMP accumulation observed in hypertrophic myocytes, cell samples were exposed to the phosphodiesterase inhibitor 3-isobutyl-1-methyl xanthine (IBMX), 1 uM, for 5 min or 1 h. In all cell samples, adenylyl cyclase activity was stimulated with 10 uM forskolin for 1 min. In all samples, IBMX treatment for 5 min or 60 min increased overall cAMP concentration. ATII inducement of increase in cAMP accumulation in hypertrophic Dahl S myocytes was not dependent upon the presence of IBMX (Figure 15a). In the absence of IBMX, accumulation of cAMP in the presence of ATII was increased significantly compared to non-ATII samples ($P<0.05$) by $110\pm4\%$ (SEM). After 5 min treatment with IBMX, ATII treatment induced an elevation of accumulated cAMP by 4%, which was not significant. However, after 60 min treatment with IBMX, cAMP accumulation was elevated significantly.
Figure 14. \[^{35}\text{S}]\text{methionine incorporation into immunoprecipitated Gs alpha.}\n
Ventricular myocytes from hypertrophic Dahl R hearts (lanes 1-6) and Dahl S hearts (lanes 7-12) were pre-labelled with 60 uCi/ml \[^{35}\text{S}]\text{methionine for 2 hr prior to immunoprecipitation, 10.5\% polyacrylamid gel electrophoresis, and autoradiography.}\n
Two polyclonal antibodies were used in the immunoprecipitation: RM-1, raised against rat brain Gs alpha, lanes 1-3 and lanes 7-9 and MS-1, raised against rat brain G beta sub-unit, lanes 4-6 and lanes 10-12. The 45 kDa marker indicates the molecular weight of Gs alpha subunit. Experiments were done 3 times in triplicate, with similar results.
compared to IBMX treated, non-ATII treated samples (P<0.05) by 140 ± 12%.

Treatment with IBMX did not affect the lowering of cAMP accumulation in Dahl R myocytes. In non-IBMX treated Dahl R myocytes, ATII lowered cAMP accumulation by 20 ± 3% whereas after 5 min IBMX treatment, ATII lowered cAMP accumulation significantly (P<0.05) by 35 ± 6% compared to non-ATII-treated samples. After 60 min pre-treatment with IBMX, ATII treatment lowered accumulated cAMP by 9 ± 2%, which was not significant (Figure 15b).

vi. Effect of Muscarinic Receptor Stimulation on Dahl R and Hypertrophic Dahl S Ventricular Myocyte cAMP Generation

The ATII effect on cAMP accumulation in the hypertrophic myocyte was compared to the effect of muscarinic receptor stimulation, since both ATII receptor and muscarinic receptor have been observed to operate through an adenylyl cyclase inhibitory pathway in cardiac myocytes (Allen et al., 1988).

In all samples in this series, adenylyl cyclase activity was stimulated with 10 uM isoproterenol for 1 min. Treatment of hypertrophic Dahl S myocytes with 1 uM carbachol increased the level of cAMP by 138 ± 12% significantly (P<0.05) when compared to samples without carbachol treatment (Figure 16). ATII was less effective than carbachol at lowering cAMP accumulation, but the difference between the ATII and carbachol effect was not significant. ATII, 1 uM, significantly (P<0.05) increased cAMP accumulation by 102 ± 2% compared to samples without ATII treatment.

Treatment of isolated Dahl R myocytes for 10 min with 1 uM carbachol resulted in significant (P<0.05) lowering of cAMP generation by 23 ± 7% compared to samples not treated with carbachol. ATII treatment of these samples was more effective than carbachol in lowering cAMP accumulation in Dahl R myocytes, lowering cAMP accumulation significantly (P<0.05) by 46 ± 6% compared to samples not treated with ATII (Figure 16).

Carbachol plus ATII treatment did not result in additive elevations or lowering of cAMP accumulation in hypertrophic Dahl S or Dahl R myocytes. Carbachol plus ATII treatment of hypertrophic Dahl S myocytes significantly (P<0.05) increased cAMP
accumulation by $123 \pm 10\%$ compared to samples treated with neither ATII nor carbachol. This was not significantly different ($P>0.05$) from the value obtained using ATII or carbachol alone. Carbachol and ATII together significantly ($P<0.05$) lowered intracellular Dahl R myocyte cAMP generation by $38 \pm 5\%$ compared to samples with neither carbachol or ATII (Figure 16). This was not significantly different from the value obtained using ATII alone. This suggests that the component conferring depression of stimulation and reversal of inhibition of cAMP response in the hypertrophic myocytes was downstream of the ATII and muscarinic receptors.

**vii. Pertussis Toxin Sensitivity of Dahl R and Hypertrophic Dahl S Ventricular Myocytes**

Since alterations in the function of G proteins have been observed in other models of cardiac hypertrophy (Foster et al., 1991; Anand-Srivastava et al., 1983), the following hypothesis was tested: that the reversal of adenylyl cyclase activation in Dahl S rats was due to functional alteration in the pertussis toxin sensitive inhibitory G protein (Gi), which has been linked to both ATII (Allen et al., 1988; Anand-Srivastava, 1989) and muscarinic receptors (Allen et al., 1988) in rat ventricle.

All the samples in this series were stimulated with 10 uM isoproterenol for 1 min. Those treated with 1 uM ATII were treated for 5 min before the isoproterenol treatment. Hypertrophic Dahl S myocytes which were treated for 1 h with 50 ng/ml pertussis toxin displayed a 3-fold increase in cAMP accumulation compared to samples without pertussis toxin. The presence of ATII made no difference to cAMP accumulation after 1 h pertussis toxin treatment (Figure 17a). Further treatment with pertussis toxin for 4 h (Figure 17b) had no further effect on abolishing the ATII increase of cAMP accumulation in hypertrophic Dahl S rats.

In contrast, Dahl R myocytes which were incubated with pertussis toxin for 1 hr still displayed a lowering of cAMP accumulation by 30% in the presence of ATII (Figure 17a). However, after 4 h pertussis toxin treatment, inhibition of adenylyl cyclase activation by ATII was abolished in Dahl R ventricular myocytes (Fig.17b).
Figure 15. Effect of phosphodiesterase inhibition on reversal of cAMP inhibition in the hypertrophic ventricular myocyte.

Ventricular myocytes from hypertrophic Dahl S hearts (panel a) or a Dahl R heart (panel b) were incubated for 5 or 60 min in 1 uM isobutylmethyl xanthine (IBMX) without 1 uM ATII (filled area) or with 1 uM ATII (diagonal lined area) for the last 5 min. Samples were then exposed to 10 uM isoproterenol for 1 min. Cellular cAMP and protein were determined as described in Materials and Methods section. Experiments were done three times in triplicate. Each bar represents mean ± SEM.
Figure 16. Reversal of inhibition of cAMP generation by muscarinic receptor stimulation in the ventricular myocyte from hypertrophic hearts.
Ventricular myocytes from Dahl R hearts (filled areas) or from hypertrophic Dahl S hearts (hatched) were incubated for 5 min in 1 μM ATII or 1 μM carbamyl choline before being stimulated with 10 μM isoproterenol for 1 min. Experiments were performed in triplicate on three pairs of matched animals. Each bar represents the mean ± SEM.
Figure 17. Effect of pertussis toxin incubation on enhancement of cAMP generation in the hypertrophic heart.
Ventricular myocytes from Dahl R (filled areas) or hypertrophic Dahl S (open areas) hearts were incubated with 50 ng/ml pertussis toxin for 1 hr (panel a) or 4 hrs (panel b) prior to 1 uM ATII incubation for 5 min plus 10 uM isoproterenol incubation for 1 min. Cellular cAMP and protein were determined as described in Materials and Methods section. Experiments were in triplicate on two pairs of matched animals. Each bar represents the mean ± 1/2 the range of means.
viii. **In vitro-ADP Ribosylation of Gi alpha**

Based on the functional assessment of the pertussis toxin sensitive inhibitory G proteins, analysis of Gi alpha was carried out. Two observations prompted this experiment: first, that the response of the hypertrophic Dahl S myocytes was more sensitive to pertussis toxin than Dahl R myocytes. Second, that the level of cAMP after pertussis toxin treatment and ATII inhibition/enhancement of adenylyl cyclase, was equal in Dahl R and hypertrophic Dahl S myocytes. The basis of the action of pertussis toxin is the ADP-ribosylation of a cysteine residue four removed from the carboxyl terminus of the Gi alpha subunit (Gilman, 1987). To quantitate for Gi protein, membrane proteins were prepared from ventricular myocytes from Dahl R and hypertrophic Dahl S hearts and used as substrates for in vitro $[^{32}P]$ADP-ribosylation by pertussis toxin.

Pertussis toxin catalyzed ADP-ribosylation of a diffuse band between $M_r$ = 41,000 and 43,000 band in both Dahl R and hypertrophic S myocytes (Figure 18a). By varying the concentrations of pertussis toxin it was possible to generate concentration-response curves of the ADP-ribosylation reaction in Dahl R and hypertrophic S myocytes. Lower levels of ADP-ribosylated substrates, as quantitated by video densitometry, were observed in the hypertrophic Dahl S myocyte from concentrations of 10 ng/ml to 50 ng/ml pertussis toxin. An $M_r$ = 31,000 band was used as an internal reference (Figure 18b). At 50 ng/ml pertussis toxin, the level of ADP-ribosylated substrate was found to be 40 % less in the hypertrophic Dahl S membranes in comparison to Dahl R.

ix. **Contractile Properties of R and Hypertrophic S Hearts in Response to ATII**

The hypothesis underlying this series of experiments was that the observed differences in cAMP response between Dahl R and hypertrophic Dahl S hearts had functional ramifications. To test this, isolated hearts were assessed in a modified Langendorf preparation. Perfused hearts received a range of forskolin concentrations, in the absence or presence of ATII. In Dahl S hearts ATII displayed a significant (P<0.05) increase in the response to forskolin (Figure 19a) with respect to left ventricular developed pressure over
Figure 18a. **In vitro** ADP ribosylation of Gi alpha of ventricular myocytes from hypertrophic heart.

Sarcolemmal membranes from ventricular myocytes of Dahl R or hypertrophic Dahl S hearts were incubated in different concentrations of pertussis toxin and ADP ribosylation mixture as described in Materials and Methods section. 5 ug of ADP ribosylated proteins were electrophoresed on 10.5% SDS gels and autoradiographed. Lane 1 - 5: Dahl R, Lanes 6-9: Dahl S. Samples contained the following concentrations of pertussis toxin: Lane 1 and 6 - none, 2 and 7 - 50 ug/ml, 3 and 8 - 25 ug/ml pertussis toxin, 4 and 9 - 12.5 ug/ml, Lane 5 contained both cholera toxin and pertussis toxin. Experiments were performed on four pairs of animals with similar results.

Figure 18b. Densitometric analysis of ADP-ribosylation pertussis toxin concentration-response curves.

R42 and R31 designate, respectively, the 42 kDa and 31 kDa pertussis toxin substrates from Dahl R ventricular myocytes. S42 and S31 designate, respectively, the 42 kDa and 31 kDa pertussis toxin substrates from hypertrophic Dahl S ventricular myocytes.
Figure 18c. Comparison of optical density of ADP ribosylation 45 kDa cholera toxin substrate in Dahl R and Dahl S sarcolemmal membranes with ADP ribosylation of 42 kDa pertussis toxin substrate.

In vitro ADP ribosylation of sarcolemmal membranes from Dahl R and S ventricular myocytes was carried out in the presence of 25 ug/ml cholera toxin (upper panel) or 50 ug/ml pertussis toxin (lower panel) as described in Materials and Methods. Dahl R and Dahl S are indicated by 'R' and 'S', respectively. Points are means ± SEM.
Figure 19. Analysis of contractile function of isolated hearts from Dahl R and Dahl S rats.

Isolated hearts from Dahl S (panel A) and Dahl R (panel B) rats were prepared as described in detail in Materials and Methods section. Hearts were treated with a range of forskolin concentrations without (open circles) or with (closed circles) ATII and left ventricular developed pressure was measured. Experiments were carried out on three pairs of matched animals with similar results. Each point is the mean ± SEM.
a range of forskolin concentrations. In the Dahl R rat ATII treatment significantly depressed left ventricular developed pressure (P < 0.05) in comparison to non-ATII treated, forskolin-stimulated developed left ventricular pressure (Figure 19b).

x. **Phorbol 12,13 Myristyl Acetate Effects on ATII Reversal of cAMP Regulation**

The phorbol ester PMA was used to probe for the involvement of protein kinase C in the inhibition or enhancement of cAMP generation. This was based in part on results in 3T3 fibroblasts in which inhibition of adenylyl cyclase by carbachol was found to be dependent on protein kinase C (Diaz-Laviada et al., 1991), and in part on the reported role of protein kinase C in the signal transduction of ATII in heart (Allen et al., 1988; Dosemici et al., 1988; Hoh et al., 1990).

In this experiment cells were treated with PMA with the objective of down-regulating protein kinase C. All the samples were treated with 10 uM forskolin for 2 min. In the absence of PMA, 1 uM ATII elevated cAMP accumulation significantly by 50 % in hypertrophic Dahl S myocytes (Figure 20). However, after pre-incubation for 4 h in 100 nM PMA, the effect of ATII on cAMP accumulation was insignificant. In the Dahl R myocytes, ATII lowered cAMP accumulation significantly 50 % but after PMA treatment the ATII effect was abolished. The overall forskolin-stimulated cAMP accumulation was lower after PMA treatment. However, the only changes brought on by the presence of PMA were in the ATII-treated samples.

d) **Discussion**

The effect of ATII on cAMP in the Dahl S hypertrophic ventricular myocyte was studied. The accumulation of cAMP was observed to be distinctively controlled in two ways in the hypertrophic Dahl S ventricular myocyte compared to the ventricular myocyte from the non-hypertrophic heart. Firstly, in the hypertrophic Dahl S cardiac myocyte cAMP, the response to stimulation was depressed compared to the response in the non-hypertrophic myocyte from either the Dahl R or Dahl S rat. This may be due in part, to the diminished presence of ADP-ribosylated Gs alpha in the hypertrophic heart. Secondly, ATII, which
Figure 20. PMA Effects on Reversal of ATII Effect on cAMP in the Hypertrophic Myocyte.

Samples of ventricular myocytes were stimulated with forskolin for 2 min. Hypertrophic Dahl S (diagonal lines) or Dahl R (solid) ventricular myocytes were pre-treated with 1 uM ATII for 5 min prior to forskolin treatment where indicated. PMA, 100 nM, treatment was for 4 hrs prior to ATII and forskolin treatment. Intracellular cAMP was assayed as described in Materials and Methods. Experiments were done two times in triplicate. Each bar is the mean ± 1/2 the range of means.
inhibits cAMP generation in non-hypertrophic ventricular myocytes, increased cAMP generation in the hypertrophic Dahl S ventricular myocyte to above the level provoked using only forskolin, a direct activator of adenylyl cyclase (Seamons et al., 1981). These activities of ATII were blockable by using Saralasin, indicating that both actions were mediated by the ATII receptor. The pattern of these results was not altered by treatment with IBMX, a phosphodiesterase inhibitor. This suggests that adenylyl cyclase activity, rather than phosphodiesterase activity, was altered in cardiac hypertrophy in the Dahl S rat. Similarities in the response of ATII and carbachol indicate that the response was not unique to ATII and suggests a common signal transduction pathway used by the ATII and muscarinic receptors. Because the adenylyl cyclase activity of hypertrophic myocytes appeared to be more sensitive to pertussis toxin than non-hypertrophic myocytes, the presence of pertussis toxin-sensitive and cholera toxin-sensitive G protein subunits in control and hypertrophic ventricular myocytes was probed for. Diminished quantities of ADP ribosylated Gs and Gi alpha subunits were apparent in the hypertrophic myocyte, using this technique. The alterations in cAMP generation were reflected in the contractile performance of the isolated hypertrophied heart, in which left ventricular developed pressure was increased by ATII and forskolin stimulation in contrast to the normal heart in which ATII depressed forskolin-induced increase in contractility.

Diminution of adenylyl cyclase activation in response to beta receptor stimulation has been observed in several experimental models of cardiac hypertrophy (Sharma et al., 1982; Foster et al., 1991; Anand-Srivastava et al., 1983). Myocardial adenylyl cyclase activity is lower in the Spontaneously Hypertensive Rat (SHR) (Blumenthal et al., 1982; Limas and Limas, 1978) and in the renal hypertensive rat (RHR) (Sharma et al., 1982), compared to Wistar Kyoto controls. In SHRs, adenylyl cyclase activity decreased during development of left ventricular hypertrophy (Blumenthal et al., 1982). The mechanism of the lowered cAMP response has been attributed to reduced beta receptor density in the Spontaneously Hypertensive Rat (Limas and Limas, 1978) and alterations in G protein mRNAs and proteins in hamsters and humans (Feldman et al., 1988; Feldman et al., 1990). The
depressed response to isoproterenol stimulation, which was directly related to the degree of hypertrophy, has been observed in the inbred Dahl S rat heart (see section 1 of this chapter) (Sunga and Rabkin, 1991).

Limas and Limas (1985) studied cardiac beta receptors in the outbred Dahl S and Dahl R rat and reported higher receptor density with increased time on high NaCl diet in the Dahl S rat heart. The absence of a reduction of adenylyl cyclase activity in Limas and Limas study is in contrast with the depressed cAMP generation found in Dahl rat ventricular hypertrophy (Sunga and Rabkin, 1991) and in contrast to the depression of adenylyl cyclase found in other studies of cardiac hypertrophy (Sharma et al., 1982; Foster et al., 1991; Anand-Srivastava et al., 1983; Upsher and Khairallah, 1985; Blumenthal et al., 1982). The reason for the absence of an alteration in adenylyl cyclase response in the high salt Dahl S myocyte in Limas' study can be attributed to the moderate degree of ventricular hypertrophy of the outbred Dahl rats used in the experiments. Limas and Limas (1985) reported a 10% increase in heart to body weight ratio of hypertrophic Dahl S compared to Dahl R. In contrast, in the present study of inbred Dahl rats, Dahl S rat ventricles displayed a more than 50% increase in heart to body weight hypertrophy, compared to matched Dahl R rats. As previously demonstrated for this model, the depression in cAMP response in the inbred Dahl is related to the degree of hypertrophy (Sunga and Rabkin, 1991). A further, important point of difference between these two studies is that in the Limas and Limas study in vitro assays of adenylyl cyclase were performed, whereas in the present study, cAMP accumulation in intact cells was studied with the aim of examining cellular control of the adenylyl cyclase-cAMP axis. Furthermore, the present study indicates that the differential control of cAMP generation in hypertrophic and control ventricular myocytes appeared to be independent of beta-adrenergic receptor density, because forskolin, which by-passes the beta receptor and directly activates adenylyl cyclase (Seamons et al., 1981), produced the same diminutive response as does isoproterenol in the hypertrophic inbred Dahl S ventricular myocyte.

The control of cAMP generation was also probed by means of in vitro ADP
ribosylation of membranes from Dahl R and Dahl S myocytes using cholera toxin. This aspect of the study revealed lower levels of cholera toxin-sensitive ADP ribosylation substrates in the hypertrophic hearts. The primary cholera toxin substrate in heart is the alpha subunit of the trimeric stimulatory G protein, designated Gs (Longabough et al., 1986). The cholera toxin-sensitive substrate observed in these studies was an Mr = 45,000 protein, which is consistent with previous studies showing that of the four isoforms of Gs alpha, the Mr = 45,000 isoform predominates in heart (Holmer and Homcy, 1991). Gs protein in heart links a number of types of membrane receptors to adenyl cyclase, and is thought to directly activate Ca^{2+} channels (Yatani et al., 1987). Gs is thus part of the central pathway in the control of cardiac activity since both Ca^{2+} and cAMP regulate cardiac myocyte phosphorylation-dephosphorylation cycles.

The results of the present ADP ribosylation study suggest G protein alterations that differ from G protein changes in failing human hearts. Feldman et al. (1988; 1990) have demonstrated increases in mRNA and protein levels of Gs alpha. This contrast likely reflects fundamental differences in the cellular basis of hypertrophy as compared to heart failure, as well as possible G protein differences between the rat and human ventricular myocyte.

The second, major aspect of the distinct signal transduction pathway in hypertrophic myocytes was the reversal of the inhibitory action of ATII and carbachol on cAMP generation in hypertrophic Dahl S myocytes. ATII and carbachol, in normal cardiac myocytes, depressed cAMP generation. In contrast, in the hypertrophic Dahl S myocyte, both ATII and carbachol elevated cAMP generation. Since both ATII and carbachol stimulation produce the same effect in the hypertrophic Dahl S ventricular myocyte, the mechanism of the reversal is likely downstream from the ATII receptor and muscarinic receptor and at a convergent point in the transduction of these two signals. An early point of convergence of these two signals is the guanine nucleotide activation of an inhibitory G protein (Gi), which inhibits adenyl cyclase activity (Hildebrandt et al., 1982; Katada et al., 1984) and is linked to both ATII and muscarinic receptors in the ventricular myocyte (Allen et al., 1988; Anand-Srivastava, 1989).
Neither ventricular ATII nor muscarinic receptors have been examined in Dahl R and S rats. However, previous studies have indicated that the vascular response of Dahl S rats to ATII bolus injection is greater than that of Dahl R rats. This is likely due to low circulating and tissue concentrations of ATII (Iwai et al., 1970; 1973) which may be linked to differences in ATII receptor or better coupling between ATII receptors and intracellular signalling pathways in Dahl S compared to Dahl R rat and which may manifest as differences in the myocardial cAMP response to ATII. Potential support for this can be found in a recent study using the non-peptide ATII receptor inhibitor DuP753, which reduced mortality among Dahl S rats on high salt diet without markedly lowering blood pressure (von Lutterotti et al., 1991).

Pertussis toxin inactivates Gi alpha by catalyzing the ADP ribosylation of Gi alpha which blocks the interaction between the G protein and receptors (Gilman, 1987). Cultured Dahl R cardiac myocytes that were briefly exposed to pertussis toxin, exhibited an increase in forskolin-stimulated cAMP. However, ATII inhibition of adenylyl cyclase was not abolished in these cells. In contrast, pertussis toxin abolished the ATII-stimulated enhancement of forskolin-stimulated cAMP in cardiac myocytes from hypertrophic Dahl S rats. This indicated that in the hypertrophic myocyte there is divergence of the pathway of ATII signal transduction either within the G protein complex itself or in a component of the pathway that impinges on G protein function.

Changes in the levels of functional Gi alpha expression in the heart have been observed during rat heart development (Allen et al., 1988), in the failing heart (Feldman et al., 1989), and as a result of chronic exposure to converting enzyme inhibitors (Horn et al., 1988), norepinephrine (Reithmann et al., 1989) and pertussis toxin (Watkins et al., 1989). In view of this and the differences in pertussis toxin sensitivity that we observed in the hypertrophic Dahl S heart, the pertussis toxin-sensitive G protein complex was further examined. Our ADP ribosylation studies indicated that one or more pertussis toxin substrates of Mr = 39,000 to Mr = 42,000 are present in adult ventricular myocytes from Dahl R and Dahl S rats, and furthermore, that diminished levels of ADP-ribosylated Mr = 41,000
substrates are present in the hypertrophic Dahl S myocyte. Furthermore, the difference in ADP-ribosylation substrate between hypertrophic Dahl S cardiac myocytes and non-hypertrophic Dahl R myocytes may be partly due to differences in the de novo synthesis of Gi alpha since the rate of $[^{35}\text{S}]\text{methionine}$ incorporation into immunoprecipitable Gi alpha appeared to be lower in cells from the hypertrophic strains.

Allen et al. (1988) examined the functional alterations of the Gi proteins in rat heart cultures and showed a link between both the functional expression and quantity of two pertussis toxin-sensitive proteins, and the developmental stage of heart cell. Neonatal cells did not express functional inhibitory G protein, yet acquired it after 11 days in culture. These results taken together with the functional alteration of adenylyl cyclase produced by pertussis toxin in the hypertrophic Dahl S myocyte, support the hypothesis that the function of Gi is altered by an upstream component of the signal transduction pathway in hypertrophic cell. Other tissues also have displayed alterations in the function and quantity of the G protein subunits. These include human neutrophils (Bokoch, 1987) and 3T3-L1 cells (Watkins et al., 1987), which display attenuated inhibition of adenylyl cyclase by alpha2-receptors. The 3T3 cells have a high beta to alpha G protein ratio which may be a basis for the absence of a functioning inhibitory mechanism (Watkins et al., 1987). These mechanisms may be of importance for the hypertrophic Dahl S model of cardiac hypertrophy, although the quantitative determination of the beta to alpha subunit ratio in the hypertrophic Dahl S heart has not been made.

Functional analysis of isolated intact hearts from Dahl R and hypertrophic Dahl S rats reflected the alterations in cAMP generation observed in the isolated myocyte. ATII treatment enhanced or produced a further positive inotropic effect compared to that of forskolin treatment alone in hypertrophic S hearts. In contrast ATII had a negative inotropic effect on forskolin-stimulated cardiac contractile force generation in isolated hearts from Dahl R rats. Although we have not examined whether ATII alone has a positive inotropic effect, this has been widely observed in hearts of several species such as cat (Dempsey et al., 1971), rabbit, guinea pig, chick (Freer et al., 1976) and adult rat ventricular myocytes.
(Neyses and Vetter., 1989). Others (Peach, 1977) have reported an inability to stimulate the adult rat heart with ATII.

These results indicate that control of adenylyl cyclase is radically altered in the hypertrophic cardiac myocyte of the Dahl S rat. The mechanism of this functional alteration may be partly due to lowered expression of the Gi alpha subunit of the G protein complex, and altered function of the pertussis toxin-sensitive component of the G protein complex. However this mechanism alone cannot completely account for the observed reversal of cAMP inhibition. The reversal of cAMP inhibition likely depends upon profound alterations in the signal transduction of ATII in the hypertrophic myocyte. One may therefore speculate that other elements upstream of the Gi complex play a role in converting inhibition to enhancement of adenylyl cyclase activity.

This latter concept led to investigation of other signalling pathways that might interact with the control of adenylyl cyclase. Protein kinase C is known to be stimulated by ATII in the rat heart (Allen et al., 1988; Dosemici et al., 1988; Hoh et al., 1990). Furthermore, the regulation of Gi activity may depend upon protein kinase C activity in rat heart cells (Moscat et al., 1989; Moscona-Amir and Henis, 1989) and cultured fibroblasts (Diaz-Laviada et al., 1991). Therefore experiments were performed with the objective of examining "cross-communication" between the phospholipase C-protein kinase C axis and the cAMP reversal by ATII in the hypertrophic rat heart myocyte. PMA was used to down-regulate protein kinase C (Castagna et al., 1983), thus removing this potential component in the control of cAMP generation. The abolition of both the inhibition and elevation of cAMP in the presence of long-term treatment with PMA suggest that protein kinase C is a required element in control of adenylyl cyclase.

3. CONCLUSIONS

In conclusion, the diminution of cAMP generation by adenylyl cyclase in cardiac myocytes from the hypertrophic heart of the Dahl S rat compared to non-hypertrophic controls may be due in part to lower Gs alpha in the hypertrophic Dahl S heart. In the
hypertrophic myocyte, ATII or carbachol enhanced adenylyl cyclase stimulation, while in the normal heart ATII and carbachol inhibit adenylyl cyclase stimulation. These results indicate that control of adenylyl cyclase is altered in the hypertrophic cardiac myocyte of the Dahl S rat. The mechanism of this functional alteration may be partly due to lowered expression of the Gi alpha subunit of the G protein complex, and altered function of the pertussis toxin-sensitive component of the G protein complex. This mechanism, however, alone cannot completely account for the observed reversal of inhibition of cAMP increase. One might therefore speculate that other elements upstream of the Gi complex may also play a role in converting inhibition of adenylyl cyclase into enhancement.

The consequence of alterations of Gs and Gi activity in the hypertrophic cardiac myocyte is potentially important to the performance of the hypertrophic heart. While the heart becomes progressively less responsive to beta stimulation during the development of hypertrophy, it may become more responsive to ATII (Baker et al., 1990). In reverting to locally-generated inotropes such as ATII, the hypertrophic cardiac myocyte may establish inotropic homeostasis thereby compensating for adrenoreceptor exhaustion. An intriguing aspect of our results is that the level of cAMP in hypertrophic myocytes produced after ATII pre-treatment consistently approximated the same value of cAMP generated after forskolin alone in non-hypertrophic cells. The coupling of the two phenomena, the adenylyl cyclase stimulation through Gs and the enhancement of adenylyl cyclase stimulation through stimulation of other receptors such as ATII may be a key aspect of control of contractile performance by the hypertrophic heart.
CHAPTER IV
PROTEIN PHOSPHORYLATION AND
PROTEIN KINASE ACTIVITY INDUCED BY ATII
IN THE HYPERTROPHIC HEART

1. ANGIOTENSIN II INDUCED PHOSPHORYLATION OF A 42 kDa PROTEIN IN
THE HYPERTROPHIC DAHL S RAT

a) Summary

ATII-induced phosphorylation of proteins was examined in isolated myocytes from hearts
of Dahl rats. High salt diet induced cardiac hypertrophy in Dahl S rats. ATII-induced
phosphorylation of a 42 kDa protein (pp42) was detected by two-dimensional electrophoresis
in hypertrophic but not normal ventricular myocytes. ATII stimulation was time-dependent with
a peak effect at 30 min. The half-maximal and maximal concentrations of ATII that stimulated
pp42 phosphorylation were 1 and 10 nM respectively. Phosphorylation of pp42 was a function
of the degree of cardiac hypertrophy. PMA induced phosphorylation of pp42 indicates the
possibility of an association between protein kinase C and the signal transduction pathway of
ATII-induced pp42 phosphorylation. Ionomycin and A23187 at 1 uM, did not stimulate
phosphorylation of pp42. ATII produced a small increase in synthesis of myocyte proteins in
both normal and hypertrophic cells as shown by [35S]methionine incorporation. However, this
increase could not account for the increase in phosphate in pp42. pp42 was not an isoform of
actin, nor of platelet origin. These results raise the possibility that ATII may play a role in
activation of factors in the hypertrophic myocyte, however, further study is required to define a
link between phosphorylation of pp42 and the hypertrophic process.
b) Introduction

In adult rat ATII produces a small increase in contractile force in right ventricular strips (Doggrell, 1989) and an increase in contraction and decrease in relaxation rate in isolated ventricular myocytes (Neyses and Vetter, 1989). The cellular mechanism of ATII action in the neonatal cardiac myocyte includes increased calcium current, increased inositol monophosphate and biphosphate accumulation (Allen et al., 1988), stimulation of the Na⁺/K⁺ pump (Moorman et al., 1989) and is mimicked by protein kinase C stimulation (Dosemici et al., 1988). In adult ventricular myocytes ATII stimulates intra-cellular calcium release (Urabe et al., 1990). In the vasculature, ATII increases systemic vascular resistance by eliciting contraction of vascular smooth muscle (Peach, 1977). Cellular responses in the vasculature include phospholipase C stimulation (Smith et al., 1988), mobilization of Ca⁺ (Nabika and Vellitri, 1985), activation of Na⁺/H⁺ exchange (Berk et al., 1987), induction of expression of nuclear oncogenes such as c-fos (Kawahara et al., 1988; Taubman et al., 1989) and phosphorylation of nuclear and cytoskeletal intermediate filaments (Tsuda and Alexander, 1990; Tsuda et al., 1988). Although some of these responses are common to those elicited by mitogens such as PDGF (Brock et al., 1985; Griendling et al., 1986), ATII causes vascular smooth muscle hypertrophy, rather than cell proliferation (Berk et al., 1989). Thus, while the intra-cellular mechanism of action of ATII on the cardiac myocyte has not been entirely elucidated, important similarities between vascular smooth muscle and cardiac muscle cells are apparent.

A role of ATII as a signal for growth stimulation, as determined by cell proliferation or by DNA, RNA or protein synthesis has been described in 3T3 fibroblasts (Ganten et al., 1975; Schelling et al., 1979), adrenal cortical cells (Gill et al., 1977), in rat heart (Khairallah and Kanabus, 1983; Dzau et al., 1989) and, more recently, in chick cardiomyocytes (Aceto and Baker, 1990). Myocardial hypertrophy has been linked to the action of ATII by the ability of angiotensin converting enzyme inhibitors to prevent and reverse it (Nagano et al., 1991; Kromer and Riegger, 1988).

Based on these results as well as the other parallels between vascular smooth muscle
and cardiac muscle, phosphoproteins in ATII-treated isolated ventricular myocytes from normal and hypertrophic hearts were analyzed. The underlying hypothesis of these experiments was that ATII-induced protein phosphorylation is distinctive due to differential signalling in the hypertrophic compared to the normal cardiac myocyte. In this section, the ATII-induced phosphorylation of an \( M_r = 42,000 \) protein in the hypertrophic heart is characterized.

c) Results

i. Ventricular Hypertrophy in the Dahl Rat

Inbred SS/Jr and outbred DS rats fed high salt diet developed ventricular hypertrophy as determined by increased ventricular weight relative to total body weight (Figure 21). In contrast outbred DS rats displayed a greater variability in degree of cardiac hypertrophy than inbred SS/Jr rats. Age and sex matched inbred SR/Jr or outbred DR rats fed the identical diet maintained a constant ventricular weight to body weight ratio with no signs of left ventricular hypertrophy. In the inbred SS/Jr rats, there was a significant (\( P < 0.05 \)) relationship (\( r = 0.811 \)) between duration of high NaCl diet and the degree of ventricular hypertrophy.

ii. Viability of Ventricular Myocytes

Viability of cells was usually between 75 and 85% as determined by trypan blue exclusion. The difference in viability between normal and hypertrophied myocytes isolated in the same experiment was less than 5%. Phosphoprotein differences between the hypertrophied and normal myocytes were not attributable to viability differences. Isolated cells were characteristically rod-shaped and quiescent. The yield of viable cells was 15-20 million per heart.

iii. Electrophoretic Analysis of Angiotensin II Stimulated Cardiac Myocytes

ATII increased phosphorylation of a number of proteins in hypertrophic S myocytes but not in R myocytes. This was evident after 30 min of 1 uM ATII exposure by isoelectric focusing (Figure 22) and by two-dimensional gel electrophoresis of phosphoproteins (Figure 23, H and D, respectively). Especially prominent was a \( M_r = 42,000 \) protein (pp42) pp42
Dahl rats were fed an additional 6% NaCl mixed with powdered Purina chow from 21 days of age and allowed free access to drinking water. After various times on 6% NaCl diet total body weight was determined and animals sacrificed. Ventricular weights were determined after retrograde perfusion of the ventricles. Dahl R and Dahl S strains had been inbred for more than 60 generations. The regression line shows the relationship between ventricle/body weight ratio and time on 6% NaCl diet.

Figure 21  Ventricular Hypertrophy in the Dahl Rat
Figure 22. Isoelectric focusing of ATII-stimulated phosphoproteins from Dahl R and hypertrophic Dahl S myocytes.

Isolated ventricular myocytes from age matched and diet-matched male Dahl R and hypertrophic Dahl S rats were pre-labelled with 250 uCi/ml $[^{32}\text{P}]$orthophosphate for 2 h, then stimulated with 100 nM ATII for 10 min or 30 min. Cell pellets were prepared for isoelectric focusing. Experiments were done 4 times with similar results.
resolved as a series of at least 7 discrete points within a pH range of 5.0 to 5.5 and displayed an acidic isoelectric shift with ATII stimulation. No differences were evident in phosphoproteins from unstimulated Dahl R and S myocytes (Figure 23, B and F). Myocardial proteins from Dahl R and hypertrophic S rat hearts were similar when analyzed by O'Farrell gels and stained with Coomassie Brilliant Blue (Figure 23, A and E). No apparent alteration in protein profile occurred after 30 min of ATII stimulation (Figure 23, C and G).

iv. **Time Course and Concentration Dependence of Angiotensin II Stimulated Phosphorylation of pp42**

The phosphorylation of hypertrophic S myocyte pp42 was detected by an increase in two-dimensional autoradiographic density and a shift in the isoelectric point of the predominantly phosphorylated isoforms. Phosphorylation of pp42 was evident after 5 min of exposure to ATII (Figure 24). The peak phosphorylation level of 1.7-times basal levels was reached after 30 min of ATII stimulation, after which it declined gradually in parallel with a retreat of the isoelectric point of the phosphorylated isoforms by 120 min. R myocytes showed only slight stimulation of pp42 phosphorylation to 1.3-times basal levels by 5 min followed by a rapid decline to below basal levels by 30 min (Figure 24, A and B). Over the observation period, ATII produced a significant (F=5.99, p < 0.05) increase in amount of pp42 phosphorylation in the hypertrophied heart as assessed densitometrically compared to Dahl R rat heart. The prominent Mr = 32,000 protein (pp32) was quantitated for reference and displayed constant density through the time course of ATII stimulation in both R and S myocytes (Figure 24B). ATII produced a significant (p < 0.05) increase in phosphorylation of pp42 compared to pp32.

The phosphorylation of pp42 in S rat myocytes was a function of ATII concentration and was evident at a concentration of 0.1 nM, with the maximal concentration of 10 nM ATII and half-maximal concentration (EC50) of 1 nM (Figure 25). Prior treatment of S myocytes with the ATII receptor antagonist Saralasin, 10 uM, for 5 min, markedly
Isolated ventricular myocytes from age-matched and diet-matched male Dahl R and Dahl S rats were pre-labelled with 250 uCi/ml $[^{32}P]$orthophosphate for 2 h, then stimulated with 100 nM ATII for 30 min. Cell pellets were prepared for isoelectric focusing. Dahl R and Dahl S rats had ventricular to body weight ratios of 5.6 and 8.9 g/kg respectively after 4.5 weeks of high NaCl diet. Coomassie Brilliant Blue stained gels are shown on the left (A,C,E,G) and the respective autoradiographs on the right (B,D,F,H). A,B,C,D are from Dahl R. E,F,G,H are from Dahl S. C,D,G,H are ATII stimulated. The arrow indicates the $M_r=42,000$ phosphoprotein.
Figure 24-1. Time course of ATII stimulation of phosphorylation.
Isolated rat ventricular myocytes from Dahl R and Dahl S rats with ventricle to body weight indices of 6.3 and 9.1 g/kg, respectively, were pre-labelled with 250 uCi/ml for 2 h followed by stimulation with 100 nM ATII for the times indicated. All samples were harvested at the same time. The amount of protein loaded on each isoelectric focusing gel was 30 ug. Arrow 1 indicates pp42. Arrow 2 indicates pp32. A - Dahl R B - Dahl S
The data are from a representative experiment.
Figure 24-2. **Densitometric analysis of time course of phosphorylation.**
Phosphorylation levels of pp42 (Dahl S - circle, Dahl R - open square) and pp32 (Dahl S - triangle, Dahl R - closed square) were quantitated by video densitometry. Data are expressed as fold change from basal level. Bars indicate the range from two separate experiments.
Figure 25. Concentration dependence of ATII-induced phosphorylation of pp42 and inhibition in the presence of [Sar¹ala⁸]angiotensin

Isolated ventricular myocytes from hypertrophic Dahl S rats were pre-labelled with 250 uCi/ml [³²P]orthophosphate for 2 h followed by 10 min stimulation with various concentrations of ATII (circles) or various concentrations ATII in the presence of 10 uM [Sar¹ala⁸]angiotensin (squares). The phosphorylation of pp42 was quantitated by video densitometric scanning of radiographs. Data are expressed as fold change over basal levels. Data shown are from two separate experiments and the bars indicate the range.
attenuated the induction of phosphorylation with ATII (Figure 25). Saralasin, 1 uM, alone had no effect on either the isoelectric point or the $^{32}$P content of pp42.

v. **pp42 is neither Actin nor of Platelet Origin**

It was conjectured that pp42 was a minor phosphorylated component of soluble G actin or nascent actin chains (Ohta et al., 1987; Steinberg, 1980). To test this hypothesis cells were pre-labelled with $[^{32}P]$orthophosphate, extracted with 1% Triton X-100, and immunoprecipitation of cell extracts was performed using monoclonal antibodies specific for actin and muscle actin. Although G actin fractions were isolated, as assayed by SDS gel electrophoresis, no phosphoprotein was detected on autoradiographs (Figure 26a) or by scintillation counting of gel slices. Pellets of extracts, which contained 1% Triton X-100 insoluble F actin, were also analyzed by SDS gel electrophoresis and autoradiography and were found not to contain phosphoprotein at the 42 kDa band (Figure 26b).

Since pp42 bears isoelectric and molecular weight similarities to the prominent platelet phosphoprotein pleckstrin (Tyers et al., 1987) the hypothesis that pp42 originated from adherence of platelets to myocytes during the isolation procedure was tested. Rat platelets were therefore isolated, allowed to take up $[^{32}P]$orthophosphate and total proteins were subjected to two-dimensional gel electrophoresis and autoradiography. Samples were prepared separately and combined with myocyte samples. The platelet phosphoprotein profile was distinct from that of myocytes. pp42 was not apparent among platelet phosphoproteins (Figure 27).

Rigorous attempts were made to identify pp42 as tyrosine phosphorylated protein by using anti-phosphotyrosine antibodies in immunoblot analysis as well as immunoprecipitation of metabolically labelled proteins. The inability to immunologically identify an ATII-responsive 42 kDa phosphoprotein in either the Dahl R or hypertrophic Dahl S rat heart suggests that pp42 may not be phosphorylated on tyrosine residues.

The question of identity of pp42 is still unresolved, in part due to abundance of the protein, and in part due to proximity on electrophoretic gels to the actin series of proteins, which contaminated samples prepared for amino acid sequence analysis.
Figure 26. Absence of ATII stimulation of phosphate incorporation into immunoprecipitated soluble G actin and Triton X-100 insoluble F actin.

Isolated ventricular myocytes from Dahl R and Dahl S rats were pre-labelled with $[^{32}]$orthophosphate for 2 h then exposed to 1 uM ATII for 30 min. Cells were lysed with 1% Triton X-100 and samples of supernatant immunoprecipitated with anti-muscle actin antibody. Upper left hand gel (a) shows 10.5% polyacrylamide SDS gels of immunoprecipitated protein. The 42 kDa position is indicated. Upper right hand (a) shows 5 day autoradiograph of the same gel.

The 1% Triton X-100 insoluble material was centrifuged at 13,500 x g, 5 min and pellet dissolved in SDS gel buffer and analyzed on 10.5% SDS gels. Left lower side shows Coomassie Brilliant Blue staining of pellet proteins (b). Right lower side is a 5 day autoradiograph of the same gel (b). The 42 kDa position is indicated. The experiment was repeated five times with anti-actin antibodies to skeletal and cardiac alpha actin, as well as gamma actin.
Figure 27.  Comparison of platelet and myocyte phosphoproteins
Platelets and ventricular myocytes from Dahl S rats were isolated and pre-labelled with $[^{32}\text{P}]$orthophosphate for 2 h. Platelet and myocyte protein samples were analyzed separately (20 µg/gel) or mixed together (10 µg platelet protein plus 10 µg myocyte protein) for two dimensional gel analysis and autoradiography. Arrow indicates pp 42. This experiment was done twice in triplicate.
vi. **Relationship Between Angiotensin II Induced Phosphorylation of pp42 and Cardiac Hypertrophy**

ATII-induced phosphorylation of pp42 was a function of the amount of ventricular hypertrophy as measured by ventricle to body weight index (Figure 28). There was little response to ATII in S rats on normal salt diet that did not have left ventricular hypertrophy and displayed heart to body weight indices similar to R rats.

vii. **Effect of PMA and Calcium Ionophores on pp42 Phosphorylation**

The phorbol ester, PMA, induced phosphorylation of pp42 in both R and S rats (Figure 29). pp32 was unaffected by PMA stimulation but a number of other proteins, including an acidic M<sub>r</sub> = 85,000 protein, was time-dependently phosphorylated by PMA (Figure 30). Time course of PMA-induced phosphorylation of pp42 in R myocytes followed a rapid increase of 1.6-fold over basal levels up to 30 min followed by a slight decline. By 120 min of PMA stimulation there was still a 1.3-fold increase in incorporated [<sup>32</sup>P]phosphate. S myocytes, in contrast, displayed a modest rise in phosphorylation of pp42 which reached 1.3 fold over basal level by 10 min then decreased to basal levels (Figure 30). PMA produced a significant (P < 0.05) increase in phosphorylation of pp42 compared to pp32, which did not appear to be altered by PMA. Cardiac myocytes exposed to 1 uM ionomycin or 1 uM A23187 over a 2 h period did not show detectable increases in phosphorylated pp42.

viii. **Identification of the Intracellular Location of pp42**

Phosphoprotein pp42 was soluble in 1% Triton X-100 and present in the sarcolemmal fraction. After hypotonic shock and 0.4 M LiBr treatment of Dahl R myocytes, which has been shown to isolate and preserve sarcolemmal structure (Anand-Srivastava, 1988), pp42 remained in the low speed centrifuge pellet containing the sarcolemmal fraction (Figure 32A).

Dahl R myocytes that were exposed to 160 nM PMA and treated with 1% Triton X-100 lysis buffer, released pp42, as shown by gel electrophoresis analysis of the supernatant and the pellet (Figure 31B).
Figure 28. Effect of cardiac hypertrophy on ATII-induced phosphorylation of pp42. Myocytes from Dahl S rats at various stages of cardiac hypertrophy were isolated and pre-labelled with \( ^{32}P \) orthophosphate for 2 h followed by exposure to 100 nM ATII for 10 min. Relative phosphate content of pp42 was determined by dividing video densitometric readings of phosphoprotein radiographic images from ATII-treated myocytes, by readings from untreated myocytes. The regression line \( (r = 0.615) \) indicated the relationship between the degree of phosphorylation and the degree of ventricular hypertrophy (ventricle/body weight) is displayed.
Figure 29. Phorbol ester stimulation of phosphorylation of Dahl R and hypertrophic Dahl S myocyte proteins.
Isolated ventricular myocytes from Dahl R and Dahl S rats were pre-labelled with 250 uCi/ml $^{32}$Porthophosphate for 2 h followed by exposure to 160 nM PMA in 0.01% dimethylsulfoxide for 10 min. Panels a and c are from Dahl R myocytes and panels b and d are from Dahl S myocytes. Panels a and b were not treated with PMA. Panels c and d were treated with PMA. Arrow 1 indicates pp42, arrow 2 indicates pp32 and arrow 3 indicates the 85 kDa protein referred to in text.
Figure 30. Time course of PMA-induced phosphorylation of pp42 and pp32 from Dahl R and hypertrophic Dahl S myocytes.

Isolated ventricular myocytes from Dahl R and Dahl S rats were pre-labelled with 250 uCi [32P]orthophosphate for 2 h followed by exposure to 160 nM PMA in 0.01% dimethylsulfoxide for various times. pp42 (S - circle, R - open square) and pp32 (S - triangle, R - closed square) phosphate content were quantitated by video densitometry. Bars show the range of data from two experiments.
Figure 31. Phosphorylation and fractionation of PMA-treated Dahl R myocytes.
Dahl R ventricular myocytes were pre-labelled with 250 uCi/ml $[^{32}P]$orthophosphate for 2 h followed by exposure to 160 nM PMA in 0.01% dimethylsulfoxide. Cells were subjected to 0.4 M LiBr-hypotonic shock treatment to isolate sarcolemmal fractions. Sarcolemmal proteins were analyzed (panel A) by two dimensional gel electrophoresis as described in Materials and Methods.

In a separate procedure $[^{32}P]$orthophosphate-labelled and PMA treated Dahl R myocytes were treated with 1% Triton X-100 containing lysis buffer. Panel B shows 10.5% polyacrylamide gel electrophoresis of 1% Triton supernatants (lanes a,c,e,g) and pellets (lanes b,d,f,h).

- a and b - no PMA
- c and d - 5 min PMA
- e and f - 10 min PMA
- g and h - 30 min PMA
ix. $^{35}$S Methionine Incorporation into Myocyte Proteins

Ventricular myocytes from Dahl R and hypertrophic Dahl S hearts were pre-labelled with 60 μM $^{35}$S methionine for 2 h during which time 1 μM ATII was added for the last 30 min.

Two dimensional gel analysis and autoradiography reveal relative de novo synthesis of cardiac proteins over a 2 hr time course. ATII stimulated R and S myocytes (Figure 32) had visibly increased $^{35}$S methionine incorporation in many proteins. pp42 was poorly visible, indicating that it is of relatively low abundance and that synthesis of pp42 was not increased relative to other cell proteins by ATII stimulation. Slightly more $^{35}$S methionine was incorporated into R compared to hypertrophic S myocyte, however the relative incorporation into pp42 was the same.

d) Discussion

These results have demonstrated that ATII induces protein phosphorylation in hypertrophic Dahl S ventricular myocytes. Of special interest was a protein of $M_r = 42,000$ which was evident in radiographs from two-dimensional electrophoresis. This action of ATII in Dahl S myocytes was likely mediated through ATII receptors since, firstly, the response of pp42 phosphorylation was dependent upon the ATII concentration and displayed an EC$_{50}$ of $10^{-9}$ M which is in accord with studies of the contractile response of isolated adult myocytes to ATII (Neyses and Vetter, 1989). Secondly, the competitive inhibitor Saralasin blocked ATII-induced phosphorylation of pp42.

The present study has found that the ATII-sensitive pp42, was not actin nor of platelet origin. The protein, although present in low abundance in the myocyte, is a prominent feature of the ATII-stimulated, phosphoprotein profile, and is characterized by a series of at least 7 discrete points which display a charge shift on autoradiographs of two dimensional gels. A number of diverse mitogens such as epidermal growth factor, PDGF and PMA phosphorylate a $M_r = 42,000$ protein in a variety of cell types (Cooper et al., 1984; Gilmore and Martin, 1983; Kohno, 1985; Rossomando et al., 1989; Sanghera et al., 1990;
Figure 32. Effect of ATII on protein synthesis.

Ventricular myocytes were isolated from Dahl R and Dahl S rats and pre-labelled with 60 uCi/ml [35S]methionine for 2 h followed by a further 1 h exposure to 100 nM ATII. Proteins were analyzed by two dimensional gel electrophoresis and autoradiography. Panels a and b are controls, panels c and d, ATII-stimulated. Arrow indicates the position of pp42 as inferred from [32P]phosphate radiographs. Experiments were done four times with similar results.
Iwasa and Hosey, 1984; Yuan and Sen, 1986; Hunter et al., 1985). An important difference may be due to difference(s) in the cell types used in those studies and the present ones: whereas the previous studies were done in established fibroblast cell lines, the present study was in primary cultures of isolated cardiomyocytes. A Mr = 42,000 protein in chick and mouse fibroblasts is phosphorylated in response to PMA but has an isoelectric point of pH 6.8 (Hunter et al., 1985) which distinguishes it from the pp42 described here which has pH 5.0 to 5.5. Another difference is in the number of isoforms. We have observed seven isoforms, which is distinct from the pp42 previously observed in 3T3 cells which apparently has two or three isoforms (Rossomando et al., 1989). A Mr = 42,000 cardiac sarcolemmal protein has also been reported as a major substrate for protein kinase C in chicks hearts (Iwasa and Hosey, 1984) and an Mr = 46,000 protein is among the proteins phosphorylated by protein kinase C in canine cardiac sarcolemma (Yuan and Sen, 1986). The present study indicates that pp42 is associated with the sarcolemmal fraction.

The function of pp42 in the heart is unknown. However, it is possible that pp42 is a component of the signalling pathway mediating the action of ATII in cardiac hypertrophy (Hunter et al., 1985; Schelling et al., 1991). The issue of the causal relationship between ATII-induced pp42 phosphorylation and cardiac hypertrophy has not been resolved and it is possible that pp42 phosphorylation occurs concomitent with but is not causally related to the development of left ventricular hypertrophy. However, some data suggest an involvement in the ATII signal transduction in the hypertrophic heart of the Dahl S rat: increased sensitivity of the hypertrophic myocyte to ATII-induced phosphorylation of pp42 was evident in contrast to the limited degree of phosphorylation of the same protein in normal myocytes from age-, sex- and diet-matched Dahl R rats or in low salt-fed Dahl S myocytes with no hypertrophy. Furthermore, the degree of phosphorylation of pp42 was proportional to the degree of cardiac hypertrophy. Thirdly, other factors such as age or high salt feeding alone are likely to play direct roles in the ATII-induced phosphorylation of pp42, since high salt-fed, age-matched Dahl R rats display no increase in pp42 phosphorylation. It is unlikely that pp42 is involved in the positive inotropic action of ATII.
(Koch-Weser, 1965; Dempsey et al., 1971; Bonnardeaux and Regoli, 1974; Moravec et al., 1990) which is believed to be mediated through stimulation of slow Ca\(^{2+}\) channels (Allen et al., 1988; Freer et al., 1976). Neither calcium ionophores A23187 nor ionomycin, which increase intracellular calcium, altered phosphorylation of pp42.

The phosphorylation of pp42 is partially mimicked by the phorbol ester PMA, suggesting that ATII-induced phosphorylation involves activation of protein kinase C (Castagna et al., 1983). However, this is not the only mechanism involved as PMA did not mimic phosphorylation of pp42 to the same extent or with the same time course as ATII. Furthermore, there was a small degree of phosphorylation of the widely distributed acidic M\(_r\) = 83,000 - 87,000 protein substrate of protein kinase C (Albert et al., 1986) in ATII-stimulated S myocytes, while noticeably more phosphorylation of the same protein occurred in both R and S myocytes stimulated with the phorbol ester PMA. The phosphorylation of pp42 by PMA was rapid and attained a higher peak in Dahl R rat hearts compared to hypertrophic S rat hearts. This is consistent with studies demonstrating that Dahl R rats have significantly more phorbol ester receptor sites per cell than Dahl S rats (Limas and Limas, 1986). This may also indicate that a component conferring altered ATII-sensitivity in S myocytes is upstream from protein kinase C in the ATII transduction pathway (Nishizuka, 1988). At the present, to my knowledge, ventricular ATII receptors have not been compared in Dahl R and S rats, however, previous studies have indicated that the vascular response of Dahl S rats to ATII bolus injection is greater than that of Dahl R rats (Iwai et al., 1973. We speculate that this may be due to differences in ATII receptor in vascular smooth muscle or better coupling between ATII receptors and intracellular signalling pathways in Dahl S compared to R rat which may also be manifest as parallel differences in the myocardial response to ATII.

Although protein synthesis was increased in ATII-treated myocytes compared to untreated myocytes from both Dahl R and hypertrophic Dahl S hearts, this was not sufficient to account for the increased phosphate content of pp42 due to ATII stimulation. The appearance of increased quantities of pp42 was not due to ATII stimulation of de novo
synthesis of the protein, since the small, non-specific increase in cardiac proteins did not parallel pp42 changes in quantity nor in time course. Two-dimensional gel analysis of [$^{35}$S]methionine labelled proteins also revealed the low abundance of pp42 relative to other actively synthesized cardiac myocyte proteins, and furthermore, demonstrated that similar levels are found in R and hypertrophic S rat ventricular myocytes.

In conclusion, ATII induces phosphorylation of a $M_r=42,000$ protein in myocytes from hypertrophic hearts of Dahl S rats. Pending its further identification and delineation of its functional role(s) in the heart, the data suggest that pp42 maybe a component of the transduction of a trophic signal in the heart (Schelling et al., 1991; Freer et al., 1976), since the amount of phosphorylation of the protein was related to the degree of cardiac hypertrophy and the demonstrated potential for ATII to regulate growth in the heart (Khairallah and Kanabus, 1983; Aceto et al., 1990), aortic (Geisterfer et al., 1988) and other vascular smooth muscle (Berk et al., 1989).
2. ANGIOTENSIN II INDUCED ALTERATIONS OF MITOGEN ACTIVATED PROTEIN KINASE ACTIVITY IN THE MYOCARDIUM

a) Summary

In this study, the regulation of mitogen-activated protein (MAP) kinases in the normal and hypertrophic heart was examined. Isozymes of MAP were identified and the response to ATII determined. Ventricular tissue was incubated in ATII or PMA or Compound 3 before being fractionated by MonoQ anion exchange chromatography. Incubation of ventricular tissue from normal Dahl R hearts for 30 min with ATII or PMA, led to a 2-fold increase in the phosphotransferase activity of MAP kinases. Identical treatment of ventricular tissue from Dahl S hypertrophic hearts resulted in a 50% reduction in MAP kinase activity. Immunoblotting with MAP kinase antibodies revealed the predominant p44^erks1 isoform, and a less abundant p42^erks2 isoform in rat ventricle. The abundance of these isoforms was not different in hypertrophic Dahl S ventricle compared to normal Dahl R ventricle.

These results demonstrate that MAP kinases are detectable in the rat ventricle and, moreover, are activated by both ATII and PMA via a post-translational modification in Dahl R rat. However, in the hypertrophic Dahl S ventricle, the signal transduction leading up to MAP kinase activation is radically altered.
b) Introduction

This series of experiments was designed to study the role of ATII as a regulator of MAP kinases in the hypertrophic heart compared to the normal heart. The impetus for this study arose in part from the detection of an 42 kDa ATII-sensitive, PMA-sensitive phosphoprotein (see section 1 of this chapter) which corresponds in molecular weight and isoelectric point to a major MAP kinase isoform (p42mapk). A more general rationale is based on the growing recognition that MAP kinases are key components in the intracellular signalling for a variety of mitogens and growth factors (Sturgill and Wu, 1991; Cobb et al., 1991; Pelech and Sanghera, 1991). Since ATII may play a role in regulating the status of the normal and hypertrophic heart (Schelling et al., 1991) it is possible that ATII may utilize MAP kinase as an intracellular component. MAP kinase recruitment by ATII had not previously been reported.

The MAP kinases are serine/threonine protein kinases which are themselves activated by the phosphorylation of tyrosine moieties by tyrosine kinases. The tyrosine kinases are, in turn, activated by growth factors which stimulate receptor-tyrosine kinases but also by guanine-nucleotide binding protein p21ras and phospholipase C. Activated MAP kinases target still other protein kinases such as p90rsk and p74mkl and DNA transcription factors, c-myc encoded protein, and c-jun encoded protein (for review see Pelech and Sanghera, 1990).

Thus, since MAP kinases are important in the regulation of growth, and evidence has been presented of ATII activation of intracellular protein kinases, ATII induction of MAP kinase was carried out in order to gain yet another perspective on alterations of signal transduction in the Dahl S hypertrophic heart.
c) Results

i. **ATII Stimulation of MBP Kinase Phosphorylation in Dahl R Ventricular Extracts**

Extracts from Dahl R ventricles which had been treated with 1 uM ATII for 30 min were chromatographed on MonoQ anion exchange resin. The resulting column fractions were then assayed for phosphotransferase activity towards the substrate, myelin basic protein (MBP). ATII stimulated MBP kinase activity at a peak which was eluted from MonoQ with 0.3 M NaCl (Figure 33A) at a salt concentration from which the MAP kinase isoforms p42mapk and p44erk have previously been shown to be released (Rossomando et al., 1991; Sanghera et al., 1992; Weinstein et al., 1992). The ATII-stimulated fraction contained approximately two times the phosphotransferase activity of the non-ATII treated ventricular tissue.

Since PMA is known as a potent activator of protein kinase C (Castagna et al., 1983) and MAP kinase (Pelech and Sanghera, 1992; Sturgill and Wu, 1991) and since protein kinase C is thought to be part of the signal transduction pathway of ATII (Dosemici et al., 1988) ventricles were also exposed to PMA and assayed for MAP kinase activity. PMA was found to stimulate phosphotransferase activity in the Dahl R ventricle, although the level of stimulation was less than that displayed with ATII, and was approximately one and an half times the activity of untreated samples.

ii. **ATII Depression of MBP Kinase Phosphorylation in Hypertrophic Dahl S Ventricular Extracts**

Hypertrophic Dahl S ventricular tissue was treated identically to Dahl R ventricle. After 30 min of 1 uM ATII treatment, extracts were prepared, the protein content was assayed and the extracts fractionated on MonoQ anion exchange resin. The basal level of phosphotransferase activity of hypertrophic Dahl S extracts was comparable to that in normal Dahl R myocytes. However, the phosphotransferase activity in hypertrophic Dahl S was inhibited by approximately 50% at the same salt concentration at which a peak occurred in the normal Dahl R (these are the known p42mapk and p44erk fractions),

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Figure 33. MonoQ chromatography of extracts from ATII and PMA-treated Dahl R rat ventricles.

Cytosolic protein (1.0 mg) from control extracts (open circles) and those treated for 20 min with 1 uM ATII (filled circles, panel A) or 100 nM PMA (filled triangles, panel B) were subjected to MonoQ chromatography, as described in Materials and Methods, and the column fractions assayed for phosphotransferase activity towards 1 mg MBP/ml. Similar results were obtained in three experiments.
indicating a reversal of ATII stimulation of MAP kinase activity (Figure 35A). PMA treatment also resulted in a 50% reduction in phosphotransferase activity (Figure 35B).

The results of three experiments were averaged (Figure 36) and indicate that the stimulation of MBP kinase in Dahl R ventricle is significant (P < 0.05). The inhibition of MBP kinase activity in hypertrophic Dahl S ventricle was observed twice of the three times the experiment was carried out. In the third of these, there was a much smaller lowering of the phosphotransferase activity. This rendered the mean of these experiments not statistically significant at the 5% level.

iii. Immunoblotting of MonoQ Fractionated Ventricular Extracts with MAP Kinase Antibodies

The ventricular MAP kinase present in rat ventricle were cross-reactive with a peptide based on the carboxyl terminal 35 amino acids of rat MAP kinase p44\textsuperscript{erk}\textsuperscript{-1}. This was determined by immune identification by performing immunoblots of electrophoretic gels of MonoQ fractions. Fractions containing the highest phosphotransferase activity were found to contain the greatest detectable amount of p44\textsuperscript{erk}\textsuperscript{-1} (Figure 34). The 44 kDa band was also identified using a second antibody raised against the purified sea star MAP kinase, pp44\textsuperscript{mpk}.

Analysis of fractions of extracts by polyacrylamide gel electrophoresis and immunoblotting with MAP kinase antibodies indicated that the levels of the known 42 kDa and 44 kDa MAP kinases were similar in Dahl R and hypertrophic Dahl S ventricles (Figure 34A and Figure 35). However, a potentially important difference was evident: ATII treatment induced an upward band shift in the 44 kDa MAP kinase in the Dahl R extracts, but failed to do so in the hypertrophic Dahl S extracts. This suggests that, although normal levels of MAP kinase are present in the hypertrophic heart, the ATII activation pathway is uncoupled from it.

iv. Compound 3 Effect on MAP Kinase Activity

A potent and specific protein kinase C inhibitor, Compound 3, was used to determine the role of protein kinase C in mediating the ATII activation of MBP kinase. Ventricular tissue was exposed to 200 uM Compound 3, for 1 hr. During the last 30 min,
Figure 34. Immunoblotting analysis of Dahl R ventricular extracts with MAP kinase antibodies.

MonoQ column fractions from untreated (panels A and B) and ATII-treated (panels C and D) ventricles shown in Figure 33, were subjected to immunoblotting analysis with anti-erkl CT peptide antibodies (panels A and C), and anti-p44\textsuperscript{mpk} antibodies (panels B and D). The electrophoretic migrations of the pre-stained marker proteins, phosphorylase A (106 kDa), bovine serum albumin (80 kDa), ovalbumin (50 kDa), carbonic anhydrase (33 kDa), soybean trypsin inhibitor (28 kDa) and lysozyme (19 kDa), are shown. In panel B, only the region of the immunoblot between the migration positions of ovalbumin and carbonic anhydrase, are shown. The positions of the 42 and 44 kDa MAP kinases are indicated with open and solid arrows, respectively. Similar results were obtained in two experiments.
Figure 35. MonoQ chromatography of extracts from ATII and PMA-treated Dahl S rat ventricles.

Cytosolic protein (1.0 mg) from control extracts (open circles) and those treated for 20 min with 1 uM ATII (filled circles, panel A) or 100 nM PMA (filled triangles, panel B) were subjected to MonoQ chromatography, as described in Materials and Methods, and the column fractions assayed for phosphotransferase activity towards 1 mg MBP/ml. Similar results were obtained in three experiments.
Figure 36.  ATII and PMA effects on MAP kinases in Dahl R and hypertrophic Dahl S ventricle.

ATII and PMA effects on MAP kinase were assayed in 8 Dahl R and 8 Dahl S ventricles. In some experiments 3 ventricles of the same strain were pooled. In others ventricular tissue from a single animal was assayed. The results were analyzed by the t-test to determine significance at the 5% level. Asterisk denotes significant difference compared to the control for that group.
1 uM ATII or 100 nM PMA was added where indicated. In the Dahl R ventricle, Compound 3 alone was found to have the effect of stimulating phosphotransferase activity slightly (Figure 37A). Compound 3 had only a slight effect in lowering ATII activation (Figure 37A). PMA stimulation of activity was unaffected by Compound 3 in the Dahl R ventricle.

In the hypertrophic Dahl S ventricle, however, Compound 3 was found to reverse the inhibitory actions of ATII and PMA (Figure 38A and B, respectively). Compound 3 alone had no stimulatory or inhibitory effect on phosphotransferase activity in this tissue. This indicates a divergence in the role of protein kinase C in signal transduction in the hypertrophic Dahl S ventricle when compared to the Dahl R ventricle.

v. ATII Activation of Protein Kinase C

In order to confirm possible differences in protein kinase C sensitivity to ATII in the Dahl R and hypertrophic Dahl S ventricle, protein kinase C activity assays were performed on ventricular homogenates using Histone H1 as a substrate. Basal levels of protein kinase C activity were significantly (P < 0.05, n = 6) higher in hypertrophic Dahl S, than Dahl R ventricles. Dahl R protein kinase C activity was stimulated by 1 uM ATII for 30 min by approximately 2-fold, whereas hypertrophic Dahl S was stimulated by one and a half times from the basal level (Figure 39). Compound 3, 200 nM, exposure for 60 min, was effective in depressing both ATII and basal protein kinase C activity in both Dahl R and hypertrophic Dahl S ventricles. Compound 3 alone had a slight stimulatory effect in both strains.

d) Discussion

MAP kinase in rat ventricle is activated by ATII. This was demonstrated in the Dahl R rat. Two MAP kinases of 42 and 44 kDa were immunologically demonstrated. Of these the 42 kDa MAP kinase is identified as p42mapk or ERK2 (Pelech and Sanghera, 1992; Sturgill et al., 1991; Cobb et al., 1991). The 44 kDa MAP kinase is most likely p44erk1. Since both antibodies employed in this study are known to display similar degrees of immunoreactivity, the 44 kDa MAP kinase isoform is evidently predominant in rat ventricle.
Figure 37.  Compound 3 effect on ATII and PMA stimulation of MAP kinase in the Dahl R ventricle.

Ventricular tissue from Dahl R rats was incubated in 200 nM Compound 3 for 30 min prior to incubation in ATII (panel A) or PMA (panel B). MonoQ fractionation of cytosolic extracts was followed by assay of phosphotransferase activity towards MBP.
Figure 38. Compound 3 effect on ATII and PMA stimulation of MAP kinase in the hypertrophic Dahl S ventricle.

Ventricular tissue from Dahl S rats was incubated in 200 nM Compound 3 for 30 min prior to incubation in ATII (panel A) or PMA (panel B). MonoQ fractionation of cytosolic extracts was followed by assay of phosphotransferase activity towards MBP.
Figure 39.  ATII activation of protein kinase C in the Dahl R and hypertrophic Dahl S ventricle.

Ventricular tissue was treated with 200 nM Compound 3 for 60 min with 1 μM ATII added during the last 30 min of incubation. Other samples were treated with neither ATII or Compound 3 (control), ATII alone, or Compound 3 alone. Tissue was then homogenized and the total homogenate assayed for protein kinase C phosphotransferase activity towards Histone H1, as described in Materials and Methods. Experiment was performed in sextuplicate. Bars represent SEM.
2In other tissues such as human A431 cells (Sanghera et al., 1992) and rat lymphocytes (Gold et al., 1992) these antibodies reveal higher levels of p42\textsuperscript{mapk} than p44\textsuperscript{erk1}.

It was evident that ATII stimulation of MAP kinase Dahl R was directed primarily towards p44\textsuperscript{mapk} since this isoform was predominant and since ATII failed to cause a band shift in p42\textsuperscript{mapk}, identified with the erk1-CT antibody. However, immunoblot analysis with the anti-p44\textsuperscript{mapk} antibody indicated that some of the p42\textsuperscript{mapk} may have been modified and activated in response to ATII. Since ATII has been linked to phospholipase C-catalyzed hydrolysis of phosphatidylinositol-4,5-bisphosphate (Smith et al., 1988) it is highly possible that a protein kinase C-dependent pathway is involved in ATII-induced p44\textsuperscript{erk1} activation. This was confirmed by the comparable degree of activation of MAP kinase by PMA, which is a potent stimulant of protein kinase C.

The ATII-induced inhibition of MAP kinase activity in hypertrophic Dahl S ventricles was reminiscent of the reversal of cAMP inhibition reported in chapter 3 of this dissertation. Immunoblot analysis indicated that the reversal was not due to loss of protein by proteolysis of p42\textsuperscript{mapk} or p44\textsuperscript{erk1}. Thus it is apparent that a post-ATII receptor binding step in the MAP activation pathway diverges from that in the normal ventricle. One possibility is that an ATII-induced negative regulatory step is active in the hypertrophic Dahl S ventricle, but not the normal ventricle. Furthermore, the inability of PMA to activate MAP kinase in the hypertrophic Dahl S implies that the divergent step is at protein kinase C itself, or further downstream in the signalling pathway. The insensitivity of the hypertrophic Dahl S rat to protein kinase C stimulation is consistent with studies demonstrating fewer phorbol ester sites in outbred Dahl S rat hearts than outbred Dahl R hearts (Limas and Limas, 1986), and with the lower PMA-induced phosphorylation of a 42 kDa protein in the hypertrophic Dahl S myocyte compared to the Dahl R myocyte demonstrated in section 1 of this chapter (Sunga and Rabkin, 1992).

The difference in signal transduction through protein kinase C activation is also evident in the differential sensitivity of the hypertrophic Dahl S ventricle and Dahl R ventricle to the protein kinase C inhibitor, Compound 3, which is related to staurosporine.
Compound 3 completely abolished the ATII or PMA-induced inhibition of MAP kinases in the hypertrophic ventricle. In the Dahl R ventricle, however, Compound 3 was only marginally effective in blocking the stimulation of MAP kinase activity by ATII or PMA. Furthermore, Compound 3 alone may have a stimulatory action on the Dahl R ventricle, but this too was not evident in the hypertrophic ventricle. This difference in sensitivity supports other evidence indicating that the role of protein kinase C in the regulation of MAP kinase, is altered in the hypertrophic Dahl S ventricle. Assays to determine the activity and ATII sensitivity of protein kinase C confirm a difference in the basal level of activity. However, fractionation of the extract to determine translocated sarcolemmal, versus cytosolic protein kinase C activity should improve the resolution of this result.
3. CONCLUSION

In this chapter the ATII activation of protein kinases in Dahl R and hypertrophic Dahl S ventricle was studied in two ways. ATII induction of phosphorylation was examined by using metabolic phosphorylation in isolated myocytes, and ATII activation of MAP kinase activation was analyzed and compared. These two perspectives on the intracellular signalling of ATII have provided the basis for several important conclusions to be drawn. Firstly, ATII induces the phosphorylation of cardiac proteins. Secondly, a 42 kDa phosphoprotein is prominently phosphorylated in response to ATII stimulation in isolated ventricular myocytes. Thirdly, the ATII induction of pp42 phosphorylation has protein kinase C as a component of the signalling pathway yet is a focal point for the divergence between normal and hypertrophic myocytes. These results may be reflected in the ATII effects on MAP kinase activity. MAP kinases have been identified in the ventricle and have been found sensitive to ATII. However, the Dahl R ventricle responds oppositely to hypertrophic Dahl S ventricle; whereas ATII stimulates MAP kinase in Dahl R, it inhibits MAP kinase in hypertrophic Dahl S.

Although it is not known whether pp42 phosphorylation is a component of the MAP kinase control pathway, it is possible that pp42 phosphorylation is a negative signal which inhibits MAP kinase activity in the hypertrophic Dahl S ventricle.
CHAPTER V
SUMMARY AND CONCLUSIONS

1. Methodological Considerations

1. This study demonstrates the first use of the Dahl S rat as a model in which ATII activity on the hypertrophic ventricular myocyte has been examined.

   The advantages of this model are

   - increased ATII sensitivity of Dahl S tissues
   - availability of closely related strain, the Dahl R rat, which serves as a control
   - control and reproducibility of ventricular hypertrophy through simple dietary regulation of NaCl intake
   - consistent high yield of viable isolated ventricular myocytes from both Dahl R and Dahl S strains
2. Summary of Conclusions - ATII Intracellular Signalling

a) Adenylyl cyclase stimulation of hypertrophic Dahl S myocytes is depressed compared to Dahl R myocytes. This correlated with the degree of hypertrophy and appears to be independent of beta receptor density, as evidenced by similar results with isoproterenol or forskolin stimulation.

b) ATII induced inhibition of adenylyl cyclase on the normal Dahl R ventricular myocyte. The inhibition was pertussis toxin sensitive.

c) In the hypertrophic Dahl S myocyte the ATII effect on isoproterenol stimulated adenylyl cyclase was the reverse of that in the Dahl R ventricular myocyte, namely, an enhancement of activity. The pertussis toxin sensitivity of hypertrophic Dahl S myocytes differed from that of Dahl R.

d) The reversal was not dependent on differences in phosphodiesterase activity, since phosphodiesterase inhibition did not abolish the reversal of ATII action on cAMP.

e) Both inhibition of adenylyl cyclase in Dahl R and stimulation of adenylyl cyclase in hypertrophic Dahl S were blocked by Saralasin, an ATII analogue which competes with ATII for ATII receptor sites. The divergence in ATII activity on adenylyl cyclase was not due to ATII receptor differences between the strains since the muscarinic receptor agonist, carbachol, had a similar action to ATII in Dahl R and hypertrophic Dahl S.

f) The reversal of adenylyl cyclase activity was not due to alterations in the
amount of cholera toxin or pertussis toxin G protein subunit substrates, although some differences in the ADP ribosylation substrates were apparent.

g) The ATII effect on Dahl R and hypertrophic Dahl S myocyte was reflected in differences in ATII action on the contractile function of isolated hearts. In Dahl R, ATII inhibited forskolin stimulation of contraction, whereas in hypertrophic Dahl S, ATII induced elevation in forskolin-stimulated contractile increase.

h) A role for protein kinase C in the divergence of ATII effect on adenylyl cyclase is suggested by the action of the protein kinase C activator PMA. Long term PMA treatment abolishes the ATII stimulation of adenylyl cyclase in the hypertrophic Dahl S myocyte, but has only a small effect on the ATII inhibition of adenylyl cyclase in the Dahl R.

i) ATII induced phosphorylation of proteins in the hypertrophic Dahl S, and to a lesser extent, in Dahl R ventricular myocytes. The most prominent of these was a 42 kDa protein (pp42) which was concentration- and time- dependently phosphorylated in response to ATII treatment.

j) pp42 was shown not to be actin, nor pleckstrin, nor to be tyrosine phosphorylated. A large fraction of the protein resides in the sarcolemma, but was released by 1% Triton X-100.

k) pp42 phosphorylation was also induced by PMA but the sensitivity of Dahl R myocytes to PMA was greater than hypertrophic Dahl S myocytes.
1) *De novo* synthesis of pp42 was not stimulated by ATII sufficiently to account for the appearance of increased levels of the phosphoprotein.

m) ATII was shown for the first time to induce the activation of protein (MAP) kinases in the cardiac tissue. MAP kinase p44<sub>erkl</sub> and p42<sub>mapk</sub> were stimulated by ATII in Dahl R, but inhibited in hypertrophic Dahl S ventricular tissue.

n) PMA was able to mimic the stimulation in Dahl R and inhibition in hypertrophic Dahl S of MAP kinase activity.

o) Dahl R and Dahl S ventricular MAP kinase activation was differentially sensitive to the protein kinase C inhibitor, Compound 3. In Dahl R ventricle, Compound 3 was ineffective in blocking the ATII effect and produced a minor stimulation MAP kinase by itself. In the Dahl S ventricle, Compound 3 was effective in blocking the ATII inhibition of MAP kinase activity.

p) Protein kinase C activity was stimulated by ATII in both Dahl R and hypertrophic Dahl S myocytes and the stimulation blocked by Compound 3. However, the basal level of protein kinase C activity of hypertrophic Dahl S ventricle was significantly higher than Dahl R.
3. Implications

These experiments sought to elucidate the effect of ATII on the hypertrophic myocardium. The results indicated that ATII has several distinct actions on the hypertrophic myocardium compared to the non-hypertrophic myocardium. The mechanism of this divergence of ATII activity was studied with reference to known signal transduction pathways.

The first aspect of this work focused on the beta-receptor-adenylyl cyclase axis. The depressed cAMP response to beta-receptor stimulation in the hypertrophic Dahl heart agrees with a number of studies which show that adreno-sympathetic control is altered under a number of conditions including hypertrophy (Upsher and Khairallah, 1985; Limas and Limas, 1985; Foster et al., 1991). This suggests that, under some conditions, cardiac cells revert to a state of lowered reliance on sympatho-adrenal control. In place of this, it is possible that other means of contractile control come to the fore. Circulating or local renin-angiotensin II systems may participate as one of these alternative control systems. In the present study, this hypothesis is supported by the results obtained by assaying cAMP responses for the known inhibitory action of ATII on adenylyl cyclase. The hypertrophic Dahl rat heart myocyte not only lacked the normal inhibitory response to ATII, but displayed just the reverse: the enhancement of stimulated cAMP accumulation. Other work has suggested that while the heart becomes progressively less responsive to beta stimulation during the development of hypertrophy, it may become more responsive to ATII (Baker et al, 1990). In reverting to locally generated inotropes such as ATII, the hypertrophic cardiac myocyte may establish inotropic homeostasis thereby compensating for adrenoreceptor exhaustion. An intriguing aspect of present results is that the level of cAMP in hypertrophic myocytes produced after ATII pre-treatment consistently approximated the same value of cAMP generated after forskolin alone in non-hypertrophic cells.

This suggests a general dynamism in the control over fully differentiated cardiac myocytes. It also indicates a shift in the potential significance of ATII as the heart becomes
hypertrophic. The question of whether this shift is advantageous in the moment to moment response of the heart to hemodynamic conditions, or a reaction to a state of pathological growth, is important here. But present observations cannot provide an answer.

Further experiments on intact myocytes did, however, shed light on possible contributing mechanisms of this radical change in cAMP regulation. Since phosphodiesterase inhibition did not affect the reversal of ATII inhibition of cAMP, the locus of the divergent path can be concluded to be in the cAMP generating mechanism. G-proteins were candidates for this divergence since they play roles in both stimulus and inhibition of adenylyl cyclase (Gilman, 1984,1987) and because they have been demonstrated to be compliant with respect to the altered state of the myocardium in hypertrophy and failure (Feldman et al., 1988,1989). This contention is supported by the observation that pertussis toxin abolished the difference between normal and hypertrophic myocytes with respect to ATII effect on cAMP. The consequence of alterations of Gs and Gi activity in the hypertrophic cardiac myocyte is potentially important to the performance of the hypertrophic heart. The coupling of the two phenomena, the adenylyl cyclase stimulation through Gs and the enhancement of adenylyl cyclase stimulation through stimulation of other receptors such as ATII, may be a key aspect of control of contractile performance by the hypertrophic heart.

Since the mechanism of reversal of ATII inhibition of adenylyl cyclase cannot be fully accounted for by the G protein alteration observed thus far, other areas of signal transduction were investigated. This leads to the other major aspect of this investigation: protein kinase activation and protein phosphorylation.

The role of cAMP is not, of course, restricted to contractile control, but is, among other things, involved in cell cycle control. In other words it is possible that the alteration in adenylyl cyclase activity observed in the Dahl S rat is linked to the accelerated growth of these cells. One of the approaches used to probe the signal transduction of growth cycle control, is to examine protein phosphorylation and protein kinase activity. The protein kinase "cascade" is now known to be a complex intracellular network "wired" to inform
the cell of mitogen and growth factor presence at surface receptors by ultimately regulating
gene expression (Pelech et al., 1990). Changes in the "wiring" of the system could potentially
alter the effect a ligand, such as ATII, has on a cell. The latter part of this study focused,
then, on the protein kinase cascade by comparing phosphoprotein profiles in hypertrophic
Dahl S and normal Dahl R ventricular myocyte, and by examining specific mitogen activated
protein (MAP) kinases.

These experiments revealed further divergences in ATII signal transduction pathway
of the hypertrophic myocyte. The phosphoprotein study revealed a prominent 42 kDa
protein which was differentially phosphorylated in response to ATII in the Dahl S
hypertrophic myocyte. This protein then served as a landmark by which to assay ATII
intracellular signalling and allowed the determination of the low sensitivity of the
hypertrophic Dahl S myocyte 42 kDa protein to protein kinase C stimulation with PMA.
Although the identification of pp42 has proven difficult, it was thought that it may be
identical with p42\textsuperscript{mapk}, a known component of the protein kinase cascade linked with growth
activation in rat lymphocytes (Gold et al., 1992). p42\textsuperscript{mapk} activation was specifically assessed
and was demonstrated to be activated by ATII in the normal rat ventricle but inhibited in
the hypertrophic Dahl S ventricle, which was a reversal reminiscent of the divergences in
cAMP control. Another prominent MAP kinase, p44\textsuperscript{erk1} was also activated by ATII in Dahl
R but inhibited by ATII in hypertrophic Dahl S ventricle.

Thus, the protein kinase cascade is indeed activated differentially by ATII in normal
and hypertrophic ventricle. This not only supports the notion that ATII plays a distinct role
in hypertrophic myocardium but also provides a link between ATII signalling and the
mechanism of growth regulation in the ventricular myocardium. Since ATII stimulates MAP
kinases in the normal ventricular myocyte, it might be concluded that ATII can play the role
of growth factor. Yet, in the hypertrophic ventricle, ATII inhibits MAP kinase activity,
suggesting that other roles, some of them as a regulatory factor, predominate.

Taken together, these studies suggest that ATII signals through the protein kinase C
axis, which implicates protein kinase C as a possible area for further divergences between
hypertrophic Dahl S and Dahl R ventricle.

The Dahl rat, as a model of cardiac hypertrophy, has thus provided the basis for further investigation into the role ATII on the hypertrophic ventricular myocardium. The data presented here is of potential importance in the elucidation of the role of ATII in cardiac hypertrophy.
REFERENCES

CHAPTER I REFERENCES


Brockman SK (1965) Cardiodynamics of complete heart block. Am.J.Cardiol. 16:72-83


Claycomb WC (1976) Biochemical aspects of cardiac muscle differentiation: possible control of deoxyribonucleic acid synthesis and cell differentiation by adrenergic innervation and cyclic adenosine 3',5'-monophosphate. J.Biol.Chem. 251:6082-6089


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141


Garwitz ET, Jones AW (1982) Aldosterone infusion into the rat and dose-dependent changes in blood pressure and arterial ion transport. Hypertension 4:374-381


Goss CM (1938) The first contractions of the heart in rat embryos. Anat. Rec. 70:505-524


Kent RL, Hoober JK, Cooper G IV (1989) Load responsiveness of protein synthesis in adult mammalian myocardium: Role of cardiac deformation linked to sodium influx. Circ.Res. 64:74-85

Kessler PD, Cates AE, Van Dop C, Felman AM (1989) Decreased biactivity of the guanine nucleotide-binding protein that stimulates adenylate cyclase (Gs) in hearts from cardiomyopathic Syrian hamsters. J.Clin.Invest. 84:244-252

Keung EC (1989) Calcium current is increased in isolated adult myocytes from hypertrophied rat myocardium. Circ.Res. 64:753-763


144


Meerson FZ (1961) On the mechanism of compensatory hyperfunction and insufficiency of the heart. Cor Vasa 3:161-177


Peterson MB, Lesch M (1972) Protein synthesis and amino acid transport in isolated rabbit right ventricular muscle. Circ. Res. 31:317-327


Rakusan K (1971) Quantitative morphology of capillaries of the heart. Number of capillaries in animal and human hearts under normal and pathological conditions. Methods Achiev. Exp. Pathol. 5:272-286


Re RN, Michalik RJ, Dzau VJ (1983) Cardiac myocytes contain renin (abstr.) Clin. Res. 31:845A


RobishawJD, FosterKA (1989) Role of G proteins in the regulation of the cardiovascular system Ann.Rev.Physiol.51:229-244


Schneider MD, Parker TG (1990) Cardiac myocytes as the targets for the action of peptide growth factors. *Circulation* **81**:1443-1456


Strauer BE (1980) Hypertensive Heart Disease, Springer-Verlag, New York; pp 26


CHAPTER II REFERENCES


CHAPTER III REFERENCES


Seamons K, Daly JW (1981) Activation of adenylate cyclase by the diterpene forskolin does not require the guanine nucleotide regulatory protein. J. Biol. Chem. 256:9799-9801


Urabe Y, Kent RL, Mann DL (1990) The positive inotropic effect of angiotensin II is modulated by protein kinase C. *Circ.* (suppl.) 82:I11216


4. CHAPTER IV REFERENCES


Smith SH, Kramer MF, Reis I, Bishop SP, Ingwall JS: Use of isolated adult cardiac myocyte technique to calculate per cell activity of creatine kinase in normal and hypertrophied myocardium, in Clark WA, Decker RS, Borg TK (eds): Biology of Isolated Adult Cardiac Myocytes, Elsevier Science Publishing Co., New York, 1988, pp.228-


Sunga PS, Rabkin SW (in press) Angiotensin II induces phosphorylation of a Mr=42,000 protein in myocytes from hypertrophic hearts from Dahl rats. Hypertension (in press)


Zak R: Cardiac hypertrophy and atrophy, in Opie LH (ed): The Heart Grune and Stratton, London, 1984, pp 198-209
CHAPTER V REFERENCES


