ROLE OF ATRIAL NATRIURETIC PEPTIDE IN DIABETES-INDUCED CONGESTIVE HEART FAILURE

BY

ANIMESH SAHAI

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of

MASTER OF SCIENCE

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Winnipeg, Manitoba

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ISBN 0-315-86088-X

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TO

my Father and Mother
for teaching me the values of life

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ABSTRACT

$Ca^{2+}+Mg^{2+}$ATPase which serves as a Ca^{2+} pump in the kidney basolateral membrane (BLM) is essential to the maintenance of an intracellular Ca^{2+} concentration optimal for kidney function. Since atrial natriuretic peptide (ANP) is known to participate in Ca^{2+} homeostasis and is involved in direct stimulation of the Ca^{2+} pump in kidney BLM, experiments were initially designed to see if $Ca^{2+}+Mg^{2+}$ATPase is linked to the ANP-receptor system. ATPase activity was measured by release of ^{32}P from [γ-^{32}P]ATP into the medium. Incubation of BLM, prepared by differential centrifugation of rat kidney cortex, in the presence of ANP, increased $Ca^{2+}+Mg^{2+}$ATPase in a dose-dependent manner. However, activation of guanylate cyclase, a well-known receptor for ANP, by GTP and Mg^{2+} inhibited ATPase activity. Non hydrolysable GTP, such as GTP-γ-S, had no effect on the Ca^{2+} pump system indicating that substrate utilization is a prerequisite for inhibition. Tissue specificity of this inhibitory effect of GTP for Ca^{2+} + Mg^{2+} ATPase was examined in liver plasma membranes, and it was shown that the inhibition of the Ca^{2+} + Mg^{2+} ATPase was not significant. Studies were also done to ascertain for similar effects of GTP on the renal cortical Na^{+} + K^{+} ATPase. No inhibition could be documented implying that the effect of GTP was specific for Ca^{2+} + Mg^{2+} ATPase. Inclusion of cGMP in the incubation media did not modulate the Ca^{2+} + Mg^{2+} ATPase activity indicating that the restraining effect of the guanylate cyclase on the Ca^{2+} + Mg^{2+} ATPase was not a post-receptor mediated phenomenon. Ca^{2+} + Mg^{2+}ATPase was assayed in the presence of various stimulators and inhibitors of guanylate cyclase. Addition of sodium azide, a

powerful stimulator of guanylate cyclase, caused marked depression of Ca^{2+} + Mg^{2+} ATPase activity. Studies with guanylate cyclase antibody resulted in a marked elevation of $Ca^{2+} + Mg^{2+}$ATPase. The data suggest that ANP mediates its cellular effects in part by changes in Ca^{2+} transport in kidney cortex and that $Ca^{2+} + Mg^{2+}$ ATPase is linked to ANP receptors being reciprocally modulated by the guanylate cyclase system.

In the second phase of the study, $Ca^{2+} + Mg^{2+}$ATPase and guanylate cyclase were tested in hypertensive-diabetic rats (D+H) in congestive heart failure (CHF). Animals were divided into four groups: control, diabetic (D), hypertensive (H) and diabetic plus hypertensive (D+H). Diabetes was induced by an intravenous injection of streptozotocin (65 mg/kg) and hypertension by abdominal aortic constriction.The animals were monitored and sacrificed at 1 and 6 weeks post treatment for subsequent determination of circulating ANP and kidney enzyme systems. Plasma ANP and Ca^{2+} + Mg^{2+} ATPase activity in the kidney basolateral membrane increased in all groups after 1 week of treatment. Ca^{2+} + Mg^{2+} ATPase activity, on the other hand, was significantly decreased only in the D+H group after 6 weeks. This decrease was also associated with a decrease in plasma ANP, increase in kidney ANP receptor number and a reduced guanylate cyclase activity. However, the sensitivity of the Ca^{2+} pump to ANP was increased at 1 week and unchanged at 6 weeks. Based on the present results, it is suggested that a compensatory stage of CHF may exist in D+H group at 1 week where renal $Ca^{2+} + Mg^{2+}$ATPase is more sensitive to ANP in order to maintain intracellular Ca^{2+} homeostasis. As Ca^{2+} + Mg^{2+}ATPase is an efflux mechanism for the Ca^{2+} absorbed

into the cell from the glomerular filtrate, a depression in its activity at 6 weeks would lead to an increase of cytosolic Ca^{2+} concentration. The inability to eject the excess Ca^{2+} would eventually result in Ca^{2+} overload which will compromise cellular function and integrity. We conclude that a defect in coupling between the Ca^{2+} pump and kidney ANP-receptor system as observed in the D+H group may contribute to the development of Ca^{2+} overload, nephropathy and CHF.

ACKNOWLEDGEMENT

I would first like to express my gratitude to Dr. P.K.Ganguly, for providing me with the laboratory facilities at the St. Boniface Research Centre to conduct my studies. His valuable advice and constructive criticism have been immensely helpful to me. He knew when to put the pressure (" do it right away") and when to take things easy (" do it first thing tomorrow morning"). His constant reminders of "jump to the pump" have been very helpful in my studies. I also wish to express my thanks to our laboratory technicians, Yi Jing Chen and Kallol Mukherjee both of who have assisted me in my work. Thanks are also due to Gilbert Kirouac and Nobby Woo for their constructive criticisms regarding my thesis. I am also grateful to the colleagues and friends, too numerous to mention, at the St. Boniface Research Centre who have been a source of constant input.

I an indebted to my committee members, Dr. J.A. Thliveris, Dr. P.K. Singal and Dr. J. E. Anderson for their contribution both in terms of my experiments and in helping me bring my thesis to its present shape.

Finally, thanks are due to my wife, Madhu, for her encouragement and help in finishing my thesis.

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LIST OF ABBREVIATIONS

ANP	Atrial Natriuretic Peptide
ATPase	Adenosine Triphosphatase
BLM	Basolateral Membrane
cGMP	cyclic Guanosine Monophosphate
Ca^{2+}	Calcium
CHF	Congestive Heart Failure
GC	Guanylate Cyclase
GFR	Glomerular Filtration Rate
K^+	Potassium
Mg^{2+}	Magnesium
Na^+	Sodium
DDH_2O	Double distilled water

INTRODUCTION

The incidence of severe cardiomyopathy and congestive heart failure has been found to be significantly higher in diabetic patients with long-standing hypertension. Hypertension was first linked to diabetes by Hitzenberger in 1921 (1). However, a causal relationship between diabetic cardiomyopathy and hypertension was demonstrated from the studies of Factor et al. (2-4) on hypertensive rats. The additive effects of hypertension and diabetes mellitus resulted in a significantly greater degree of myocardial fibrosis and degeneration, than with either disease alone. These structural changes were seen at both the light microscopic (2) and ultrastructural levels (3), and were similar to their earlier findings on hypertensive-diabetic patients (4). It has been recognized that hypertension is important not only for ultimate renal risk, but also for the development of cardiovascular complications of diabetes mellitus. The incidence of cardiovascular complications is elevated primarily in diabetics who developed nephropathy (5,6). This would reemphasize a widely prevalent view that diabetes has a priming effect on the myocardium so that supplemental hypertension would result in progressive myocyte damage and dysfunction (4-6).

Myocardial dysfunction results in reduced tissue perfusion which activates a series of neurohumoral compensatory mechanisms. In this regard, a cardiac hormone, atrial natriuretic peptide (ANP) has been identified and shown to have a controlling influence on the regulation of plasma volume (7). Considerable correlation has been noted between ANP levels and congestive heart failure (8). ANP is now beginning to be recognised as a prognostic factor in congestive heart

failure comparable to the New York Heart Association (NYHA) classification and ventricular hyperexcitability (9). While research has focused in identifying the role of ANP in hypertension and documented heart failure, relatively little information exists on the status of this peptide in chronic diabetes. Diabetes mellitus (which is a grouping of anatomic and chemical factors in which an absolute or relative deficiency of insulin or its function usually is present) is associated with extracellular fluid expansion (10,11) which is a well known entity for the release of ANP (12). Since ANP with its properties of vasodilatation, salt and water loss provides a counterregulatory mechanism to maintain plasma volume in congestive heart failure (7-9), this hormone may have a cardinal influence on the genesis of congestive heart failure in diabetes.

Body fluid volume regulation is critical to life and has been a subject of intense research for a substantial period of time. In the words of the nineteenth century physiologist, Claude Bernard, "The constancy of the internal environment is the condition of free and independent existence" (14). Yet many conflicting opinions and apparent paradoxes have clouded the issue of body fluid volume regulation. Research from different laboratories has led to the hypothesis that cardiovascular homeostasis is achieved by the orchestration of cardiac and renal systems referred to as the cardio-renal axis. Although research has flourished in elucidating the role of cardiac system in body fluid volume regulation and its alterations in pathological states, the kidney has long remained the Cinderella of fluid physiology. However, the kidney is increasingly being recognised as a major determinant of body fluid status, and alterations in renal structural and functional

integrity can effectively alter fluid disposition of the body.

Kidney function may have a considerable significance in volume-related disorders such as diabetes and congestive heart failure. Increased cardiovascular mortality of type I diabetics is generally confined to the population segment with proteinuria (5,6). This suggests a positive correlation between cardiovascular mortality and evolving renal dysfunction. Therefore renal integrity, structural and functional, is the undercurrent theme in the effective maintenance of body fluid status in volume disorders such as congestive heart failure. And if the body fluid status is considered as a dynamic system, where the heart and the kidney can be described as the "pump" and "filter" respectively, loss in the filtrable surface area due to a compromised kidney, could seriously jeopardize fluid volume status.

Kidney cortex basolateral membrane contains a high-affinity, low capacity calcium (Ca^{2+}) + magnesium (Mg^{2+}) adenosine triphosphatase (ATPase) that serves as a Ca^{2+} pump in maintaining optimal cytosolic Ca^{2+} concentrations at a lower level than in the extracellular fluid (15). Because the enzyme is localised only to the basolateral membrane (15), the authors suggested that this pump participates in the exit of Ca^{2+} from the tubular cells. As a corollary, it can be said that defective pumping of this ATPase might lead to a diminished exit of Ca^{2+} from the cell with an overall build-up of Ca^{2+} in the cytosol. This condition, referred to as the "calcium overload" could result in the death of the cell and thus a loss kidney function (16). Since ANP is known to participate in Ca^{2+} transport mechanisms (17), it is possible that ANP may exert considerable influence on the kidney cytosolic Ca^{2+} concentration through its action on the Ca^{2+} + Mg^{2+} ATPase

pump.

Furthermore, it has been shown that ANP binds to specific receptors on the plasma membrane to elicit its physiological responses (18). In this regard, guanylate cyclase has been identified as a transmembrane protein with dual activities of ligand binding and synthesis of the second messenger, cGMP from guanosine triphosphate (GTP) (19). ANP receptors are modified under various pathophysiological states (20). It is possible that when hypertension is superimposed on to diabetes, the receptor-mediated responses influenced by these two membrane-bound enzymes in the kidney are altered. The purpose of this study was to investigate the hypothesis that it is this alteration in enzyme activity which represents the crucial development in the genesis of congestive heart failure in the setting of chronic diabetes with hypertension. The present study was therefore designed, 1) to investigate the link between these two membrane-bound enzymes, both of which represent a target site for ANP's action, and, 2) to assess how alterations in their activity may influence the development of congestive heart failure in chronic diabetes superimposed with hypertension.

REVIEW OF THE LITERATURE

2.1 ATRIAL NATRIURETIC PEPTIDE

2.1.1 Synthesis, storage and degradation

The discovery by DeBold and co-workers of the potent diuretic and natriuretic properties of atrial extracts (21-23) led to the identification of a cardiac hormone, aptly termed atrial natriuretic peptide (ANP). Elucidation of the molecular structure of circulating ANP and its precursors showed that it is synthesized as a 151-amino acid molecule called prepro-ANP (24). The human sequence (25) shares strong homology with those in rat (26), dog (27), and rabbit (27). It must be pointed out however that human ANP is identical to that in rats except for substitution of methionine for isoleucine at position 12 (28).Cleavage of the "signal" peptide, a region rich in hydrophobic residues, yields pro-ANP (1-126), which is the principal storage form of the peptide (29). Bioactive peptides are derived from the carboxy-terminus, with the predominant circulating form being ANP (1-28), (30). Proteases [atrioactivase (31), thrombin (?) (31)] present in the atrial tissue and serum are responsible for conversion to the active peptide.

In addition to atrial myocytes, ANP has been shown to be synthesized and stored in a wide variety of tissues, albeit at levels far lower than those found in atria, although the cardiac tissue ANP contribution of the body fluid homeostasis is still under investigation. Atrial natriuretic gene expression has been observed in fetal ventricles (32), aortic arch (33), lung (34), anterior pituitary (34), hypothalamus (34), brain (35), adrenals (36) and kidney (37). It may be mentioned

that in the fetus, ANP content and mRNA levels are lower in the atria than in the ventricles, and these distributions reverse in early post-natal life (38). Similarly, in adults with normal cardiovascular hemodynamics, the ANP gene is expressed in both the ventricles and atria, but the concentration in the ventricles appears very low. However, a reinduction of the ANP gene occurs in ventricles "under stress" such as in congestive heart failure (39,40).

ANP is a short-acting peptide with a half-life of 2 to 4 minutes in animals and humans, and is primarily degraded in the kidney (41). The brush border of the proximal tubule in the kidney is very rich in degradative enzymes and exhibits one of the two ways in which ANP is removed from the circulation. Cleavage of the disulfide bond (Cys-Phe) at positions 7-8 by a metalloendopeptidase variously termed enkephalinase or neutral endopeptidase EC 24.11, disrupts the ring structure of ANP (42,43). This endopeptidase can be inhibited in vivo by phosphoramidon or thiorphan (44,45), UK69578 and UK79300 (46) with corresponding increases in plasma ANP, urinary cGMP (the second messenger of ANP) and U_{Na} V (urinary Na^{+} excretion rate). Thus inhibition of this endopeptidase leads to an augmentation of the hypotensive, natriuretic, and urinary cGMP responses to ANP, suggesting a therapeutic potential for selective inhibitors of EC 24.11. In addition to degradation by enzymes, ANP is also removed from the circulation by binding to the so-called "clearance" receptor. This plasma membrane protein is expressed in large abundance on vascular endothelial cells (47-49). It has a long extracellular ANP binding domain and a very short intracellular domain and inactivates circulating plasma ANP.

2.1.2 Secretion and function

ANP is secreted in response to a variety of mechanical and humoral stimuli. Cardiac stretch has been suggested as the principal mediator of ANP release (50). While heart rate per se has no effect on ANP secretion in isolated, perfused, beating atria, inflation of a balloon in the left atrium of dogs induced a marked increase in urine flow (51), whereas prevention of atrial stretch in volume-expanded animals abolished this renal response (52). Similarly Langendorff heart-lung preparations in rats release bioactive or immunoreactive ANP in response to atrial stretch induced by volume expansion (53). Ledsome et al. (54) observed increased plasma ANP levels in intact dogs subjected to mitral valve obstruction. Atrial distension which accompanies acute and chronic volume overloading in rats (55-57) and humans (58) is responsible for the raised plasma ANP levels. Similar increments in plasma ANP levels have been documented in pathological states associated with increased atrial pressure including rapid tachyarrhythmias, congestive heart failure and various disorders associated with expansion of extracellular fluid volume. However, very little information exists in the literature on the levels of ANP and their role in diabetes-induced congestive heart failure.

Apart from these mechanical stimuli, ANP is also released in response to humoral stimuli. Glucocorticoids and mineralocorticoids have been shown to have a priming effect for ANP release (59). Dexamethasone, a long acting steroid, can increase ANP gene transcription in keeping with evidence of glucocorticoid binding site on the ANP gene and with the known regulatory effect of glucocorticoids on

transcription of mRNAs encoding other hormones (60). Several other factors also influence ANP secretion. Acetylcholine, epinephrine and vasopressin all cause release of a natriuretic substance from rat atrial tissue in vitro, as detected by bioassay (61,62). In vivo, intravenous administration of vasopressin, angiotensin II or phenylephrine raises plasma ANP levels in rats, possibly due to their systemic vascular effects, since the rise in plasma ANP correlates closely with elevations in mean arterial blood pressure (63). Endothelin is a newly discovered potent vasoconstrictor peptide made by endothelial cells in response to increased shear stress, and a variety of chemical agonists (e.g. thrombin, epinephrine, phorbol esters) (64). Furthermore, it also has been shown to augment plasma ANP levels (65) as well as ANP release from isolated cardiac myocytes (66), from isolated contracting right atria (67) and from isolated heart preparations (68).

2.1.3 Biological effects

The definitive effect of ANP is the enhancement of renal sodium and water excretion (68-72). This natriuresis and diuresis are accompanied by similar marked increases in phosphate, calcium, magnesium, chloride, and cGMP excretion (73-79). Studies utilizing inhibitors of ANP degradation (80,81) as well as specific anti-ANP antisera (82,83) have shown that elevation of circulating ANP levels is accompanied by increased salt and water excretion.

The observed solute and water excretion could be explained as a response to increased renal blood flow. However, in studies showing augmented renal blood flow, the increase was transient, lasting less than a minute (75,84). Moreover, in both conscious and anesthetized rats, ANP tends to reduce renal blood flow. Since

prior denervation of the kidney also tends to reduce renal blood flow (85), it is possible that changes in sympathetic nerve tone and the circulating levels of vasoconstrictor can modify overall vascular responsiveness to ANP. It is evident that changes in renal blood flow per se are not the major factors responsible for enhanced renal solute excretion (86,87).

Many studies have demonstrated that ANP increases GFR and filtration fraction (88-90). ANP dilates preglomerular (afferent) arterioles and constricts postglomerular (efferent) arterioles, effectively increasing hydraulic pressure within glomerular capillaries (91) while offsetting effects on glomerular blood flow. Using quantitative video microscopy, Marin-Grez et al. (92) observed dose-dependent dilatation of arcuate, interlobular, and proximal afferent vessels and constriction of efferent arterioles in response to ANP infusion. Calculated resistances in afferent and efferent arterioles were in accord with those obtained from video image analysis in that afferent arteriolar resistance fell while efferent arteriolar resistance rose. Fried et al. (93) examining isolated perfused dog glomeruli, reported significant increases in glomerular hydraulic pressure and efferent resistance when ANP was perfused.

In addition, ANP may act to relax glomerular mesangial cells (94,95). This effect may alter GFR in two ways. First, relaxation of the mesangial cells results in expansion of capillary surface area available for filtration (94). Second, these cells increase the glomerular capillary ultrafiltration coefficient, K_f at least in cultured glomerular mesangial cells (96). A direct effect of ANP on K_f has also been seen in studies of cultured glomerular mesangial cells (96,97).

Although it is attractive to postulate that the increase in GFR may be responsible for the observed natriuresis and diuresis after ANP administration, this is not always the case. Many studies have detected no changes in GFR on low-dose ANP infusion, while natriuresis and diuresis occurred (98,99). At higher doses, the increase in GFR is more marked (100,101). Several investigators also believe that ANP also directly alters tubule Na^+ and water reabsorption and thereby causes the observed effects (102,103). Moreover, increases in Na^+ and water delivery to the distal nephron, after ANP administration, are usually insufficient to account fully for the observed natriuresis and diuresis (104). Therefore, as the renal tubule consists of several highly specialized segments arranged in series, actions of ANP at several sites contribute to the final amount of natriuresis and diuresis.

Experimental studies have shown that high doses of ANP, injected into man, increase urine volume and electrolyte excretion. ANP also lowers arterial pressure and reduces renin and aldosterone concentrations, while raising heart rate, haematocrit and plasma norepinephrine (105). These data, along with information from animal experiments (79,80,88) have suggested that atrial peptides protect against fluid overload, and counterbalance the renin-angiotensin system and perhaps the sympathetic system in normal situation. However it is uncertain whether ANP plays an important pathophysiologic role in man. If indeed atrial peptides are essential in the maintenance of fluid volume and arterial pressure in the healthy individual man and in patients with disorders of fluid balance, then several predictions might be made. First, ANP levels should respond predictably to volume-loading or pressor stimuli. It is well-known that manoeuvres which

increase the volume of the central circulation stimulate ANP release (105) in a dose-response manner (106). Second, a physiological and pathophysiological role would be supported by evidence that minor increases in plasma ANP, to levels seen in clinical disorders, and preferably within the range seen in healthy volunteers, induced clear-cut biological effects. Cuneo et al. (107) demonstrated that low-dose infusion of ANP inhibits the normal aldosterone response to angiotensin II in sodium-restricted subjects, while also suppressing renin levels. Similarly Morice et al. (108) reported an increase in urinary sodium excretion with low-dose infusion of ANP.

In pathophysiological states such as congestive heart failure, plasma ANP levels are 5 to 10 fold higher as compared to controls (109,110). Normal individuals and patients with heart disease who do not suffer from CHF exhibit plasma ANP levels ranging from 10 to 50 pmol/L, whereas patients with CHF typically exhibit levels in excess of 100 pmol/L, with wide individual variation (111-113). The plasma level of ANP correlates closely with indices of the severity of the CHF, varying directly with right atrial and pulmonary capillary wedge pressures and inversely with cardiac index, stroke volume, blood pressure and New York Heart Association class (114-116). Animal models of CHF show that high plasma ANP levels also correlated inversely with atrial tissue concentrations, denoting prompt secretion and little tissue storage despite high ANP mRNA levels in the atria (117). It is noteworthy that effective therapy for CHF leads to reduction in plasma ANP levels usually in proportion to improvement in clinical status and cardiac performance (113), but still does not provide a definitive cure.

2.1.4 Mechanism of action

ANP binds to stereospecific cell surface receptors and thereby evokes physiological responses in target cells (18). This hormone-receptor interaction induces the plasma membrane associated guanylate cyclase which converts MgGTP to cGMP (19). The cGMP activates cGMP-dependent protein kinases, which in turn are capable of phosphorylating a large number of intracellular proteins (118), thereby expressing the physiological actions induced by ANP.

a) ANP Receptors

Autoradiography has identified specific ANP binding sites in various target tissues, most notable of which are the kidney, adrenal and the vasculature (119). Receptors have also been identified in the central nervous system, pigmented epithelium and ciliary process of the eye, hepatocytes, gall bladder, colonic smooth muscle and lung parenchyma (120-125). In the kidney, binding sites are shown to be concentrated in large renal vessels, glomeruli and renal medulla (126-130). Receptors have also been identified in a number of cell types including adrenal glomerulosa cells, renal inner medullary collecting duct cells, renal glomerular mesangial and endothelial cells, arterial smooth muscle and endothelial cells and the pig kidney epithelial cell line LLC-PK_1 (131-136).

Radioreceptor assay systems and affinity cross-linking experiments have suggested the presence of several distinct cell surface ANP binding sites in most cells and tissues (137-142). However, there is still considerable debate over the nature, function and even the existence of these receptors amongst investigators. However, a consensus of opinions agrees on the existence of at least three different

receptors viz. ANP-receptor 1 (ANP-R1), ANP-R2 and ANP-R3.

ANP-R2, also referred to as the clearance receptor or C-receptor, is a plasma-membrane associated protein that binds ANP with high affinity. SDS-PAGE analysis has shown it to be a 120-130 kDa molecular mass under non-reducing conditions (65-70 kDa under reducing conditions) (143-145). This receptor does not have guanylate cyclase activity, the enzyme via which ANP generates cGMP, the second messenger for expression of its biological activities. In fact, there is no evidence whatsoever to suggest that binding of ANP to this receptor is capable of eliciting the specific cellular responses. Moreover, this receptor not only binds ANP (1-28) or the bioactive ANP but also binds ANP fragments and internally ring-deleted ANP analogues with equal affinity (146-148). These data along with the presence of abundant ANP-R2 on the vasculature suggest that it probably has a role in endogenous degradation/inactivation of ANP.

ANP-R3 is also a distinct receptor for ANP sharing over 70% homology with the guanylate cyclase and kinase domains. It has been identified in human placenta and rat brain (149,150). However, no specific function has yet been ascribed to it.

ANP-R1 also is a membrane-associated protein with an apparent molecular mass of about 130 kDa (144,145). It has a selective affinity for ANP (1-28) (135). The binding of ANP at R1 activates particulate guanylate cyclase (151). Studies have confirmed that the ANP binding site and guanylate cyclase activity are on the same transmembrane protein of R1 (152). It must be mentioned that another 180-kDa membrane protein with guanylate cyclase activity has been purified to

homogeneity from rat adrenocortical carcinoma cells (153). The 1:1 ANP-receptor stoichiometry suggests that this protein is also a bifunctional protein (153). However, further investigation including the sequencing of the gene that encodes this 180-kDa ANP binding site is required to decide whether it is a distinct receptor or the same as currently known ANP receptors associated with other membrane-bound proteins.

b) Guanylate cyclase

Guanylate cyclase belongs to a family of proteins involved in cell signalling mechanisms. It has been shown to exist in various cellular compartments, and its different forms are yet to be identified (154). Different locations have been recognized based on their presence in the plasma membrane, cytosol or a detergent-insoluble cytoskeletal fraction (155;156). Studies have shown that Ca^{2+} via an intermediary binding protein regulates guanylate cyclase (157-159), which in this case probably resides in the plasma membrane or cytoskeleton. Ca^{2+} has been shown to be capable of modulating the activity of guanylate cyclase. In several species it has been conclusively proved that addition of Ca^{2+} stimulates plasma membrane associated guanylate cyclase (157,158). A role for calmodulin has been suggested in mediating this response though the exact nature of the intermediary binding protein is under dispute (158,159).

Another group of guanylate cyclases exist which are under the regulatory control of extracellular peptides. Plasma membrane-associated forms of guanylate cyclase, known to be transmembrane proteins (160),can be regulated

by various peptides and on SDS-PAGE analysis have a molecular weight ranging from 120-180 kDa (161). They can be distinguished from the soluble form of guanylate cyclase in that while the soluble form exhibits linear kinetics as a function of substrate concentration (154,155), the particulate or plasma-membrane form characteristically displays positive cooperative behaviour as a function of the substrate (154,155,161).

The physiological effects of ANP involve interactions at the target cell surface resulting in the activation of particulate guanylate cyclase and the elevation of intracellular levels of cGMP (162). However, a bifunctional role has been envisaged for guanylate cyclase (19). The binding of a ligand to an extracellular domain of the guanylate cyclase transmits a signal to an intracellular catalytic site. Indeed, ANP stimulates particulate guanylate cyclase in a concentration-dependent manner in responsive tissues where it exclusively stimulates the maximum enzyme activity (V_{max}) without altering the Michaelis constant (K_m) of the enzyme (163,164). ANP binding to the receptor domain of guanylate cyclase induces conformational changes in an adjacent catalytic domain of guanylate cyclase thereby increasing the rate of cGMP formation. Cyclic GMP then acts as the "second messenger" coupling ANP-membrane interactions to the ultimate physiological response.

In vascular smooth muscle, cGMP activates cGMP-dependent protein kinase and phosphorylates a number of intracellular proteins (151). cGMP also dephosphorylates myosin light chains thereby inducing relaxation of vascular muscle (165). In the kidney tubular cell phosphorylation by cGMP-dependent protein kinase inhibits an amiloride-sensitive cation Na^+ channel (166-168), thereby

inducing natriuresis. In other cell types, phosphorylation by cGMP-dependent protein kinases appears to mediate the actions of ANP, but by mechanisms that are poorly understood (169,170).

In keeping with its role in mediating smooth muscle relaxation, ANP acting via cGMP has been postulated to decrease the intracellular Ca^{2+} concentration (171,172). The fact that cGMP may play a role in the exit of Ca^{2+} from the cell is suggested by the finding that cytosolic Ca^{2+} stores, normally depleted with repeated agonist stimulation, are depleted more rapidly in the presence of agents that elevate cytosolic cGMP (173). Studies have shown that ANP decreases cytosolic Ca^{2+} concentrations in rat aortic smooth muscle cells (95) and rat glomerular mesangial cells (174,175). The mechanism by which ANP alters Ca^{2+} mobilization could involve regulation at the level of Ca^{2+} release from intracellular stores, reuptake of Ca^{2+} into these stores, or Ca^{2+} influx or efflux across plasma membranes.

This physiological role of ANP in Ca^{2+} transport cannot be overstressed, especially at sites where considerable efflux/influx occurs e.g. the kidney. The available evidence thus favors the view that ANP has a role in mediating cytosolic Ca^{2+} concentrations in order to maintain an optimal intracellular Ca^{2+} concentration.

2.2 RENAL HANDLING OF CALCIUM

Calcium has only been recently recognised as an universal cell messenger. It was the classical work by Ringer in 1882 (176) on the role of Ca^{2+} in muscle

contraction which showed that Ca^{2+} was fundamental to cell signalling mechanisms. Subsequently, the developments in the field of Ca^{2+} ionophores, the discovery of calmodulin and other intracellular Ca^{2+} binding proteins, the progress in studies on membrane transport of Ca^{2+} and the development of precise techniques for measuring intracellular Ca^{2+} concentration have contributed to the continued expansion of interest in the role of Ca^{2+}. Paralleling these developments, there has been an autocatalytic growth in the understanding of mechanisms that are involved in the regulation of cytosolic Ca^{2+} concentration.

Considering the importance of calcium as an intracellular messenger, it is conceivable that mechanisms exist to maintain, an optimal cytosolic Ca^{2+} concentration. Calcium homeostasis is maintained by the integrated actions of the intestine, bone and kidneys. Interchange of Ca^{2+} between skeleton and blood, its absorption from the intestine, and renal reabsorption of filtered Ca^{2+} are all involved, but the relative contribution of each is still controversial. Although the gut and skeleton do play significant roles, Nordin and Peacock (177) and Nordin et al. (178) have shown that the kidney is the chief regulator of calcium.

In the blood, 35 to 45% of total calcium is bound to plasma proteins, and only the remaining fraction passes freely across the glomerular basement membrane. In the healthy man, this free fraction of calcium approximates 11,000 mg. It is obvious from this that there are effective mechanism(s) for tubular reabsorption of Ca^{2+}. Data from micropuncture and microperfusion studies (179-181) also indicate active Ca^{2+} reabsorption along the tubule. Both micropuncture studies and the evaluation of urine samples collected following transient ureteral

occlusion reveal tubular fluid/ultrafiltrable plasma ratios of Ca^{2+} well below 1.0 (177,182-184), despite an electric potential difference across the tubule that should lead to the appearance of tubular fluid/ultrafiltrable plasma ratios above 1.0. These findings suggest that a very effective active transport mechanism exists for Ca^{2+} in the renal tubule.

Under basic conditions, nearly all the Ca^{2+} in the glomerular filtrate is reabsorbed along the nephron. Though the maximal load of the filtered Ca^{2+} is absorbed along the proximal portion of the tubule, reabsorption is considerable along the distal segments of the nephron (185). Both active and passive modes of transport have been postulated to exist for tubular reabsorption of Ca^{2+}, even along in the same segment, though the cellular events responsible for active Ca^{2+} transport by the renal tubule remain undefined.

However, it is important to understand a few essential concepts in the area of cellular Ca^{2+} homeostasis. Most of the Ca^{2+} is reabsorbed from the tubular lumen into the cell across the brush-border membrane along an electrochemical gradient by a facilitated diffusion transport mechanism which is powered by an electrogenic sodium transport system (186,187). In other words, Ca^{2+} reabsorption into the cell is a passive mechanism. Ca^{2+} is maintained at low concentrations in the cell with the background concentration of free Ca^{2+} in most cytosol oscillating between 0.1 and 0.2 μM (188-190). However, Ca^{2+} as important as it is in cell signalling and other biochemical processes, the situation of Ca^{2+} overload could jeopardise cellular function and integrity (16). Indeed, inundation of the cytosol with Ca^{2+} is a frequent and early event in cell pathology (16). It might be even

challenging to say that eucaryotic cells have elected to live in a permanent state of controlled risk, a dynamically convenient but nevertheless dangerous choice where the margin separating cells from Ca^{2+} catastrophe may, on occasion, be very narrow. This obviously necessitates the presence of systems that eject Ca^{2+} from the cells thereby offsetting its downhill penetration. Moreover, the electrochemical gradient at the basolateral side is reversed, requiring energy-dependent mechanisms for the extrusion of Ca^{2+} (15,17). And it is by concerted actions of the importing system (Ca^{2+} channel) and the exporting systems (Ca^{2+} pump and Na^+-Ca^{2+} exchanger that an optimal cytosolic Ca^{2+} concentration is maintained. The Na^+-Ca^{2+} exchanger is a large-capacity, low-affinity electrogenic antiporter (191). However, kinetic studies have shown that it is in equilibrium at cytosolic free Ca^{2+} levels and does not contribute greatly to Ca^{2+} extrusion (192). The Ca^{2+} pump on the other hand is a low capacity system with a high affinity for Ca^{2+} (17). Its high affinity enables it to interact with Ca^{2+} even at the very low background intracellular concentration of resting cells. It therefore functions continuously, satisfying the demands for the fine tuning of intracellular Ca^{2+}.

2.3 RENAL Ca^{2+}-ATPASE

Cytoplasmic free Ca^{2+} concentration (0.1 to 0.2 μM) is several orders of magnitude lower than in the extracellular milieu. Though the diffusion of Ca^{2+} into the cell is passive, the electrochemical gradient at the basolateral side is reversed, requiring energy-dependent mechanisms for Ca^{2+} exit (15). In transporting epithelia, pump and exchanger systems participate in the vectorial translocation of

calcium for maintaining intracellular Ca^{2+} homeostasis (15,192). Thus, membrane-bound Ca^{2+} transporting enzymes have been identified in cortex homogenates (193), suspensions of cortical tubules (194) and microsomes (195-198) from rat, rabbit and hog kidneys. Strong corroborative evidence for a function of Ca^{2+}-ATPase in renal tubular Ca^{2+} transport has been obtained from two studies on plasma tubules. First, separation of vesicles from the luminal and basolateral aspect of the cell has shown that these enzymes are located exclusively in the basolateral membrane (195,197,198), where they extrude Ca^{2+} into the peritubular space against steep gradients. Second, Ca^{2+} uptake by (inside-out) basolateral membrane vesicles (corresponding in vivo to Ca^{2+} extrusion from the cell), depends on, and is supported by, ATP found in the cytosol and in concentrations optimal for Ca^{2+}-dependent ATPase activity (186).

2.3.1 General properties

Kinne-Saffran and Kinne (195) were the first to describe the presence of a Ca^{2+}-stimulated ATPase in the renal basolateral membrane. Studies showed that the enzyme was present along the entire nephron and was activated by millimolar concentrations of either Ca^{2+} or Mg^{2+}, neither of which was necessary for activation by the other cation (199). The physiologic role of this low-affinity enzyme in transmembrane calcium transport is not clear, but is activated by Ca^{2+} concentrations in the millimolar range whereas the cytosolic free Ca^{2+} concentration in kidney cells is of a lower magnitude (190). It is conceivable that the high capacity of the enzyme compensates for its low affinity (195), or that Ca^{2+} may be "compartmentalized" at the inner surface of the membrane to a higher

concentration than in the rest of the cytosol. The exact status of this low-affinity Ca^{2+}-ATPase in Ca^{2+} transport still remains to be determined.

A more acceptable hypothesis is the presence of a high-affinity Ca^{2+}-ATPase for extruding Ca^{2+} out from the renal tubular cells, activated by normal intracellular Ca^{2+} concentrations. In rat renal cortex basolateral membranes and rabbit renal tubules, a Mg^{2+}-dependent, high-affinity, low capacity calmodulin-sensitive Ca^{2+}-ATPase has been identified (191,197,200-203). Moreover, the relationship between the K_m for Ca^{2+} of this (Ca^{2+}+Mg^{2+}) ATPase (0.68 μM) and that of the ATP-dependent Ca^{2+} uptake (0.5 μM) in vesicle preparations lends further credence to the existence and involvement of this enzyme in transmembrane Ca^{2+} transport (186,197).

2.3.2 Ca^{2+} + Mg^{2+} ATPase and Ca^{2+} transport

The general mechanism of the Ca^{2+} pump follows the pattern of that of all other P-type ion pumps (204,205). The cycle begins with the Ca^{2+} dependent transfer of the terminal phosphate of ATP to an aspartic acid residue in the pump (206,207). The phosphorylated intermediate has a high Ca^{2+} affinity. The pump bearing the phosphorylated intermediate (E_1-P in the conventional formalism) undergoes a conformation transition promoted by Mg^{2+}: the new conformer is called E_2-P (208). The matter of the affinity for ATP is more complex. The hydrolysis to E_2 and P_i is assumed to be accelerated by the binding of ATP to a site on the pump that has less affinity (209,210) than the site that binds ATP to initiate phosphorylation (209,210). In the last step of the reaction cycle the

dephosphorylated E_2 conformer reverts back to the initial E_1 conformation. Analogous to other P-type ion pumps, vanadate (a pentacoordinate, trigonal bipyramid stereo analogue of phosphate), also inhibits the Ca^{2+}-ATPase at very low concentrations (211). It is generally assumed that vanadate inhibits by binding to the aspartic acid residue. Not only has it been shown to act as a non-competitive inhibitor of the binding of ATP to the high affinity site (212), but also as a mixed, partially competitive inhibitor at the low affinity site (212). Vanadate interacts with the E_2 conformer of the ATPase, blocking the last step of the reaction (i.e. E_2--E_1 transformation) thereby stabilizing the enzyme in the E_2 conformation.

One aspect of the reaction cycle which is still open to question is the step during which translocation of Ca^{2+} across the hydrophobic barrier of the membrane takes place. Two steps in the reaction cycle have been tentatively identified. The first refers to a conformational change of the pump protein, i.e. E_1-P to E_2-P, and the second, from conformer E_2 to E_1. In either case, the Ca^{2+} binding site, located on the interior of the membrane before the translocation step (E_1 conformation), will be located on the external side of the plasma membrane at the end of the translocation step, i.e. the Ca^{2+} binding site on the E_2 conformer of the pump faces the exterior of the cell (213). Apart from the dispute regarding the site of Ca^{2+} transfer, considerable debate also persists in the number of Ca^{2+} atoms translocated during the cycle. The observation of a Hill coefficient greater than one for the activation of ATPase, saturation of the pump and its rate of Ca^{2+} transfer, in erythrocytes is proportional to the square of cytosolic Ca^{2+}, indicate two high-affinity Ca^{2+} binding sites (214,215). It is possible that the sigmoidal kinetics of this

pump could be attributed to other factors, e.g. the Ca^{2+}-dependent binding of the activator calmodulin (216). Even if two Ca^{2+} binding sites existed, whether the translocation step involves one to two Ca^{2+} atoms is still an open issue. Kinetic studies under optimal conditions (217) or in purified preparations (218) favor a 1:1 stoichiometry. Others have found that Ca^{2+}: ATP stoichiometry depends on the Ca^{2+} concentration, approaching two above 500 μM (219,220).

2.3.3 Ca^{2+} transport in the diabetic kidney

The action of insulin, like other hormone systems, involves the initial binding of the hormone to a specific receptor on the cell membrane, which would initiate a series of biochemical events. The potential role of Ca^{2+} in mediating insulin action is under current study (221). A potential role for Ca^{2+} in insulin-mediated cellular responses is suggested by observations of direct modulation by insulin of high affinity $Ca^{2+}+Mg^{2+}$ATPase activity in adipocyte plasma membranes (222). If there is some regulatory role for this enzyme in mediating insulin action, it is reasonable to assume that in situations of insulin resistance, insulin's effect on $Ca^{2+}+Mg^{2+}$ATPase may be different from that found under normal conditions. Levy et al. (221) examined the direct effect of insulin on the high affinity $Ca^{2+}+Mg^{2+}$ATPase of kidney basolateral membranes in streptozotocin-induced diabetic rats. The enzyme activity in basolateral membranes from diabetic rats was higher at all Ca^{2+} concentrations tested due to a higher maximum velocity of the enzyme from diabetic rats. The enzyme was inhibited by trifluperazine and no difference was noted in the calmodulin content in membranes from diabetic and control rats. Furthermore, insulin significantly increased the activity of the

$Ca^{2+} + Mg^{2+}$ATPase in the kidney basolateral membranes though there was a lack of such response from the membranes of diabetic rats. These findings not only demonstrate a defect in the ability of insulin to regulate the $Ca^{2+} + Mg^{2+}$ATPase activity in diabetic rats but also inculpate an inherent defect in enzyme activity as responsible for the impaired insulin action in diabetic rats. It may also be pointed out that in the presence of an increased Ca^{2+} pump in diabetes, rats are resistant to the development of congestive heart failure unless superimposed with hypertension (223). However, a causal relationship has not yet been established. Thus it is clear from the foregoing discussion that an impaired intracellular Ca^{2+} homeostasis represents a crucial factor in the pathogenesis of altered insulin effects in target tissues.

2.4 DIABETIC NEPHROPATHY

Nephropathy is a serious complication in diabetes mellitus and is an important cause of morbidity and mortality in diabetic patients (224). Renal failure develops in approximately 40 percent of these patients, usually 15 to 20 years after the diagnosis is made. Kidney disease associated with diabetes is the single most cause of end-stage renal disease (ESRD) in the United States and Europe (224). Diabetic kidney disease represents the only cause of uremia requiring renal replacement therapy, either in the form of transplantation or dialysis, that is increasing in frequency (225).

Diabetic nephropathy is progressive, characterized by subtle abnormalities including an increase in GFR ("hyperfiltration") and renal hypertrophy (226). It

must be noted that the initial increase in GFR is temporary while it decreases at later stage. Blood pressure is normal or slightly elevated and urinary albumin excretion rates are less than 20 to 30 mg/24 hours (227). With the advent of sensitive radioimmunoassays (RIA) for albumin, urinary albumin excretion can be readily quantitated in a range not previously detectable by routine clinical methodology (30 to 250 mg/24 hours, referred to as "microalbuminuria" (228,229)). Microalbuminuria is described as the hallmark of incipient diabetic nephropathy and an indicator of the development of clinically significant diabetic nephropathy (82). There is a small but significant increase in blood pressure, although these patients are not necessarily hypertensive, and typically have a preservation of renal function with a normal or elevated GFR (230). With further progression of the disease, overt nephropathy characterised by the triad of clinically detectable proteinuria ("dipstick" positive, total urinary protein excretion more than 250 mg/24 hours), hypertension and a decreased GFR. Hypertension at this stage is ubiquitous (231).

Pathologically, four distinctive lesions can be recognized by light microscopy in glomeruli of diabetic patients in addition to alterations in the thickness and configuration of the basement membranes (BM) (232-234). These are the nodular and diffuse forms of intercapillary glomerulosclerosis, the capsular drop lesion, and the fibrin cap lesion. The nodular lesion or the Kimmelsteil-Wilson lesion is considered to be pathognomonic of diabetes mellitus, while the other three lesions seem generally to lack specificity, and probably represent a more advanced stage of the diffuse glomerulosclerosis. The thickening of the glomerular BM is a slow

and gradual process. Studies have suggested (235,236) that two kinds of glomerular BM abnormalities are found in diabetic patients: one occurs very early in diabetes and is probably related to the acute glomerular hypertrophy and the other type of thickening increases with the duration of diabetes (237).

Paralleling these observations, functional changes in the diabetic kidney have been documented by various studies (238,239). Micropuncture studies in diabetic rats have shown increased GFR, renal plasma flow (RPF), single-nephron GFR, and intraglomerular capillary pressure (238,239). The increased GFR that occurs is probably caused by greater reduction in afferent than efferent arteriolar resistances and increased glomerular transcapillary hydraulic pressure gradient. Hyperglycemia, growth hormone and glucagon (240), a substance termed glomerulopressin (a glucuronic acid conjugate synthesized in the liver (241)), increased kidney size accompanied by glomerular enlargement in newly diagnosed type I diabetics and increased filtration surface area are several mechanisms that have been implicated to explain the elevated GFR in diabetes (223,231). Renal function in normal humans is also influenced by catecholamines, renin, angiotensin II and prostaglandins (243). In this regard, atrial natriuretic peptide, a recently discovered hormone released from the heart, has also been identified as a potential regulator of renal function (88-90).

The increased GFR serves as a potential mediator of hyperfiltration and is an attempt by the body to maintain its fluid homeostasis. These increments in GFR average approximately 40% percent compared to age-matched non-diabetic controls (244-248). Experimental studies in rats made diabetic with streptozotocin

indicate that diabetes induces a state of intrarenal vasodilatation, with elevation of the single nephron glomerular capillary plasma flow rate. The mean glomerular capillary hydraulic pressure tends to rise as the reduction in afferent arteriolar resistance is proportionately greater than that in efferent arteriolar resistance (238,249,250). Glomerular hyperperfusion and hypertension thus contribute to the observed single nephron hyperfiltration in diabetes. However, despite extensive investigations, the precise mechanisms underlying hyperfiltration remain elusive. One of the mechanisms to explain hyperfiltration implicates a cardiac hormone, atrial natriuretic peptide (ANP) with potent natriuretic and diuretic properties (251).

2.5 CONGESTIVE HEART FAILURE DUE TO CHRONIC DIABETES

A considerable body of evidence suggests an increased incidence of hypertension in patients who are diabetic (252,253). Krolewski and co-workers have reported that 26 percent of male and 34 percent of female diabetics followed at the Joslin Clinic were hypertensive compared with 13.7 percent and 19.5 percent of men and women respectively, in the general population (254). Different surveys have reported an even higher prevalence (255-257), and a consensus of several reports suggests that the frequency of hypertension among diabetics is 30 to 55 percent, approximately twice that of the general population (253,256-258). Furthermore, studies have demonstrated that the association between diabetes and hypertension is constant in both sexes and at all ages (259). Clinical data and experimental evidence strongly indicate that the co-existence of both hypertension

and diabetes results in a more severe degree of cardiomyopathy than by either process alone (260), and that cardiomyopathy is independent of coronary atherosclerosis (261,262). Several clinical and experimental studies reflect varying degrees of left ventricular dysfunction, independent of extramural coronary atherosclerosis in diabetes mellitus (263-267).

2.5.1 Diabetic cardiomyopathy

It might be considered that diabetes in itself causes development of a specific cardiomyopathy. In fact, diabetes mellitus was first linked to cardiomyopathy over a hundred years ago (268). Diabetic cardiomyopathy was further characterized in 1972 by Rubler et al. (269) based on necropsy findings in four diabetic patients with Kimmelsteil-Wilson disease and myocardial enlargement, hypertrophy, fibrosis and failure. The contribution of diabetes to the development of cardiomyopathy was further characterized by Hamby et al. (270), who noted an increased incidence of diabetes (22%) in patients with idiopathic cardiomyopathy compared to 11% in an age-and sex-matched cohort without cardiomyopathy. Kannel et al. (271) reported data from the Framingham study showing that diabetic men had a 2.4-fold greater risk for developing heart failure than non-diabetic men over an 18-year period. The relative risk for women was 5.1 times higher. Once again these patients did not have clinically significant coronary atherosclerosis. Zoneraich et al. (272), viewing this association from a different perspective reported an increased incidence of diabetes mellitus in patients with idiopathic cardiomyopathy. Extensive epidemiological work has thus provided evidence that the diabetic population does suffer from an increased incidence of cardiac dysfunction (273-

276).

Impairment of performance during diabetes due to a lesion in the muscle itself, but not due to coronary vascular pathology, was prompted by two essential lines of evidence. Firstly, a great number of studies have documented abnormalities in the contractile function of the heart in the absence of major vessel disease in diabetic patients (269,277-284) and diabetic animals (285-297); secondly, ultrastructural derangement of the cardiac tissue in diabetes. In fact, as early as 1966, Karlefors (298) noted that diabetic individuals had lower cardiac outputs during supine exercise than did healthy volunteers. In a subsequent study (299), newly diagnosed juvenile onset diabetics were noted to have lower stroke volumes during exercise than did controls. Stroke volume and cardiac index are depressed in diabetes (279,299), as is also the ejection fraction (261,284). Several studies have reported an increased isovolumic time in diabetic patients (277,280,283) which would correspond to an observation of delayed opening of the mitral value in diabetes (281). The pre-ejection time and the pre-ejection time/left ventricular ejection time ratio was dramatically increased (277,278,280,283,284) particularly because left ventricular ejection time was also depressed (277,284). Increments in the left ventricular end-diastolic pressure (LVEDP) and its ratio to chamber volume have also been detected in diabetic patients (279,284). These parameters would point to the observation that there is a decreased compliance of the left ventricle which would significantly hamper the function of the heart as a pump (279). Shapiro et al. (283) also found a slower relaxation process in hearts from diabetic patients by digitized M-mode echocardiography.

A noninvasive evaluation of cardiac performance utilizing systolic time intervals, phonocardiography, M-mode and two-dimensional echocardiography and Doppler echocardiography has also documented subclinical left ventricular dysfunction in diabetic individuals. Left ventricular contractility as assessed by a prolonged pre-ejection period (PEP) and shortened left ventricular ejection time (LVET), which correlate with reduced resting ejection fraction, were uniformly noted in three studies (300-302). Similar impairment of left ventricular function has also been shown by M-mode echocardiography (303,304).

Response of the left ventricle to exercise is a standard method to detect latent cardiac dysfunction and has been used extensively in diabetics. Diminished stroke volume with exercise in diabetics was first documented via invasive monitoring and indicator dye dilution techniques (297,298). More recent studies (305-308) have utilized gated radionuclide ventriculography to assess left ventricular function at rest and with exercise. Ejection fraction failed to increase appropriately but actually decreased with exercise (308). Abnormalities of left ventricular diastolic function, as judged by abnormally low peak filling rates or prolonged time-to-peak filling, have also been detected by radionuclide ventriculography (309). Left ventricular diastolic function has also been assessed by Doppler echocardiography (310-312) and abnormalities of early and late components of transmitral flow velocities have been shown to be reliable means of identifying diastolic filling abnormalities (313,314).

Paralleling these clinical findings are numerous studies that explore the association of diabetes with histopathologic abnormalities, possibly accounting for

a myopathic state. A higher incidence of intramural PAS-positive material and hyaline thickening with and without endothelial cell proliferation in both intramural and extramural coronary arteries has been noted (315,316). This finding along with subendothelial cell proliferation suggests that small vessel disease may be involved in the pathogenesis of myocardial dysfunction (317). On the contrary, studies (318) have shown that interstitial infiltration with PAS-positive material and fibrosis may be more significant with a myopathic state than small vessel disease. Myocytolysis in diabetic cardiomyopathy is extensive leading to a severe interstitial fibrosis. These changes are more compatible with systolic and diastolic abnormalities than with small vessel disease.

Therefore, it is obvious from the foregoing paragraphs that abundant evidence has accumulated for the existence of a specific diabetic cardiomyopathy. Contractile abnormalities, ultrastructural evidence and derangements in cardiac metabolism all point to the development of a "diabetic" cardiomyopathy irrespective of coronary artery atherosclerosis.

2.5.2 Role of hypertension

The incidence of high blood pressure in the diabetic population with a range of 10-80% appears to be considerably higher than that observed in the general population (252). This wide-ranging variation in incidence exemplifies the difficulty in trying to attach a single numeric figure for frequency estimates when two common and heterogenous diseases coexist. Onset of renal disease seen most often in insulin-dependent diabetes mellitus is almost invariably accompanied by hypertension (220). It can be appreciated that coexisting diabetes mellitus and

hypertension act as additive factors to further accelerate vascular damage (260). The Framingham Study showed that the impact of diabetes was greatest for certain complications such as intermittent claudication and congestive heart failure (271). Not only at the macrovascular level but also at the microvascular level, elevated blood pressure may accelerate the course and severity of diabetic microangiopathy. Hypertension occurs early in the course of the disease and has been shown to accelerate the decline of renal function in patients with diabetic nephropathy (227). Treatment studies of these patients showed that an effective antihypertensive regimen was able to retard the rate of progression of the renal failure as monitored by GFR and urinary albumin excretion rate (250).

3. AIMS AND OBJECTIVES

Under normal conditions most of the Ca^{2+} in the glomerular filtrate is reabsorbed by the nephron in order to maintain Ca^{2+} homeostasis (185). The low intracellular free Ca^{2+} concentration and an electronegative cell interior suggest the passive diffusion of Ca^{2+} across the brush border membrane into the cell from the tubular lumen (186). However, the electrochemical gradient of Ca^{2+} at the basolateral side is reversed indicating the presence of an energy-dependent process for Ca^{2+} transport. Kinne-Saffran and Kinne (195) have demonstrated an ATP-dependent Ca^{2+} transporting enzyme in cell membranes prepared from rat kidney cortex. Further, it has been demonstrated that in rat cortex basolateral membranes (BLM) and rabbit renal tubules, a magnesium-dependent, high affinity, low capacity Ca^{2+}ATPase (191,200,201) serves to pump Ca^{2+} into the extracellular fluid. This energy-dependent Ca^{2+} transport mechanism may provide an optimal cytoplasmic Ca^{2+} concentration lower than that in the extracellular fluid.

Atrial natriuretic peptide (ANP) is a hormone secreted primarily by atrial myocytes in response to local wall stretch (i.e. increased intravascular volume) (53). This hormone is known to decrease cytosolic free Ca^{2+} (96), and it is possible that ANP may be acting via a mechanism which stimulates Ca^{2+} efflux in order to maintain intracellular Ca^{2+} concentration. Since ANP has been shown to mediate its effects via accumulation of cGMP in cells by stimulating plasma-membrane associated guanylate cyclase (135,152), the present study was initially designed to investigate if the Ca^{2+} pump in kidney BLM is coupled to the ANP-receptor system.

Although it has been well established that the underlying abnormality in congestive heart failure (CHF) is a failure of the myocardium itself, which in turn leads to inadequate blood flow to peripheral tissues, a growing number of reports (319,320) suggest that renal parenchyma contributes significantly to the development of CHF. This is mainly due to the fact that body fluid homeostasis and the maintenance of a constant extracellular fluid volume is dependent on an integration of cardiac and renal function. Over the past few years, we have learned that several neurohumoral mechanisms (including ANP) are activated in order to preserve circulatory homeostasis when the peripheral perfusion is decreased in CHF.

While the incidence of severe cardiomyopathy and CHF has been found to be significantly higher in diabetic patients with long standing hypertension (271), the molecular mechanisms responsible for the altered pathophysiology of cardiorenal axis in this disease are poorly understood. Therefore, it was also the purpose of the present study to examine the status of renal Ca^{2+} pump and its interaction with ANP-receptor system in CHF due to diabetes superimposed on hypertension.

As a consequence of ANP's participation in the Ca^{2+} homeostasis and its involvement in the direct stimulation of the Ca^{2+} pump and the guanylate cyclase system in the kidney BLM, experiments were initially designed to determine the precise nature of the relationship between Ca^{2+} + Mg^{2+} ATPase and the guanylate cyclase system in the renal cortical basolateral membrane. Specifically the following experiments were designed:

1. To examine the influence of guanylate cyclase on Ca^{2+} + Mg^{2+} ATPase using GTP as the substrate for guanylate cyclase.
2. To investigate the specificity of this effect, if any, by comparison with other tissues, such as the liver plasma membrane.
3. To further delineate the distinctive effect of GTP on Ca^{2+} + Mg^{2+} ATPase, the influence of GTP on other ATPases e.g. Na^{+} + K^{+} ATPase was also analyzed.
4. To explore the possibility of a receptor-mediated effect by noting the effect of cGMP on the Ca^{2+} + Mg^{2+} ATPase activity.
5. To characterize the correlation between the two enzyme systems by using various stimulators (sodium azide) and inhibitors (guanylate cyclase antibody) of guanylate cyclase.

The studies were extended to determine the pathophysiological implications of this coupling in hypertensive-diabetic animals. Diabetes was induced by i.v. injections of streptozotocin. Hypertension was produced by placing a constricting ligature suprarenally, on the abdominal aorta. A time frame of 1 and 6 weeks was used following the experimental treatments to examine the characteristics of these animals. Specifically the following experiments were designed:

1. To typify the pathophysiological state of congestive heart failure, hemodynamic assessment of the animals was done.

2. To measure the plasma glucose as a denominator of the hyperglycemia in diabetes. Plasma ANP was measured as changes in ANP levels might play a regulatory role in receptor-mediated responses.
3. To measure Ca^{2+} + Mg^{2+} ATPase in the different groups; the sensitivity of the Ca^{2+} + Mg^{2+} ATPase to ANP was also tested. These changes were correlated with changes in guanylate cyclase activity and ANP receptor binding characteristics to show their contribution in the genesis of diabetes-induced congestive heart failure.
4. To determine the possibility of nonspecific changes in the Ca^{2+} + Mg^{2+} ATPase in the D+ H group at 6 weeks, Na^{+} + K^{+} ATPase was measured to denote the specificity of the changes in the Ca^{2+} + Mg^{2+} ATPase.
5. To document alterations in the ANP receptor-effector system by measuring guanylate cyclase activity and its responsiveness to ANP in control and experimental groups.
6. To record the modifications in receptor binding studies which occur in the experimental situation as part of the pathophysiologic process. These changes would assist in explaining alterations in receptor-mediated phenomena.

4. MATERIALS AND METHODS

4.1. Experimental animals

Male Sprague-Dawley rats weighing 200 ± 10 g were used. The total number of animals in the study were 239. They were grouped as follows: control (C) (n=66), diabetic (D) (n=41), hypertensive (H) (n=41), and combined hypertensive with diabetes (D+H) (n=91). Diabetes was induced by streptozotocin injection (65 mg/kg body wt. i.v.) whereas the control animals received the buffered vehicle (0.1 M citrate, pH 4.5) (223). The animals in the hypertension group underwent a midline laparotomy with placement of a constricting ligature around the abdominal aorta above the renal arteries using a blunt 21-gauge needle as a guide. Sham control animals also underwent the same operation except that the aorta was not banded. When hypertension was superimposed with diabetes, streptozotocin was injected 1 week before the aortic constriction (223). Because values for the different parameters between control and sham control animals were overlapping, the data in these two groups were combined. It may be pointed out that we did not constrict the renal artery to produce hypertension since a dramatic and immediate increase in plasma ANP was achieved by aortic constriction. The animals were sacrificed 1 and 6 wks later. A time frame of 1 and 6 weeks was chosen after pilot studies in our laboratory showed these time periods as representative of early and late stages of congestive heart failure.

4.2. Chemicals

All the chemicals used were of reagent grade and obtained from Sigma Co.,

St. Louis.

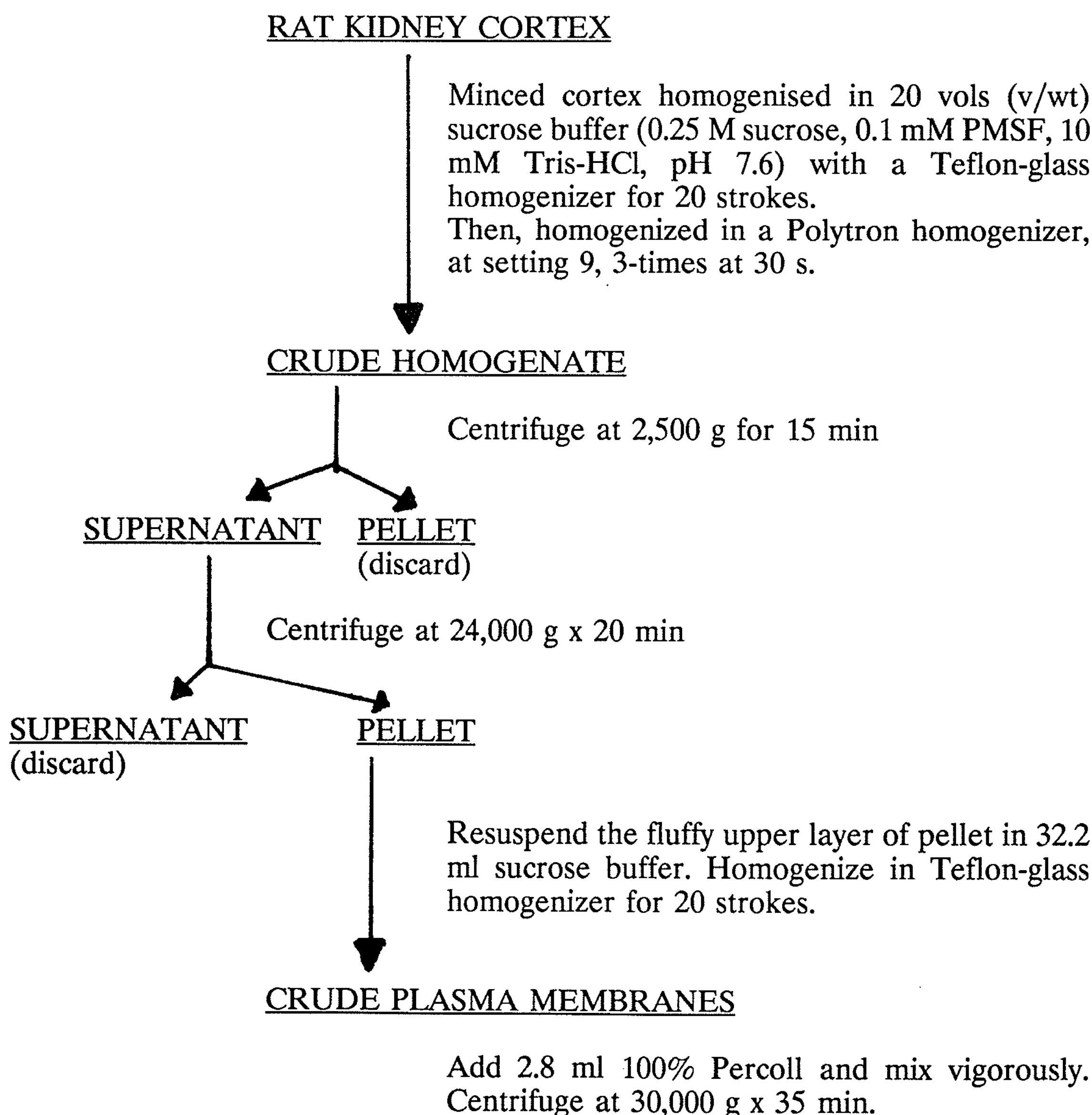
4.3. Hemodynamic assessment

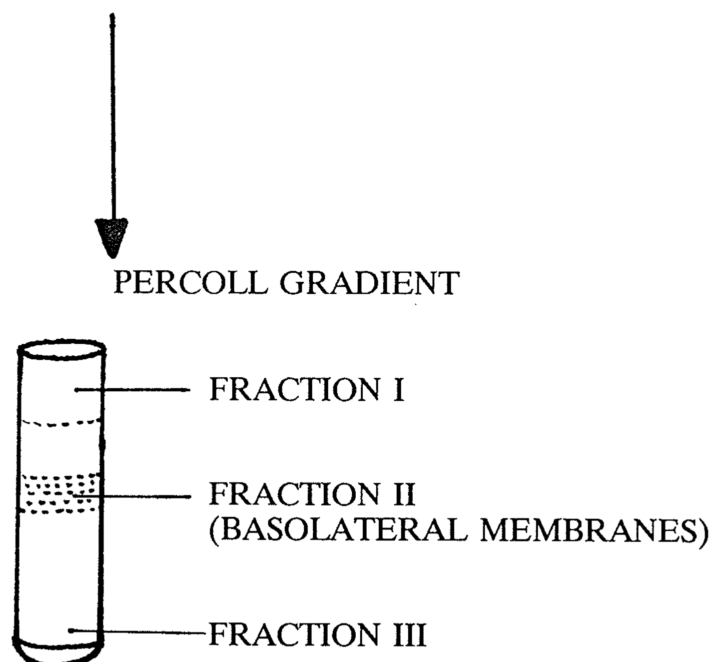
For hemodynamic assessment, animals were anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg) (223). After intubation of the trachea to maintain adequate ventilation, the right carotid artery was exposed and a microtip pressure transducer (model SPR-24, Millar Instruments, Inc., Houston, Texas) was introduced though a proximal arteriotomy. The catheter was carefully advanced through the lumen of the carotid artery until the tip of the transducer entered the left ventricle. The catheter was connected to a dynograph recorder (model RSHA, Beckman Instrument Inc., Fullerton, Calif.). After hemodynamic studies, blood samples were collected from the abdominal aorta for measurement of plasma ANP and glucose.

4.4. Membrane preparation

Basolateral membrane was isolated from kidney cortex according to Sacktor et al. (321). The kidney cortex was homogenized in 20 volumes of buffer (0.25 M sucrose, 0.1 mM PMSF (phenylmethyl sulfonyl fluoride), and 10 mM Tris-HCl, pH 7.6) at a polytron setting of 9, three times for 30 seconds each and then centrifuged (Beckman centrifuge, Model J2-21, California) at 2500 g (Beckman JA 20 rotor) for 15 min at 0-5°C. The supernatant was recentrifuged at 24,000 g for another 20 min. The pellet was resuspended in sucrose buffer, homogenized and centrifuged at 30,000 g through a density gradient of Percoll for 35 min. The fraction II obtained was diluted with 4 volumes of another buffer containing 100 mM

mannitol, 100 mM KCl (potassium chloride) and 5 mM Hepes-Tris, pH 7.2, and centrifuged at 34,000 g for 30 min. The loose fluffy pellet was isolated, resuspended in 4 volumes of Hepes-Tris buffer and centrifuged at 34,000 g for 30 min again. The final pellet was diluted in a small volume (1 ml) of Hepes-Tris buffer. The membranes were stored at -80 °C until further use (Figure I).





Fraction were collected from the top by pumping a 50 % sucrose solution into the bottom of the centrifuge tube with a peristaltic pump. Fraction were diluted with 4 vol of medium containing 100 mM KCl, 100 mM Mannitol, 5 mM Hepes-Tris, pH 7.2.
Centrifuge at 34,000 g x 30 min.

After centrifugation, the Percoll had formed a very dense glassy pellet. The membranes formed a loose fluffy pellet above the Percoll. The membranes were removed, resuspended in the same medium and, then, recentrifuged at 34,000 g x 30 min.

BASOLATERAL MEMBRANES

Membranes were suspended in a small medium.

Figure I. Protocol for preparing basolateral membranes from rat renal cortex

4.5. Plasma Glucose and ANP

a) Plasma glucose

Plasma glucose was estimated by a reagent kit (Sigma, St. Louis). Briefly, the glucose in the serum was determined by a coupled enzymatic method using the enzymes hexokinase and glucose-6-phosphate dehydrogenase (322). In principle, glucose is phosphorylated to glucose-6-phosphate by hexokinase using ATP. The glucose-6-phosphate is then reduced to 6-phosphogluconate by glucose-6-phosphate dehydrogenase in the presence of nicotinamide dinucleotide (NAD). The consequent increase in absorbance due to an equimolar reduction of NAD to NADH at 340 nm is directly proportional to glucose concentration. The concentration can thus be determined based on millimolar absorptivity of NADH (6.22 at 340 nm).

b) Plasma ANP

Measurement of plasma ANP was based on three steps:-

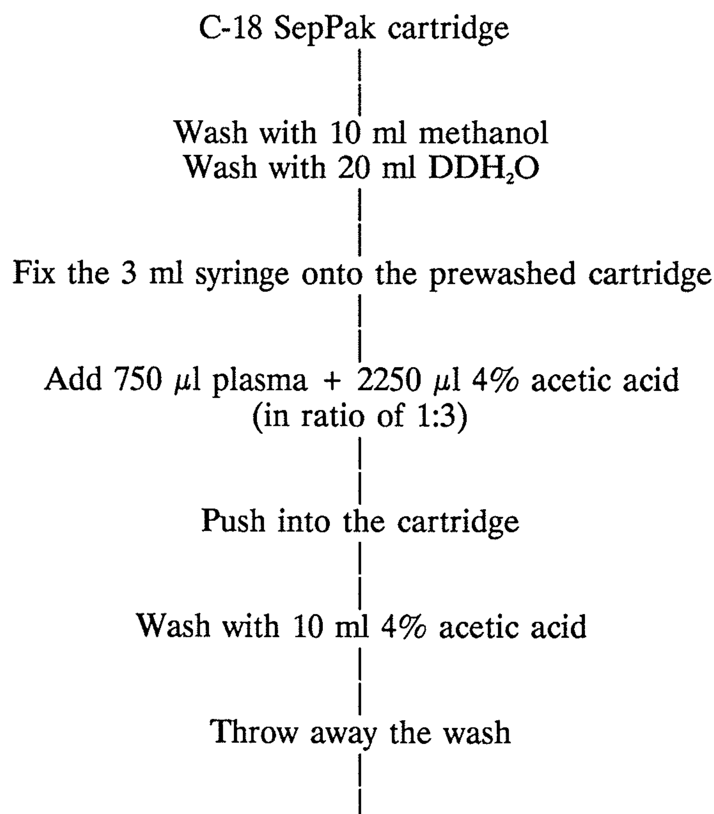
i) Affinity extraction of the samples.

ii) Plotting a standard curve using known concentrations of ANP

iii) The values of the sample are then read from the standard curve.

Plasma ANP was extracted by the method of Lang et al. (323). In brief, plasma was diluted with 4% acetic acid (1:3 vol/vol) and was passed over 1 C18 Sep Pak cartridge (Water Associates, Millipore Corp., Ontario) prewashed with 10 ml methanol, washed with 20 ml distilled water, eluted with acetonitrile and acetic acid mixture (3:1), and evaporated to dryness under nitrogen and mild heating at

50°C. ANP was then measured using a commercially available radioimmunoassay kit (Amersham, Ontario).The samples are reconstituted with assay buffer (50 mM sodium phosphate, 0.2% (w/v) gelatin, 10 mM EDTA, [(ethylenedinitrilo)-tetraacetic acid] and 0.1% (v/v) Triton X-100, pH 7.4). Antiserum and the radioactive tracer are added and allowed to incubate at 4 °C for 24 hours. After this equlibration process, charcoal and dextran (400 μl of a mix containing 0.4 gm charcoal and 0.04 gm dextran in 50 ml of the assay buffer) are mixed and centrifuged. The supernatant (free) and the pellet (bound) are counted separately in a gamma counter for 200 seconds. The standard curve is plotted using ANP in concentrations of 1 and 0.1 nM. The percentage binding of the samples is calculated and the ANP concentration of the samples read off the standard plot (Figure II)



Elute with 3 ml 3:1 acetonitrile + 4% acetic acid

|

Push into the plastic tube which is saved

|

Evaporate at 60°C under a fumehood until dry

|

Reconstitute the samples with 300 μl of assay buffer

|

Incubate with antiserum (50μl) and radioactive tracer (50μl)

|

Cap the tubes and leave at 4°C for 24 hours

|

Proceed for separation of the free and bound ANP as follows:

To 50 ml assay buffer

|

Add 0.4 gm activated charcoal + 0.04 gm dextran

|

Stir for 20 min at 4°C

|

Add 250 μl of above solution to each tube while stirring in the cold

|

Centrifuge as soon as possible at 2000 RPM for 15 min

|

Take the supernatant off into another set of plastic tubes

|

Count both free and charcoal-bound tubes in a γ-counter for 200 sec (3.33 min)

|

Calculate

Figure II. Protocol for radioimmunoassay of ANP

4.6. Ca^{2+} + Mg^{2+} ATPase estimation

The $Ca^{2+}+Mg^{2+}$ATPase was measured by a modification of the method of Pershadsingh and McDonald (324). The assay medium contained 5 μg of membrane protein, 50 mM Tris-HCl (pH 7.2), 1 mM ATP, about 0.2 μCi [γ-^{32}]ATP (New England Nuclear Corp.), 2 mM $MgCl_2$ and 10^{-10} M free Ca^{2+}. The incubation was done at 37°C for 10 minutes and the reaction stopped by 6% SDS. The released ^{32}P was converted to a phosphomolybdate complex using phosphate reagent (2 volumes of 10 N sulfuric acid, 2 volumes of 10% ammonium molybdate and 1 volume of 0.1 M silicotungstic acid). The phosphomolybdate complex was extracted into xylene: isobutanol (65:35) (325) and further quantified by liquid scintillometry. ATPase activity was expressed as nanomoles of P_i per mg protein per min and was calculated by subtracting basal Mg^{2+}ATPase from that achieved in the presence of 1 μM free Ca^{2+}.The ATPase activity was also measured as and when necessary in the presence and absence of various substrates, stimulators and inhibitors. (Figure III).

Prepare tubes

|

Add protein at 15 sec. intervals, preincubate for 3 min.

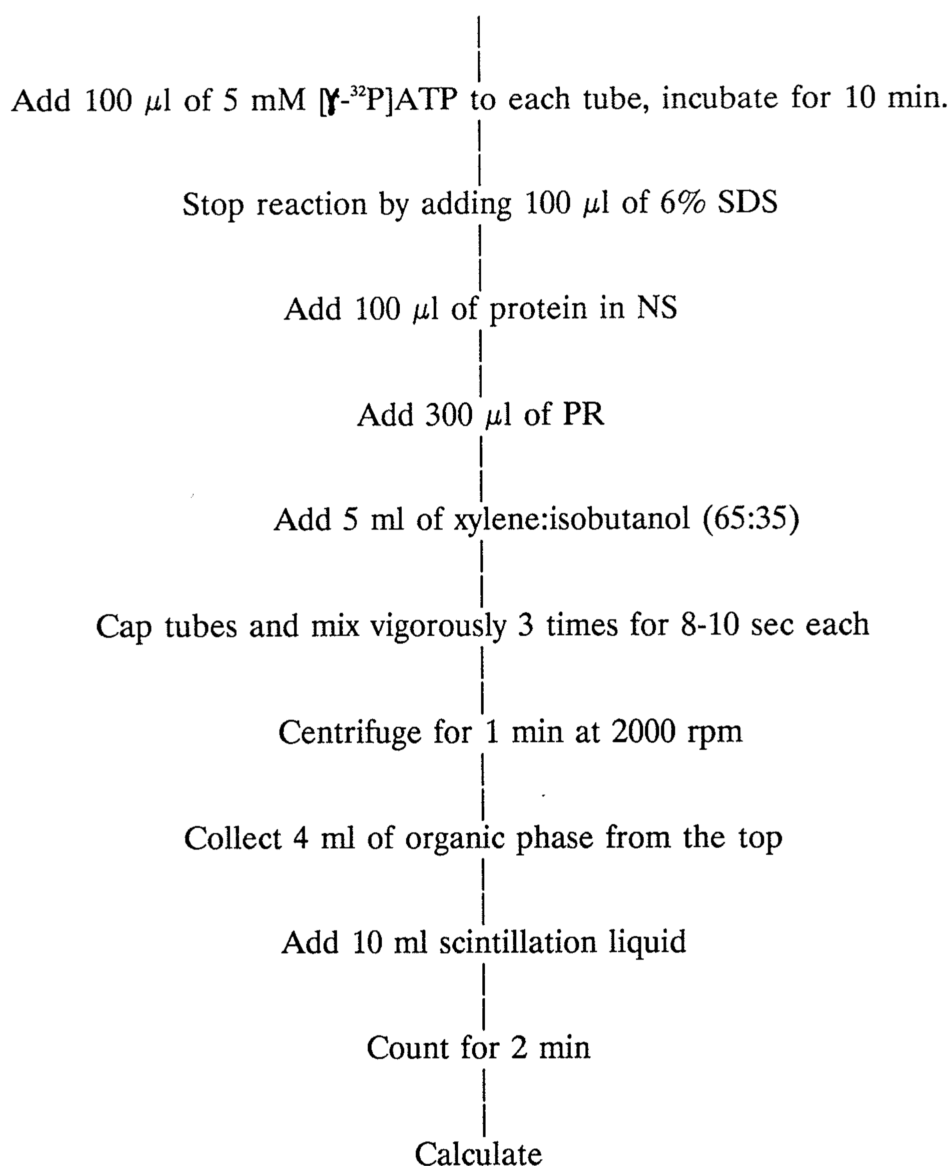


Figure III. Isotopic assay for Ca^{2+} + Mg^{2+} ATPase

4.7. ANP receptor binding

ANP-receptor binding assay was performed in kidney basolateral membranes (326). Fifty microgram of membrane protein was incubated with [^{125}I] rANP in triplicate for 60 min at room temperature in 50 mM Tris-HCl, pH 7.5, containing

1 μM aprotinin, 0.1% bacitracin, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM $MgCl_2$ and 0.4% bovine serum albumin in the presence and absence of various concentrations of unlabelled rANP (10^{-6} to 10^{-9}). The reaction was stopped by adding three ml of ice-cold Tris-HCl, pH 7.5 and the incubation mixture was filtered rapidly on 0.3% polyethylenimine treated Whatman GF/C filter. After three washes, the [^{125}I] radioactivity on the filter was determined in a gamma counter. Saturation binding assays were analyzed by the Scatchard analysis using the LIGAND program (327).

4.8. Guanylate cyclase activity

Guanylate cyclase activity measurement was based on the quantification of cGMP formed (328). The assay medium contained 80 μg of membrane protein, 50 mM Tris-HCl, pH 7.6, 10 mM theophylline, 4 mM $MgCl_2$ and 1 mM GTP. The tubes were incubated at 37 $^{\circ}$C for 10 min. The reaction was stopped with 50 mM sodium acetate, pH 4.0 and the tubes quickly immersed in a boiling water-bath for 3 min. The mixture is then centrifuged and the supernatant pipetted off for radioimmunometric determination of cGMP. The radioimmunoassay is based on the competition between unlabelled cGMP and a fixed quantity of the [^{3}H]-cGMP (4 pmol/tube, 0.08 μCi) for binding to an antiserum with a high affinity and specificity for cGMP. Standards are prepared from known concentrations of unlabelled cGMP (0.5-4 pmol/assay tube). Measurement of the antibody-bound radioactivity enables the amount of the unlabelled cGMP in the sample to be calculated. Separation of the antibody-bound cGMP from the unbound nucleotide

is achieved by precipitation with 2.95 M ammonium sulfate followed by centrifugation (Beckman centrifuge, Model J2-21, California) at 4000 rpm (Beckman JA 20.1 rotor) for 20 min. The precipitate is dissolved in 1.1 ml distilled water and the radioactivity counted using liquid scintillometry. The ratio bound (the cpm bound in the absence of unlabelled cGMP/ the cpm bound in the presence of standard or unknown) is calculated for all standards and unknowns. The ratio is plotted against pmol of inactive cGMP/tube on linear graph paper. The values of the unknown samples is the read from the standard curve.

4.9. Guanylate cyclase antibody

The guanylate cyclase antibody was a generous gift from Dr. R.K. Sharma, Cleveland Clinic Foundation, Ohio (329). Briefly, the guanylate cyclase antibody was raised in rabbits by subcutaneous injections of the 180-kDa membrane guanylate cyclase isolated from rat adrenal glands. The guanylate cyclase was emulsified in Freund's complete adjuvant and distributed subcutaneously at five sites in the nuchal region, and 1.0 ml was injected intramuscularly into each hind leg muscle. The injections were repeated at 3-week intervals using Freund's incomplete adjuvant. Rabbits were bled from the ear veins at 10 days after each injection and the serum separated. Gamma-globulins were separated by ammonium sulphate precipitation and extensive dialysis. The antibody titre was determined by solid-phase RIA using ^{125}I-labelled goat anti-rabbit IgG.

The cross-reactivity of this antibody to the guanylate cyclase in kidney basolateral membrane was then determined. The membranes were solubilized in

10 mM 3-[3-cholaimidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) and passed through a GTP-affinity column. The fraction obtained was subjected to a slot blot analysis. Initially the membranes were run on a SDS polyacrylamide gel and then transferred to a nitrocellulose membrane. The excess of protein binding sites were blocked with 2% bovine serum albumin. The nitrocellulose membrane was treated with 4-chloro-l-napthol and hydrogen peroxide to reveal the antibody cross-reactivity protein band.

4.11. Protein determination

Protein determination was done by the method of Lowry et al. (330) using bovine serum albumin as standard.

4.12. Statistics

Values are given as mean ± SEM. Analysis of variance was carried out and Duncan's New Multiple Range test was used to determine differences among the means of different groups. A p value of less than 0.05 was considered significant.

5. RESULTS

Table I shows the stimulatory effect of ANP on Ca^{2+}+Mg^{2+}ATPase. The values are expressed as the percent stimulation obtained over those in the absence of ANP. The Ca^{2+}+Mg^{2+}ATPase activity in the absence of ANP was 17.0 ± 0.8 nmoles of Pi/mg protein/min. The addition of ANP (10^{-12} to 10^{-9} M) was able to stimulate (12 to 88%) Ca^{2+}+Mg^{2+}ATPase in a concentration-dependent manner.

Table II demonstrates the influence of GTP on the Ca^{2+}+Mg^{2+}ATPase activity and the values are shown as the percent inhibition obtained from those in the absence of GTP. The addition of GTP significantly inhibited Ca^{2+}+Mg^{2+}ATPase; the maximum inhibition was achieved at 4 mM GTP. This effect of GTP was specific as no changes in the Ca^{2+} + Mg^{2+}ATPase could be detected by using GTP-γ-S, a non-hydrolysable GTP analogue (Table III). Table III also shows that cGMP was unable to induce changes in the Ca^{2+}+Mg^{2+}ATPase. The effect of GTP on the Ca^{2+} + Mg^{2+} ATPase was also examined in liver plasma membranes to determine its uniqueness to kidney BLM. Table IV shows that although GTP was able to inhibit the Ca^{2+} + Mg^{2+} ATPase in liver plasma membranes, the inhibition was minimal.

The Ca^{2+}+Mg^{2+}ATPase was assayed in the presence of various stimulators and inhibitors of guanylate cyclase. Inclusion of sodium azide, a powerful stimulator of guanylate cyclase (331), in the incubation media caused a marked depression of the Ca^{2+}+Mg^{2+}ATPase activity (Table V). Studies with the guanylate cyclase antibody resulted in a significant elevation of Ca^{2+}+Mg^{2+}ATPase (Table VI). The specificity of this inhibitory effect of GTP was examined on another ATPase in the

Table I. Effect of ANP on $Ca^{2+}+Mg^{2+}$ATPase in kidney Basolateral membrane

Conc. of ANP (M)	% stimulation
0	--
10^{-12}	12.20 ± 3.22*
10^{-11}	29.27 ± 2.72*
10^{-10}	69.76 ± 3.01*
10^{-9}	88.78 ± 3.40*

The $Ca^{2+}+Mg^{2+}$ATPase was incubated in a medium containing 5 μg of membrane protein, 50 mM Tris-HCl (pH 7.2), 2 mM $MgCl_2$, 1 mM ATP, about 0.2 μCi [γ-^{32}P]ATP and 10^{-6} M free Ca^{2+} in the presence and absence of different concentrations of ANP. The $Ca^{2+}+Mg^{2+}$ATPase was calculated by subtracting the basal Mg^{2+}ATPase from the total $Ca^{2+}+Mg^{2+}$ATPase activity. The $Ca^{2+}+Mg^{2+}$ATPase value in the absence of ANP was 17.0 ± 0.8 nmol Pi/mg protein/min. The values are expressed as the percentage stimulation over 0 ANP. Each value represents a mean of 5 experiments.

* $p<0.05$, significantly different from 0 ANP.

Table II. Effect of various concentrations of GTP on $Ca^{2+}+Mg^{2+}$ATPase

Conc. (mM)	% inhibition
0	--
0.5	20.31 ± 3.60*
1	54.17 ± 3.94*
2	66.55 ± 4.22*
4	78.02 ± 4.73*

The $Ca^{2+}+Mg^{2+}$ATPase was assayed in the presence and absence of different concentrations of GTP. The incubation media also included 10 mM theophylline, 15 mM creatine phosphokinase (135 units/mg protein), 15 mM creatine phosphate. The $Ca^{2+}+Mg^{2+}$ATPase value in the absence of GTP was 18.1 ± 0.7 nmol Pi/mg protein/min and was calculated by subtracting the basal Mg^{2+}ATPase activity from that achieved in the presence of 1 μM free Ca^{2+}.

The values are expressed as the percent inhibition from 0 GTP. Each value represents a mean of 5 experiments.

* $p<0.05$, significantly different from 0 GTP

Table III. Effect of GTP-γ-s and cGMP on $Ca^{2+}+Mg^{2+}$ATPase

Experiment	% of $Ca^{2+}+Mg^{2+}$ATPase
No addition	100
cGMP	102.92 ± 3.20
GTP-γ-S	101.83 ± 3.41

The $Ca^{2+}+Mg^{2+}$ATPase was determined by incubating the membrane protein in the presence and absence of 8 nM cGMP and 1 mM of a GTP analogue, GTP-γ-S and expressed as a percentage of absolute $Ca^{2+}+Mg^{2+}$ATPase.

The $Ca^{2+}+Mg^{2+}$ATPase activity in the absence of cGMP was 17.8 ± 0.4 nmol Pi/mg protein/min while in the absence of GTP-γ-S was 18.8 ± 0.5 nmol Pi/mg protein/min. Each value represents a mean of 5 experiments.

cGMP, cyclic guanosine monophosphate; GTP-γ-S, guanosine 5'-O-(3 thiotriphosphate).

Table IV. Effect of GTP on Ca^{2+} + Mg^{2+} ATPase in liver plasma membranes.

Experiment	% of $Ca^{2+}+Mg^{2+}$ATPase
No addition	100
4mM GTP	86.76

The $Ca^{2+}+Mg^{2+}$ATPase was determined by incubating the membrane protein in the presence and absence of 4 mM GTP and expressed as a percentage of absolute $Ca^{2+}+Mg^{2+}$ATPase

Each value represents a mean of 5 experiments.

The $Ca^{2+}+Mg^{2+}$ATPase activity in the absence of GTP was 6.32 ± 0.03 nmol Pi/mg

Table V. Effect of sodium azide on Ca^{2+} + Mg^{2+} ATPase in kidney basolateral membrane.

Experiment	$Ca^{2+} + Mg^{2+}$ATPase (%)
No addition	100
10mM sodium azide	43 ± 5*

The $Ca^{2+} + Mg^{2+}$ATPase was determined by incubating the membrane protein in the presence and absence of 10mM sodium azide and expressed as a percentage of absolute $Ca^{2+} + Mg^{2+}$ATPase.

Each value represents a mean of 5 experiments.

The $Ca^{2+} + Mg^{2+}$ATPase activity in the absence of sodium azide was 17.8 ± 0.4 nmol Pi/mg.

* $p<0.05$, significantly different from absolute $Ca^{2+} + Mg^{2+}$ATPase activity.

Table VI. Effect of guanylate cyclase antibody on $Ca^{2+}+Mg^{2+}$ATPase activity in kidney basolateral membrane.

Experiment	$Ca^{2+}+Mg^{2+}$ATPase (%)
No addition	100
GTP	49.99 ± 2.62*
GTP+GcAb	71.88 ± 3.21*

The $Ca^{2+}+Mg^{2+}$ATPase activity was assayed in the presence and absence of both ANP antibody and guanylate cyclase antibody to determine the reversal of the inhibitory effect of GTP (1 mM). The $Ca^{2+}+Mg^{2+}$ATPase activity was 15.0 ± 0.5 nmoles Pi/mg protein/min.

The values shown above are expressed as percentage of the absolute $Ca^{2+}+Mg^{2+}$ATPase and are mean of 5 experiments.

* $p<0.05$, significantly different from absolute $Ca^{2+}+Mg^{2+}$ATPase activity. GTP, guanosine triphosphate; GcAb, guanylate cyclase antibody.

Table VII. Effect of GTP on Na^+ + K^+ ATPase in kidney.

Experiment	% of Na^+ + K^+ATPase
No addition	100
4 mM GTP	94.74

The Na^+ + K^+ ATPase was determined by incubating the membrane protein in the presence and absence of 4mM GTP and expressed as a percentage of absolute Na^+ + K^+ATPase.

Each value represents a mean of 5 experiments.

The Na^+ + K^+ATPase activity in the absence of GTP was 19.37 ± 0.4 nmol Pi/mg

kidney. Table VII shows that GTP did not inhibit the Na^{+} + K^{+} ATPase, suggesting that there is no preferential treatment of the substrate (ATP and GTP) as a cause for the decreased activity of Ca^{2+} + Mg^{2+} ATPase in presence of GTP. Morever, the ATPase activity was substantially restored by using antibody against guanylate cyclase. Therefore, it is possible that the two enzyme systems viz. the guanylate cyclase and the $Ca^{2+}+Mg^{2+}$ATPase are coupled together in the renal cortical basolateral membrane of the rat . A proposed model of the coupling between the Ca^{2+} + Mg^{2+} ATPase and guanylate cyclase is shown in Figure IV.

Results of the second part describe the pathophysiologic implications of coupling between the renal $Ca^{2+}+Mg^{2+}$ATPase and ANP-receptor system. Accordingly, the general characteristics of the experimental animals were evaluated (Table VIII). Presence of diabetes in streptozotocin-injected animals at 1 wk was confirmed by the increased levels of plasma glucose compared with the control group (data not shown). Heart rate and left ventricular systolic pressure (LVSP) were increased in H (hypertensive) and D+H (diabetic plus hypertensive) group whereas heart weight/body weight ratio, liver weight/body weight ratio, kidney weight, heart norepinephrine concentration and left ventricular end-diastolic pressure (LVEDP) remained unchanged at 1 wk. Experimental animals at 6 wk showed a significantly lower body weight, higher heart weight/body weight ratio, plasma glucose, heart norepinephrine and LVEDP in diabetic animals as compared to control animals. In the H group, heart weight/body weight ratio, heart rate and LVSP were increased otherwise the animals remained healthy as a result of physiological hypertrophy. In contrast, when hypertension was superimposed on

A fourth weakness in the study surrounds the use of the video tape. Initially it was felt that it would clarify perceptions when writing up the results of the study. In this respect it was a successful tool. However, it also left the doors open for individuals to perform. As one of the independent observers reported, "Subject A was very excited about being on T.V.". To be more effective, the video camera should have been left on all the time so that the subjects would become accustomed to its presence.

A fifth weakness in the study was a difficulty in clarifying perceptions about an individual when using the Rosenberg Self-Esteem Inventory. Should the study be repeated it would be strongly suggested that the self-esteem scale be administered to a wide variety of individuals using a pilot study format. This would allow the research to test reliability and validity rather than rely on mental measurement reports. It is felt that there is substantial merit in studying the self-esteem of individuals described as trainable mentally handicapped. However, because of their limited cognitive ability it is a difficult measure to quantify.

The sixth weakness centers around the fact that it may be increased attention alone that is enough to trigger an increase in self-esteem. Considering the type of individuals used in the study and the fact that it is

Table VIII. General characteristics of experimental animals at 6 wk study

	Control (n=10)	Diabetes (n=10)	Hypertension (n=10)	Diabetes + Hypertension (n=10)
Body wt, g	449±16	229±10*	465±11	280±10*
Heart wt/Body wt ratio, mg/g	2.8±0.11	3.49±0.18*	3.33±0.14*	4.10±0.20*
Liver wt/Body wt ratio, mg/g	35.4±4.1	42.4±3.8	32.6±3.0	50.3±3.3*
Kidney wt, g	1.6±0.08	1.7±0.23	1.6±0.21	1.9±0.10*
Plasma glucose,mg/dl	140±8	450±20*	135±12	438±11*
Heart norepinephrine, ng/g	185±16	360±15*	184±12	123±12*
Heart rate, beats/min	386±12	274±10*	424±11*	430±9*
LVSP,mm Hg	140±10	135±8	230±10*	234±13*
LVEDP,mm Hg	3.2±0.36	17.6±1.2*	4.6±0.53	20.2±1.8*
Mortality (%)	0	15	20	55

Values are means ± SE of 10 experiments. LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure. Only one kidney was used for measuring weight.

* Significantly different ($p<0.05$) from control.

diabetes, an increased mortality (almost 50%) was observed. The animals which survived in the D+H group had significantly higher heart weight/body weight ratio, liver weight/body weight ratio, kidney weight and LVEDP compared with control animals, while heart norepinephrine was decreased. These animals developed ascites and the congestion in liver and lungs was also apparent.

Plasma ANP levels in different experimental groups at 1 and 6 wk of the study are shown in Figure V. Diabetic animals had increased plasma ANP level 1 wk after streptozotocin injection whereas 6 wk later the level was decreased. In D+H group a biphasic response was evident; the level was increased at 1 wk but decreased more 6 wk later. Hypertension alone resulted in an acute increase of plasma ANP level.

Since the effect of ANP is mediated via guanylate cyclase, the guanylate cyclase was tested in diabetic plus hypertensive animals to determine any alterations in its activity. Basal guanylate cyclase activity expressed in pmol cGMP/mg protein/10 min was lower in D+H (Table IX). The response to ANP was also greatly attenuated in the D+H group (both at 1 and 6 wk of the study). Guanylate cyclase activity was unaltered in D or H group at each point studied.

There was a significant increase in the Ca^{2+} + Mg^{2+} ATPase activity in kidney basolateral membrane in D, H and D+H group at 1 wk of the study (Fig.VI). At 6 wk, Ca^{2+} + Mg^{2+} ATPase was increased in H group whereas, diabetes plus hypertension resulted in a decrease in the enzyme activity. As results of part 1 showed that this enzyme is stimulated by ANP, responsiveness of this enzyme activity to ANP was also examined and the data are shown in Table X.

Responsiveness of Ca^{2+} + Mg^{2+} ATPase activity in the D+H group to ANP was markedly depressed.

In order to check the possibility of some non-specific changes in Ca^{2+} + Mg^{2+} ATPase in D+H group at 6 wk, Na^{+} + K^{+} ATPase activity was also measured and the data are presented in Figure VII. Unlike the Ca^{2+} pump activity, both D and D+H group had increased Na^{+} + K^{+} ATPase activity at 1 and 6 wk of the study. It may be pointed out that ANP is known to have minimal effect on Na^{+} + K^{+} ATPase activity (332). Table XI shows the ANP receptor binding properties in kidney basolateral membrane in different experimental groups at 6 wk. B_{max} of ANP receptors was increased significantly in D+H group. The K_d values, however, did not change.

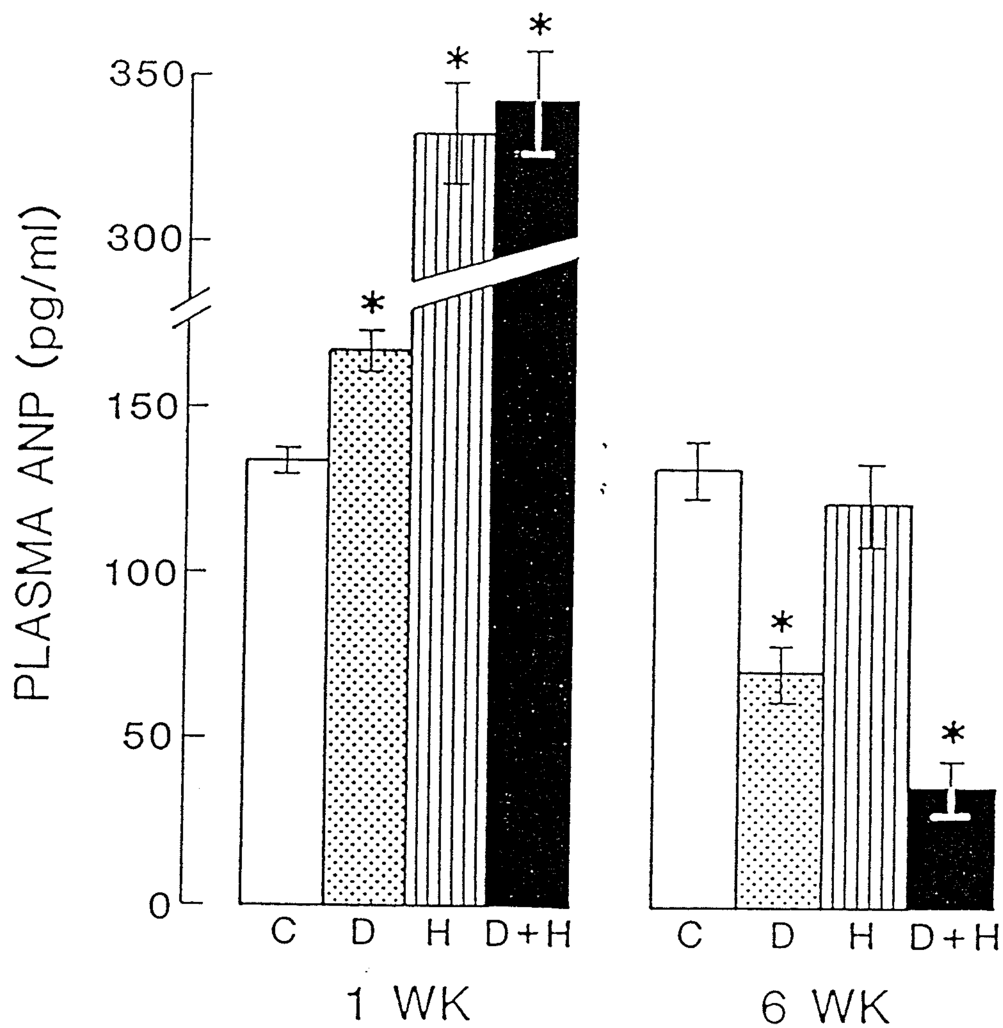


Figure V. Plasma ANP level in different experimental groups. C, Control; H, hypertension; D+H, diabetic and hypertension. *Significantly different ($p<0.05$) from control.

Table IX. Guanylate cyclase activity in kidney membrane at 6 wk study

	Guanylate cyclase activity (pmol cGMP/mg protein/10 min)
Control	
Basal	30 ± 3
With ANP	206 ± 5
Diabetes + Hypertension	
Basal	14 ± 1
With ANP	22 ± 1

Values are means ± SE of 3 experiments. 10^{-8}M ANP was used to activate guanylate cyclase activity.

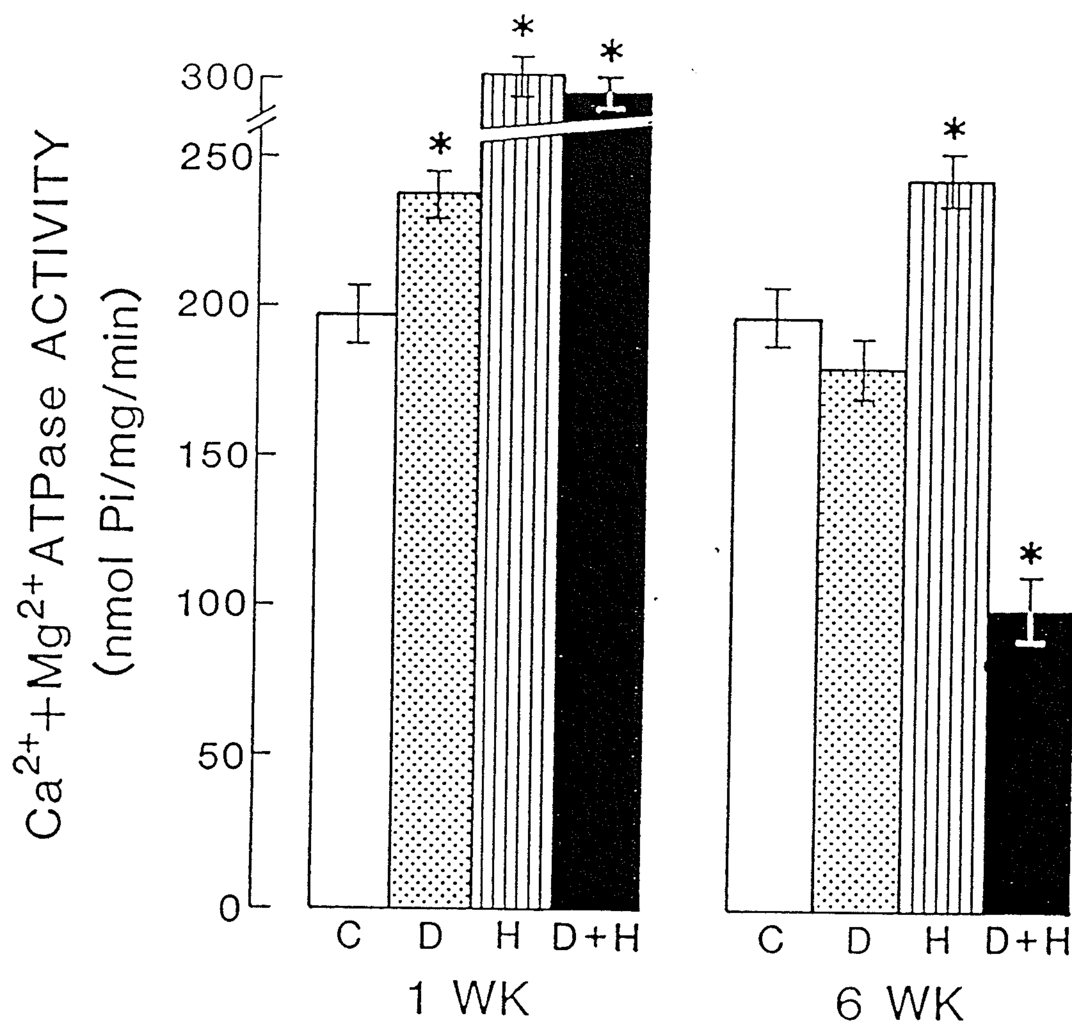


Figure VI. Ca^{2+} + Mg^{2+} ATPase activity of kidney basolateral membrane in different experimental groups. C, Control; D, diabetic; H, hypertension; D+H, diabetic + hypertension. *Significantly different ($p<0.05$) from control.

Table X.Effect of ANP on Ca^{2+} + Mg^{2+} ATPase activity in kidney basolateral membrane from control and diabetes plus hypertension.

ANP conc (M)	Ca^{2+} + Mg^{2+} ATPase (nmol Pi/mg/min)			
	Control	Diabetes	Hypertension	Diabetes and Hypertension
0	17.35 ± .04	17.77 ± 0.09	17.41 ± 0.08	8.65 ± 0.21
10^{-12}	19.78 ± 0.26*	18.01 ± 0.18	19.98 ± 0.28	8.73 ± 0.29
10^{-11}	22.99 ± 0.35*	18.74 ± 0.23	23.41 ± 0.34	9.03 ± 0.32
10^{-10}	29.49 ± 0.28*	18.53 ± 0.20	28.51 ± 0.29	8.89 ± 0.23
10^{-9}	32.97 ± 0.34*	18.43 ± 0.21	34.12 ± 0.30	8.84 ± 0.07

Values are means ± SE of 5 experiments. Ca^{2+} + Mg^{2+} ATPase was measured in the presence of 1 μM free Ca^{2+} and was the difference between the total and Mg^{2+} ATPase activity. Basal Mg^{2+} ATPase was unchanged by ANP.

* Significantly ($p<0.05$) different from 0 ANP.

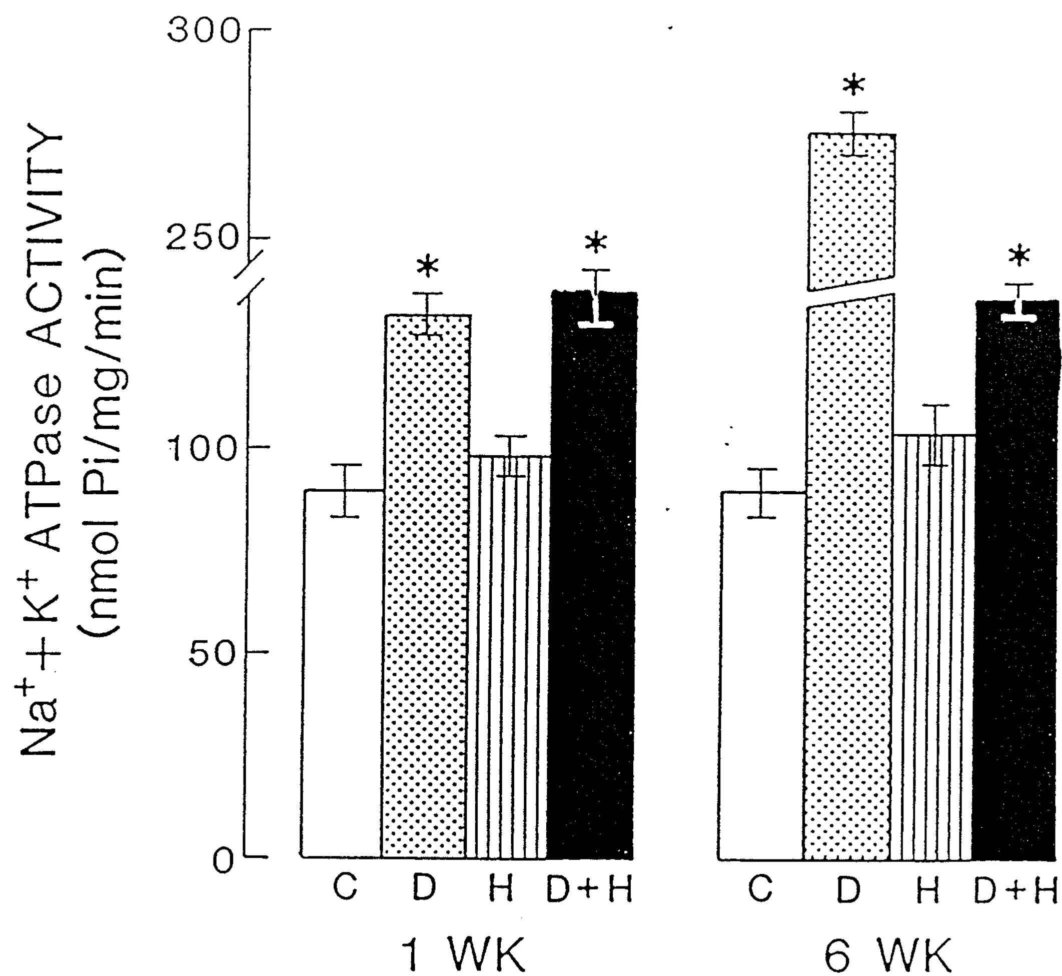


Figure VII. Na^+ + K^+ ATPase activity of kidney basolateral membrane in different experimental groups. C, Control; D, diabetic; H, hypertension; D+H, diabetic + hypertension. *Significantly different ($p<0.05$) from control.

Table XI. ANP-receptor binding in experimental animals at 6 wk study

	K_D (nM)	B_{max} (fmol/mg protein)
Control	8.1 ± 0.65	137 ± 8
Diabetes	7.3 ± 0.56	154 ± 7
Hypertension	7.6 ± 0.57	157 ± 9
Diabetes + Hypertension	7.9 ± 0.62	178 ± 9*

Values are means ± SE of 6-7 experiments done in triplicate.

* $p<0.05$ Significantly different from control

6. DISCUSSION

Consistent with the important role of Ca^{2+} in biology, active transport of this divalent cation has been demonstrated in eukaryotic cells (176). Therefore, the implication that the kidney contains a Ca^{2+} pump where hormone-related movement of Ca^{2+} occurs is of physiological importance (195). The pump is instrumental in the vectorial translocation of calcium out from the cell across the basolateral border of the membrane (193). This property of the pump is of physiological interest in as much as it provides a mechanism for the fine tuning of the cytosolic Ca^{2+} concentration. Ca^{2+} exerts a double-edged sword effect in that, though it is required as a cytosolic messenger for a variety of biochemical and physiological processes, in excess concentrations it can seriously compromise cellular integrity ("Ca^{2+} overload") (333). Hence, regulation by the Ca^{2+} pump of the cytosolic Ca^{2+} concentration provides an optimal environment for cell function.

The results of the present study showed a direct stimulatory effect of ANP on this pump suggesting that ANP may have significant bearings on Ca^{2+} transport. In spite of the fact that a considerable body of evidence suggests that ANP participates in the intracellular Ca^{2+} homeostasis by altering membrane-bound activities in other tissues such as smooth muscle (171), this is the first direct demonstration that ANP may be involved in the intracellular Ca^{2+} transport in the kidney by altering the Ca^{2+} pump activity. Furthermore, ANP is known to decrease intracellular Ca^{2+} concentration (96) and it is possible that ANP may be stimulating Ca^{2+} efflux mechanisms (Ca^{2+} pump) in order to maintain optimal levels of intracellular Ca^{2+}.

ANP has been shown to mediate its actions by stimulating particulate guanylate cyclase in responsive tissues (162). There is coexistence of the ANP receptor and guanylate cyclase activities on a single polypeptide chain suggesting that this transmembrane protein contains both the information for cell recognition and its translation into a second messenger, viz. cGMP (153). Wong et al. (78) have shown that cGMP is an excellent marker for renal biological actions of ANP. Therefore, as a consequence of its involvement in direct stimulation of the guanylate cyclase system in the kidney BLM and its participation in the Ca^{2+} homeostasis via its stimulatory action on the Ca^{2+} pump, the possibility exists that the Ca^{2+} pump and guanylate cyclase system are coupled together. Data in the present study revealed that isotopic assay of the $Ca^{2+}+Mg^{2+}$ATPase, after stimulating the guanylate cyclase system by GTP, resulted in a decreased activity. This would suggest an inhibitory role for the guanylate cyclase system on the Ca^{2+} pump. Corroborative evidence of this distinctive effect of guanylate cyclase on Ca^{2+} pump came from experiments using non-hydrolysable GTP analogues. The substitution of GTP with a non-hydrolysable analogue GTP-γ-s, was incapable of altering Ca^{2+} pump activity. This suggests that the utilisation of the substrate (GTP) by the guanylate cyclase is imperative for the modulation of the Ca^{2+} pump activity. Further evidence of the inhibitory character of the guanylate cyclase on $Ca^{2+}+Mg^{2+}$ATPase came from our studies employing different concentrations of GTP to activate the guanylate cyclase system. Our results demonstrated a dose-dependent inhibition of $Ca^{2+}+Mg^{2+}$ATPase by GTP. This would be interpreted to mean that augmentation of the guanylate cyclase system, by increasing substrate

(GTP) utilisation, showed a corresponding step-wise inhibition of $Ca^{2+}+Mg^{2+}$ATPase. It can be postulated that a reciprocal modulation exists between the activities of the guanylate cyclase and Ca^{2+} pump in which the alterations at the catalytic site of the guanylate cyclase are able to modulate Ca^{2+} pump activity. This inhibitory effect of the guanylate cyclase $Ca^{2+}+Mg^{2+}$ATPase was specific to the kidney cortex. Similar studies in the liver plasma membranes showed that though the inhibition was there, it was minimal.

A logical extension of this observation was to determine the mechanism of this inhibitory effect of the guanylate cyclase system on $Ca^{2+}+Mg^{2+}$ATPase. It is theoretically possible that this could be a post-receptor, i.e. cGMP, mediated effect. However our data showed no change in $Ca^{2+}+Mg^{2+}$ATPase activity when cGMP was included in the incubation media, thus invalidating the original hypothesis. This inability of the cGMP to alter the $Ca^{2+}+Mg^{2+}$ATPase activity would indicate that the inhibitory effect of guanylate cyclase on $Ca^{2+}+Mg^{2+}$ATPase is not mediated via cGMP, that is, it is not a post-receptor phenomenon. The possibility that $Ca^{2+}+Mg^{2+}$ATPase and guanylate cyclase are coupled together is therefore raised. Accordingly, we propose the following working model as a prospective mechanism of the coupling of these two enzymes. It is suggested according to our model, that guanylate cyclase is able to induce conformational changes in the $Ca^{2+}+Mg^{2+}$ATPase with decreased expression of the pump activity. The validity of this proposed model was examined by modulating the guanylate cyclase using various stimulators and inhibitors. Sodium azide, a well-known powerful stimulator of guanylate cyclase (331) was added to the incubation media and the

$Ca^{2+}+Mg^{2+}$ATPase assayed. The results clearly showed that $Ca^{2+}+Mg^{2+}$ATPase was depressed due to an increase in the guanylate cyclase activity. Our studies with guanylate cyclase antibody further lend credence to this model. This antibody cross-reacted with the guanylate cyclase enzyme in the kidney BLM and was able to nearly block its action. Radioisotopic assay of $Ca^{2+}+Mg^{2+}$ATPase after inhibiting the guanylate cyclase by this antibody showed an elevation of the $Ca^{2+}+Mg^{2+}$ATPase activity. Inclusion of the antibody in the incubation medium was able to block the guanylate cyclase and thereby remove the inhibitory influence of guanylate cyclase on $Ca^{2+}+Mg^{2+}$ATPase leading to increased expression of the latter enzyme.

It can be argued that depression in $Ca^{2+}+Mg^{2+}$ATPase could arise possibly as a result of competitive substrate utilisation. In other words, since the incubating media contains both ATP and GTP, the preferential utilisation of one instead of the other could lead to a decrease in enzyme activity. Accordingly, another ATPase was tested for possible effects due to preferential substrate utilisation. No inhibitory effect of GTP on $Na^{+}+K^{+}$ATPase was noted indicating that the GTP inhibitory effect was exclusive to the $Ca^{2+}+Mg^{2+}$ATPase. These data provide conclusive proof that these enzyme systems viz. guanylate cyclase and the $Ca^{2+}+Mg^{2+}$ATPase are coupled together and a reciprocal modulation exists between the two (334). Further analysis of this proposed model shows that ANP is acting on two sites to modulate the $Ca^{2+}+Mg^{2+}$ATPase. As also mentioned previously, ANP is directly stimulating the enzyme (335) while it also, paradoxically, has an inhibitory influence on the enzyme via the guanylate cyclase system. A

moment's reflection on this paradoxical situation would suggest that probably the $Ca^{2+} + Mg^{2+}$ATPase is maintained at an optimal level by these two radically opposed influences. As a consequence, the cytosolic Ca^{2+} concentration is regulated.

Important cardiovascular abnormalities have been described in animals with diabetes induced by streptozotocin or alloxan (285-287). Abnormal myocardial Ca^{2+} homeostasis and changes in myocardial contractility and relaxation have also been reported in this model (279,284). Furthermore, in spite of increased plasma norepinephrine level in streptozotocin diabetes, the animals remained normotensive even at 12 weeks of the disease process (336). Despite other hemodynamic alterations including bradycardia, increase in stroke volume, cardiac output and cardio-osmotic ratio in diabetes, rats are resistant to the development of congestive heart failure unless hypertension is superimposed as previously reported (2). The question now arises as to what protects the development of heart failure in this model? Based on the literature, it is apparent that plasma ANP may exert a pivotal influence on the protection against congestive heart failure in streptozotocin-treated rats since diabetes-induced volume expansion augments ANP release which in turn contributes to hyperfiltration (251). Clearly, there was a significant progression in the deterioration of cardiac function between 1 to 6 wk in the D+H group and a severe congestive heart failure was present in those animals allowed to live upto 10 weeks (223). Furthermore, the elevation in circulating plasma ANP appears to be inadequate to prevent the abnormal renal handling of sodium and water in hypertensive diabetic rats. Based on our results, although the plasma ANP was increased at a very early stage of the insult, it is perhaps true that as the disease

progressed the circulating level of ANP decreased, resulting in a compensatory increase of ANP receptors in kidney as shown by our biochemical studies. Furthermore, in hypertensive-diabetic rats, Ca^{2+} + Mg^{2+} ATPase, which acts as a calcium pump and plays a primary role in the Ca^{2+} homeostasis in rat kidney cortex cells (191) was significantly decreased (337). These results are interpreted to mean that an increase in plasma ANP in diabetes serves as a compensatory mechanism to maintain the circulatory homeostasis, whereas the development of congestive heart failure in this disease is associated with a decrease in plasma ANP and/or a defect in its post-receptor mechanism. This view is based on the fact that kidney ANP receptor number was increased whereas cGMP level was decreased probably as a result of a defect in the receptor-coupling system in hypertensive-diabetic group. Such a response could lead to a decrease in Ca^{2+} pump activity in kidney basolateral membrane seen in this study. These results are unlikely to be an artifact associated with the membrane preparation since Na^{+} K^{+} ATPase which is normally unaffected by ANP was increased in this group.

The fact that cGMP may be involved in the removal of Ca^{2+} from the cell is suggested by the findings that intracellular Ca^{2+} is depleted more rapidly in the presence of agents that elevate cytosolic cGMP (173). In keeping with an effect of ANP on Ca^{2+} exit, ANP reduces intracellular Ca^{2+} concentrations in rat and aortic smooth muscle cells and rat glomerular mesangial cells (171,172). The available evidence thus favors the view that ANP through stimulation of guanylate cyclase (165,168,169) modulates plasma membrane Ca^{2+} ATPase activity (335), thus regulating removal of Ca^{2+} from the cell. A lack of response of this pump activity

to ANP may lead to intracellular Ca^{2+} overload. An increased intracellular Ca^{2+} concentration may decrease oxidative phosphorylation in mitochondria (16). As a result, the capacity of the renal cell to generate ATP becomes attenuated which in turn compromises renal cellular function or precipitates nephropathy. The development of congestive heart failure may be triggered by this process as myocardium is further sensitized by volume overload (223). A diagrammatic representation of the events is shown in Figure VIII.

It may be pointed out that in a model of established congestive heart failure, plasma ANP is shown to be increased (possibly mediated via increased sympathetic activity) (115) whereas the renal sensitivity might be reduced as a result of down regulation of ANP receptors in the kidney. In fact, vascular ANP receptors are down regulated by prolonged exposure to ANP and the receptor-bound ANP is internalized (338,339). Tsunoda et al (129) have reported decreased ANP binding in renal medulla in rats with chronic heart failure. Also when the animals in the hypertensive-diabetic group were sacrificed at 10 weeks, the plasma ANP levels were, in fact, increased and ANP receptors downregulated paralleling other observations (338,340). Although a clear explanation for decreased ANP levels at 6 weeks after hypertension and diabetes is difficult at this stage, the possibility exists that at 6 weeks a decrease in plasma volume in hypertensive and diabetic rats may be responsible for the observed low plasma ANP level. Whether or not the sympathetic activity may have a modulatory role on the volume-contracted state in these animals, remains to be investigated. Our recent studies indicate that sympathetic activity is increased at 6 weeks in the hypertensive-diabetic rats (341).

Since ANP is a potent inhibitor of sympathetic activity (342), a decreased plasma ANP level would favor an increased sympathetic tone. Most likely, such a negative feedback mechanism for ANP is absent in the established stage of congestive heart failure. All these results support the view that the role of ANP is quite central to a proper understanding of the development of congestive heart failure.

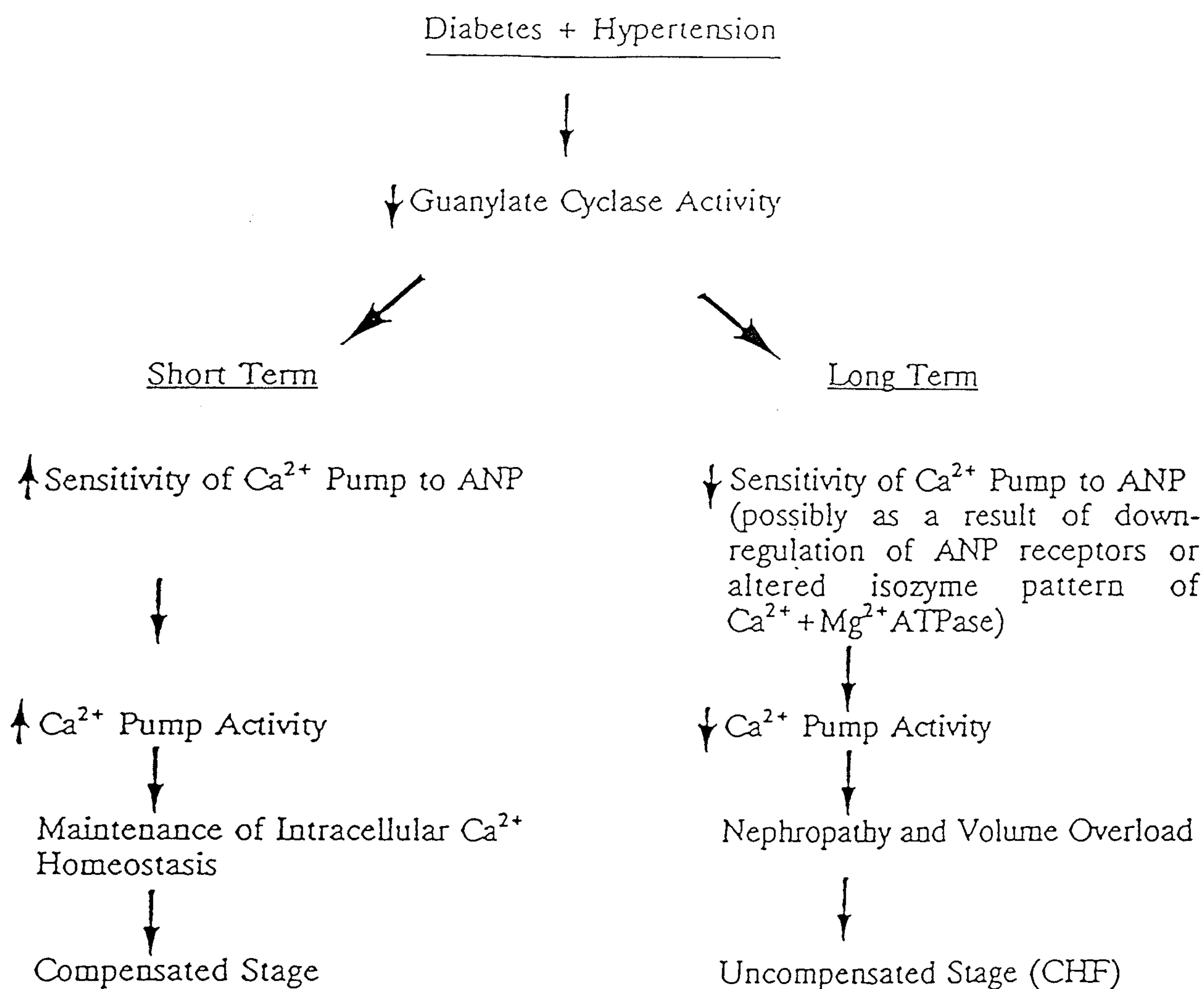


Figure VIII. Diagrammatic representation of the events leading to compensated and uncompensated stages of congestive heart failure (CHF). Initially when diabetes is superimposed with hypertension, guanylate cyclase activity is decreased, which (because of reciprocal modulation) enhances Ca^{2+} pump activity. While the same modulatory effect at a later stage may decrease the pump activity, the exact mechanism responsible for the decreased sensitivity of Ca^{2+} pump to ANP is not known at present. Down regulation of ANP receptor or altered isozyme pattern of $Ca^{2+}+Mg^{2+}$ATPase may be responsible for such alteration.

8. CONCLUSIONS

The present study indicates that the ANP, a cardiac hormone, mediates its biological functions by acting on its receptors in the kidney basolateral membrane. It has been further established that ANP receptors are coupled with Ca^{2+} + Mg^{2+} ATPase, the enzyme that participates in the vectorial translocation of Ca^{2+} from the tubular lumen in to the plasma. Chronic diabetes associated with hypertension leads to a defect in the ANP-receptor coupling system resulting in nephropathy and congestive heart failure. Although future studies are warranted to understand the exact molecular defects underlying this pathology, the present study further extends our knowledge that the interaction between ANP and Ca^{2+} + Mg^{2+} ATPase in kidney is quite fundamental to the proper understanding of congestive heart failure in diabetes.

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