1978

The Effect of Isoproterenol and Alpha- and Beta-Adrenoreceptor Blockade on Renin Release and Cyclic-35ì HOF HOF Adenosine Monophosphate Content in Rat Renal Cortical Slices

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THE EFFECT OF ISOPROTERENOL AND ALPHA- AND BETA-ADRENORECEPTOR BLOCKADE ON RENIN RELEASE AND CYCLIC-3'5'-ADENOSINE MONOPHOSPHATE CONTENT IN RAT RENAL CORTICAL SLICES

by

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A Thesis Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of

Master of Science

May, 1978

ABSTRACT

The effect of the specific beta-adrenergic receptor agonist l-isoproterenol on renin release and c-AMP content of kidney slices from sodium-replete and sodium-deficient rats was studied *in vitro*. Male, Sprague-Dawley rats weighing 210 ± 10g were fed high sodium (over 2.2 mEg/L) or low sodium (less than 0.02 mEg/L) diets for periods of time of 2-3 weeks. Following decapitation, renal cortical slices weighing 50 ± 20mg were incubated for 1 hour at 37°C in Robinson's buffer medium with or without (control) the various pharmacological agents. Isoproterenol doses ranging from 10^{-13}M to 10^{-3}M did not affect renin release or c-AMP content in slices from sodium-replete rats. In contrast, isoproterenol doses from 10^{-7}M to 10^{-3}M significantly increased renin release and c-AMP content in slices from sodium-deficient rats, whereas concentrations lower than 10^{-7}M were ineffective. Addition of the beta-adrenergic receptor blocker d,l-propranolol (10^{-4}M) to slices from sodium deficient rats prevented stimulation of renin release and c-AMP content by 10^{-7}M isoproterenol but only partially

inhibited the effect of a higher isoproterenol dose (10^{-3}M). In the same slices, addition of the alpha-adrenergic receptor blocker phentolamine (10^{-6}M) prevented the stimulatory effect of two isoproterenol doses (10^{-7}M and 10^{-3}M) on renin release. Its effect on c-AMP content, however, was only partial, since in the presence of phentolamine plus isoproterenol (10^{-7}M), tissue c-AMP content levels remained significantly elevated. It should be pointed out, however, that although phentolamine appeared to prevent the stimulatory effect of 10^{-7}M and 10^{-3}M isoproterenol on renin release, the mean renin secretory rates observed in the presence of this blocking agent plus the two doses of isoproterenol were no different from those seen with isoproterenol added alone, but the standard errors were larger in the former.

These data indicate that: 1) sodium deficiency potentiates the renin release and c-AMP responses to added isoproterenol; 2) isoproterenol appears to stimulate renin release in a dose-response relationship; 3) isoproterenol stimulates renin release in sodium deficient rats by a beta-adrenergic receptor mechanism mediated by the genera-

tion of intracellular c-AMP; 4) alpha-adrenergic receptors may partly mediate the stimulatory effect of isoproterenol on renin secretion, instead of mediating an inhibitory effect as proposed for high norepinephrine concentrations in other studies.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to the director of my thesis committee, Dr. Genaro Lopez, who, for his professional and technical advice, patience and friendship, has enriched my life with the knowledge, skill and desire to continue my future endeavor in the life sciences.

Furthermore, I thank the other members of my thesis committee, Drs. Harold Manner, Mark Goldie, and Albert Rotermund for their professional assistance in the preparation of this manuscript, and for their friendship over the past four years.

I am deeply indebted to my colleagues Vic Aletich and Fred Romano for their part as fellow researchers, and above all for their friendship.

Acknowledgment is also given to Dr. B. Braverman, Department of Physiology, Loyola University Stritch School of Medicine, for his generous donation of 24-hour nephrectomized dog plasma, and to Argonne National Laboratory for the use of their computer facilities.

Lastly, to my father...for his continuous support and encouragement provided me in my academic endeavors,

To my mother...for her love and guidance, whom without the past seven years would have been most difficult,

To my brother and sisters...for their love and emotional support allowing me to make it his far,

To my wife Claudia...who, for her love and devotion, has given me the desire to continue.

VITA

The author, Louis Michael Lissuzzo, is the son of Joseph Anthony Lissuzzo and Pauline (Sparacino) Lissuzzo. He was born February 2, 1953 in Chicago, Illinois.

His elementary education was obtained in the Sacred Heart and Jane Adams grammar school, Melrose Park, Illinois. His secondary education was obtained at the Proviso East High School, Maywood, Illinois, where he graduated in 1971.

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In September, 1976, he was granted an assistantship in the Department of Biology at Loyola University of Chicago to pursue studies leading to the Master of Science degree. In January, 1978, he became a member of the Illinois Academy of Science. In April, 1978, he received a Certificate of Recognition for his research from the Sigma Xi Research

Forum and additionally, he received a research fellowship from Argonne National Laboratory for the summer of 1978. In May, 1978, he was awarded the Master of Science degree in Biology.

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CHAPTER I

INTRODUCTION

During the past decade, the importance of neural sympathetic mechanisms in the regulation of renin secretion has been clearly demonstrated in vitro (1-10) and in vivo (11-15) and has been discussed at length in a number of review articles (16-18). Nevertheless, substantial progress has been slowed by the difficulty of separating these neural factors from the various humoral and hemodynamic ones which influence renin secretion. It is generally accepted that renin secretion is partly regulated by norepinephrine, the catecholamine released from sympathetic nerve terminals, via a beta-adrenergic receptor complex. This complex appears to be located on the membranes of the juxtaglomerular cells themselves, and may utilize cyclic-3'5'-adenosine monophosphate (c-AMP) as the intracellular mediator of renin release by norepinephrine.

Several studies (7, 9, 10, 15) have also shown that in addition to its stimulatory effect on renin release at physiological and subphysiological concentrations which

appears to be beta-receptor mediated, high concentrations of norepinephrine may exert an inhibitory effect on renin release. This inhibitory effect of norepinephrine on renin release may be mediated by renal alpha-adrenergic receptors (8, 9, 15) through a decrease in intracellularly generated c-AMP (9). The significance of these opposite effects of norepinephrine on renin release is not clear at this time.

This investigation examines some of the mechanisms by which the sympathetic nervous system regulates renin secretion from the juxtaglomerular cells of the mammalian kidney. Although this research is basic in nature it has important clinical implications as well, particularly with regard to hypertensive disorders characterized by a chronic maladjustment of the renin secretory mechanisms (i.e. high-renin hypertension) which may be neurally mediated to a significant degree (18, 19).

Overall, these experiments are designed to evaluate the effects of a specific beta-adrenergic receptor agonist, isoproterenol (20), either alone or coupled with alpha- and beta-adrenergic receptor blockade on renin release. Examination of these effects has been accomplished

by means of an *in vitro* rat renal cortical slice preparation, obtained from rats maintained on sodium-replete or sodium-deficient diets for 2-3 weeks (7, 9).

Specifically, these experiments will attempt to determine whether or not: 1) isoproterenol stimulates renin secretion primarily via a beta-adrenergic receptor pathway, mediated by c-AMP and involving new synthesis of renin as suggested for norepinephrine (4, 5, 7, 9) rather than by a preferential membrane effect not involving *de novo* synthesis of renin (21); 2) beta-adrenergic receptor blockade by d,l-propranolol inhibits isoproterenol-stimulated renin release, as suggested for norepinephrine and other catecholamines (4); 3) renal alpha-adrenergic receptors play a role in inhibiting isoproterenol-stimulated renin release, as recently suggested by some investigators regarding norepinephrine (8, 9); 4) there is a correlation between c-AMP and renin release changes in response to isoproterenol, which would constitute strong evidence for a beta-adrenergic receptor pathway mediating renin secretion via intracellularly generated c-AMP and *de novo* synthesis of renin; and 5) sodium deficiency potentiates the renin secretory responses to isoproterenol, as suggested from various *in*

vivo and in vitro studies with regard to other catecholamines (9, 12).

CHAPTER II

LITERATURE REVIEW

It is now well established that renin release is regulated by several interrelating factors. These factors, described by Davis (17), include the following: 1) the intrarenal receptors, composed of the baroreceptors of the renal afferent arteriole, and the macula densa natrio-receptors located in the walls of the distal convoluted tubule of the kidney; 2) humoral agents such as sodium, potassium, calcium, antidiuretic hormone, angiotensin-II, c-AMP and the circulating catecholamines epinephrine and norepinephrine from the adrenal medulla; 3) the sympathetic nervous system, via catecholamines released from renal sympathetic nerve endings.

A. The Renin-Angiotensin System

Renin, a hormone synthesized and released from the juxtaglomerular cells of the afferent arteriole of the kidney, is involved in the control of blood pressure, fluid volume and electrolyte balance (17, 22-26). That the jux_ taglomerular cells are indeed vascular smooth muscle

cells modified to serve an endocrine function was first suggested by Goormagtigh (27). Laragh (26) and Liddle et. al (28) have recently reviewed the renin-angiotensin system biochemical pathway, which is initiated by endogenous renin acting enzymatically on the tetradecapeptide angiotensinogen (renin substrate), a gamma globulin synthesized in the liver, to liberate the decapeptide angiotensin-I by hydrolysis of the C-terminal tetrapeptide of angiotensinogen. Angiotensin-I in turn is cleaved by a converting enzyme found in large amounts in the pulmonary circulation, liberating the octapeptide angiotensin-II, considered to be the most powerful pressor agent in the systemic circulation. In addition to its effects on blood pressure, angiotensin-II has been postulated as being a potent stimulator of the synthesis and secretion of aldosterone, a mineralocorticoid from the adrenal cortical glomerulosa cells (2, 29). However, recent evidence by Vaughan (30) have suggested that angiotensin-III, a circulating heptapeptide derived from angiotensin-II, may be the actual modulator of aldosterone synthesis and release.

B. Intrarenal Receptors

Tobian et. al (22) have suggested that a decreased stretch of the afferent arteriole as a result of changes in perfussion pressure, leads to an increase in renin release. Using a non-filtering, denervated kidney coupled with adrenalectomy, which effectively eliminated macula densa, renal nerve catecholamines and circulating catecholamine effects, respectively, Blain and Davis (25) observed a significant increase in renin release in response to a moderate hemorrhage (20ml/kg), thus supporting the baroreceptor hypothesis.

Vander and Miller (31) first suggested that the macula densa cells of the distal convoluted tubule are sensitive to changes in sodium "load" or concentration. In their experiments with anesthetized dogs, they observed that the increase in renin secretion caused by aortic constriction was no longer seen upon administration of diuretic agents. They therefore suggested that diuretic agents increase the sodium load at the macula densa thus inhibiting renin release. They further proposed that renin release is stimulated by a decreased sodium load at the macula densa, and that the macula densa cells function as effective natrioreceptors. Nash (32) has supported this macula densa theory

this macula densa theory by showing that in dogs having their ureters blocked, infusion of sodium chloride solutions resulted in a significant decrease in renin secretion.

C. Humoral and Hemodynamic Effects on Renin Release

Further experiments by Vander (33) on the effects of potassium on renin secretion have shown that infusion of potassium chloride solutions into normal or salt-depleted dogs caused a decrease in renin release. Potassium is postulated to inhibit renin release indirectly by inhibiting proximal tubule sodium reabsorption which would, in turn, increase the sodium load at the macula densa.

Furthermore, Michelakis et. al (1) have shown that c-AMP added to incubated kidney cell suspensions resulted in a greater net production of renin than cells incubated without c-AMP, suggesting a possible direct effect of this nucleotide on renin secretion. More recent investigations by Saruta et. al (34) have provided added evidence for a stimulatory role of c-AMP on renin secretion. The same workers have additionally suggested that calcium also plays a stimulatory role on renin release. However, most of the evidence concerning the possibility of a direct

effect of ions on renin release is still controversial and further studies are clearly needed.

The mechanisms by which angiotensin-II and anti-diuretic hormone (ADH) inhibit renin release have been reviewed by Shade et. al (35). Using sodium-depleted dogs with a nonfiltering kidney they found a decrease in renin secretion after infusion of angiotension-II and ADH, suggesting that the action of these humoral agents may be exerted directly on the juxtaglomerular cells, which supports previous work by Bunag et. al (11). While the possibility of a direct mechanism of action of ADH on renin secretion remains largely unclear, these studies and others (9) generally show that angiotensin-II appears to exert a direct regulatory control over renin release by a short-loop negative feedback of locally-generated angiotensin-II on the juxtaglomerular cells. It has also been suggested that this inhibitory effect of angiotensin-II is mediated by a decrease in intracellularly generated c-AMP (9).

D. The Sympathetic Nervous System and Renin Secretion

Evidence is accumulating that the sympathetic nervous system is a very important modulator of renin

secretion, not only for the minute-to-minute control in the healthy individual, but in pathological states as well (18, 19, 36).

Electron microscopic (37) and fluorometic studies (38) have provided clear evidence for a dense sympathetic innervation of the juxtaglomerular cells, suggesting the possibility of a direct stimulation of renin secretion by released norepinephrine from sympathetic nerve endings and perhaps by its released precursors (dopamine) as well. In vivo experiments by Vander (39) confirmed these early observations by showing that electrical stimulation of the renal nerves significantly increased plasma renin activity. Johnson et. al (40) have supported these findings by showing that direct stimulation of the renal nerves resulted in a concomitant increase in renin secretion. Passo et. al (41) additionally found that stimulation of the medulla oblongata increased plasma renin activity and that this response was prevented by renal dennervation.

Investigations concerning the effects of catecholamines on renin release (17) have shown that the circulating catecholamines epinephrine and norepinephrine released from the adrenal medulla, as well as norepine-

phrine released from renal sympathetic nerve endings, stimulate renin release and that this reponse may be mediated by a beta-adrenoreceptor pathway. Evidence for the existence of a beta-adrenoreceptor mechanism mediating renin release has been obtained from the studies of Loeffler et. al (42) who showed that in dogs given l-propranolol, a known beta-adrenoreceptor blocker, renin release produced by renal nerve stimulation was significantly decreased. Moreover, Assaykeen et. al (13) observed that as a result of hypoglycemia produced in dogs, which increased the levels of circulating catecholamines, renin secretion was abolished upon injection of l-propranolol. The same workers additionally showed that following administration of phentolamine, an alpha-adrenorector blocker, renin secretion was potentiated above control levels.

However, these in vivo findings cannot eliminate the possibility of other in vivo factors participating in the observed responses. Thus, in order to evaluate the possibility of a direct action of the sympathetic nervous system on renin secretion, in vitro systems have been designed which effectively eliminate the contribution of humoral, hemodynamic and other in vivo influences. Using an in

vitro renal cortical slice preparation, Nolly *et. al* (4) have shown that added norepinephrine stimulates renin release, and that this stimulation appears to be mediated by a beta-adrenoreceptor complex located on the membranes of the juxtaglomerular cells. Furthermore, Nolly *et. al.* (4) provided additional evidence for their postulation by showing that the addition of theophylline, an inhibitor of the phosphodiesterase enzyme that ordinarily degrades intracellular c-AMP (43), resulted in a potentiation of the renin release responses to added norepinephrine. This suggests that norepinephrine may exert its action on renin release via a beta-adrenoreceptor mechanism which utilizes c-AMP as the intracellular mediator. Numerous other *in vitro* workers are in agreement with this proposed mechanism of action (1, 5-7, 44). Nolly *et. al* (4) have also shown that norepinephrine-stimulated renin release is potentiated by the addition of phentolamine and phenoxybenzamine, both of which are alpha-adrenoreceptor blocking agents. This may suggest a contribution of alpha-adrenoreceptors in the regulation of renin secretion which may be inhibitory in nature.

These in vitro observations have been further extended by Ganong and Lopez (9) who showed that the significant stimulatory effect of low (10^{-12}M to 10^{-7}M) norepinephrine doses on in vitro renin secretion in sodium-deficient rats was accompanied by concomitant increases in tissue c-AMP content. This constitutes additional evidence for a beta-adrenoreceptor mechanism mediating renin secretion. Conversely, the same workers have shown that high (10^{-4}M) norepinephrine doses were inhibitory to renin release and that this response is coupled with a significant decrease in tissue c-AMP levels. They have suggested, as well as others (3, 8, 15), that this inhibitory effect of high norepinephrine concentrations on renin release may be alpha-adrenoreceptor mediated since simultaneous addition of specific alpha-adrenoreceptor blockers (e.g. phentolamine) effectively reversed the inhibition (8, 9).

On the other hand, Winer et. al (45) have presented evidence suggesting that alpha-adrenoreceptors actually mediate the stimulatory effect of catecholamines on renin secretion.

In vitro studies by Ganong and Lopez (9) have shown that the renin secretory responses to added norepinephrine

are potentiated under sodium-deficient conditions, supporting previous in vivo data (12). Most studies pertaining to renin secretion have utilized animals maintained on a commercial chow diet. However, a commercial chow diet contains approximately 12 times the recommended amount of sodium by the National Research Council. This obviously would result in a much lower basal renin release in these animals since renin release is extremely sensitive to plasma sodium levels. On the other hand, animals maintained on sodium-deficient diets show a much higher rate of basal renin release (7, 46) which appears to be due to an increased de novo synthesis; this has been shown by the elegant electron microscopic studies of Reidel (47). Moreover, in vivo (11, 12) and in vitro studies (7, 9) have suggested that sodium-deficient conditions, in addition to increasing the basal renin secretory rate, potentiate the renin secretory reponses to sympathetic stimuli. Thus, the evaluation of both stimulatory and inhibitory effects of various adrenergic agents on renin secretion in animals maintained on sodium-deficient diets may be more easily ascertained, in view of the apparent greater sensitivity of the juxtaglomerular cells coupled with the higher resting renin release rates seen in the sodium-deficient state.

E. Isoproterenol and Renin Secretion

Numerous studies on the effects of isoproterenol, a catecholamine with postulated specific beta-adrenoreceptor stimulating properties (20), have presented conflicting evidence as to the actual mechanism mediating its stimulatory effect on renin secretion.

In vitro studies by Weinberger et. al (5) have shown that isoproterenol stimulates renin release and that this effect is blocked by the addition of l- and d,l-propranolol but not by d-propranolol, which has all the properties of l-propranolol except its beta-blocking ability. Similar results have been reported by Capponi and Valloton (8) who showed that the significant stimulatory effect of isoproterenol on renin release was inhibited by d,l-propranolol, which constitute clear evidence for a beta-adrenoreceptor mechanism of action of this catecholamine. Other investigators (44, 48) have further suggested that the stimulatory effect of isoproterenol on renin secretion is mediated by changes in intracellularly generated c-AMP and involves de novo synthesis of renin; this is consistent with a beta-adrenoreceptor mechanism of action.

Assaykeen et. al (49) have also shown in anesthetized dogs that administration of phentolamine during isoproterenol infusion did not cause a reduction in renin. However, the addition of l- and d,l-propranolol effectively prevented the increase in renin secretion seen during isoproterenol infusion. These data add support to a beta-adrenoreceptor mediated mechanism of action.

In contrast to the possibility of an adenylate-cyclase complex mediating the renin release responses to isoproterenol, Johns and Singer (21) have indicated that the stimulatory effect of isoproterenol on renin release may preferentially be a membrane effect. They indicate that this leads to the output of preformed cytoplasmic renin granules without any involvement of an adenyl-cyclase-containing receptor complex; thus, without de novo synthesis of renin. Partially in agreement with this view is the work by Beck (50) who has suggested that the renin-releasing effect of isoproterenol can be dissociated from the renal increase in c-AMP.

Conversely, Reid et. al (14) have reported that although intravenous infusion of isoproterenol caused a significant increase in renin secretion, no increase was observed during intrarenal infusion. They, therefore,

suggested the existence of an extrarenal receptor mechanism mediating the renin secretory responses to the infused isoproterenol, the location of which has not been proposed.

In any case, isoproterenol may eventually be considered to be another important regulator of renin secretion, particularly in view of the finding by Lockett et. al (51) of a substance that appears to be isoproterenol in the extracts of rat adrenal glands. This suggests the interesting possibility that isoproterenol may be an endogenous secretory product of the adrenal medulla, and thus capable of participating in the minute-to-minute regulation of renin secretion in the body.

CHAPTER III

MATERIALS AND METHODS

Male, Sprague-Dawley rats (Sprague-Dawley, Co.) weighing 210±10g were utilized for the experiments. They were maintained in temperature-controlled rooms (23±2°C), two animals per cage, and had access to distilled-deionized water ad libitum. The animals were divided into two groups of 24 each, which were subsequently studied in two different experimental trials. The first group of 24 rats was further divided into two equal subgroups, each subjected to a specific dietary regime in which sodium was the only variable. Thus, one subgroup of 12 rats was fed a normal sodium chow diet (Purina) for 2-3 weeks while the other subgroup was fed a sodium-deficient diet (TEKLAD TEST, Co.) for a similar period of time. The second group of 24 rats, utilized in later experiments, was fed only a sodium-deficient diet for a period of 2-3 weeks.

At the end of the specific dietary intake period, animals from the first group were sacrificed by decapitation, and their kidneys rapidly excised, decapsulated and

gassed with a mixture of 95% O_2-5% CO_2 (52) while being maintained in Robinson's buffer medium at 4°C. Slices of renal cortex approximately 0.3mm thick were prepared using a Stadie-Riggs microtome (A. Thomas, Co.) and divided into five similar portions, each weighing approximately 50 ± 20mg, which were randomly assigned to incubating flasks to assure a homogeneous population of cells. The slices were then preincubated for 15 minutes in 2.5ml of Robinson's buffer medium at 37°C in an atmosphere saturated with 95% O_2-5% CO_2 using a Dubnoff shaking incubator (Precision Scientific, Co.). They were subsequently transferred to similarly numbered flasks containing fresh Robinson's at 37°C at which time the catecholamine was added. L-Isoproterenol (Sigma), prepared in 0.1% ascorbic acid to prevent oxidation of the catecholamine (5), was added in concentrations ranging from 10^{-13}M to 10^{-3}M in order to test for a dose response. This was followed by a 60 minute incubation period at 37°C with continuous 95% O_2-5% CO_2 gassing and shaking. One flask from each group of slices served as an untreated control. After one-hour incubation the supernatant medium was collected and stored at -20°C until assayed, while the tissue was immediately frozen on dry-ice, homogenated in 1.0ml of 8%

TCA (trichloroacetic acid) and subsequently extracted with water-saturated ether (Mallinckrodt). The ether phase was then discarded and the c-AMP containing water phase was subsequently lyophyllized and measured for c-AMP content using a modification of the competitive protein binding assay of Gilman (53). Briefly, the assay was performed as follows: fifty microliters of the unknown lyophyllized samples, previously diluted in 250 microliters of buffer (Tris/EDTA), were analyzed in duplicate along with 100 microliters of binding protein purified from bovine muscle (Amersham) and 50 microliters of ^{3}H-c-AMP (Amersham). The samples were then incubated for 90 minutes at 4°C at which time 200 microliters of charcoal suspension (Amersham) were added which separated protein-bound from free c-AMP. The samples were then vortexed for 5 seconds and immediately centrifuged in a Sorvall RC2-B refrigerated centrifuge for 10 minutes at 10,000 X G, also at 4°C. At this time 200 microliters of the supernatant were transferred into counting vials (Scientific Products) containing 10.0 ml of ACS-liquid scintillation cocktail (Amersham), shaken and then allowed to settle in the dark for approximately 30 minutes prior to counting. Counting was done in a Packard liquid scintillation

counter for 5 minutes with the background counts subtracted. The values for the unknown samples were read from a standard curve and the amount in picomoles of c-AMP (read off the curve) were multiplied by a factor of 5 (i.e. dilution factor due to 1:5 dilution of sample in Tris/EDTA buffer) and divided by the milligrams of wet tissue weight to give the final concentration in pmol c-AMP content/mg wet tissue.

In turn, renin in the incubated supernatant medium was measured by radioimmunoassay (RIA) of angiotensis-I and expressed as nanograms A-I generated/mg wet tissue/hour incubation. The supernatant samples were prepared for the RIA desribed as follows: plasma from 24-hour nephrectomized dogs was utilized as the source of renin substrate (angiotensinogen). Appropriate amounts of the converting enzyme-angiotensinases inhibitor agent, PhenylMethylSulfonylFluoride (Sigma), were added to the nephrectomized plasma to prevent catabolism of angiotensin-I or its conversion to angiotensin-II. One milliliter of this mixture was added to 25 microliters of sample (renin-supernatant), and angiotensin-I was generated for 2 hours in a water bath at 37°C. At the end of the 2-hour generation period, one ml of distilled-deionized water was added to the tubes and the samples were

then boiled for three minutes. The samples were subsequently stored at -20°C until assayed for angiotension-I by RIA (Squibb). In short, the angiotensin-I RIA was done as follows: duplicate tubes containing unknown amounts of sample (25, 50, or 100 microliters), one ml of ^{125}I-Angiotensin-I (approximately 5,000 counts per minute) and 50 microliters of angiotensin-I antiserum were incubated at 4°C for 24 hours. At this time, one ml of charcoal suspension adjusted to ph 5.5 with 0.1N HCl were added to separate antibody-bound from free angiotensin-I, vortexed for 5 seconds and centrifuged for three minutes at 10,000 X G at 4°C. The supernatant was then transferred to test tubes (Scientific Products) and counted in a gamma counter (Packard) for two minutes with the background counts subtracted. Percent-bound values for the unknown samples were used as an index to determine the picogram amounts of angiotensin-I from a standard curve. The final angiotensin-I concentration in the unknown samples were expressed as ng A-I generated/mg wet tissue/hr (see Appendix A).

Slices of renal cortex from the second group of rats were similarly prepared and divided into six similar portions with one serving as control. They were also treated in the same manner as those from the first series of studies.

However, in this instance, a specific beta-adrenoreceptor blocker d,l-propranolol (Sigma), prepared in 0.1% ascorbic acid to a concentration of 10^{-4}M, and an alpha-adreno-receptorblocker, phentolamine mesylate (Regitine, CIBA), also prepared in 0.1% ascorbic acid to a concentration of 10^{-6}M, were added both before the pre-incubation (15 min.) and incubation (60 min.) periods. These agents were evaluated when added either alone or in the presence of four different isoproterenol concentrations (10^{-11}, 10^{-9}, 10^{-7}, and 10^{-3}M). Supernatant and tissue samples were collected, prepared and stored in a manner identical to those from the first series of studies and subsequently analyzed for the same parameters as previously described.

The data collected from these series of studies were analyzed for statistical significance using a modified paired student's T test (54) with the aid of a desk-top programmable calculator (Hewlet-Packard).

CHAPTER IV

RESULTS

In a preliminary investigation, the effect of dietary sodium changes on renin release and the c-AMP content of rat kidney tissue was examined using the previously described renal cortical slice preparation. These data can be seen in Figure 1. As observed, the basal, non-stimulated, renin release rate in slices from sodium-deficient rats was significantly greater (p 0.01) than that seen in slices from sodium-replete animals. There were not significant differences, however, between both dietary groups of slices in regard to the c-AMP content of the tissue in response to dietary sodium manipulation.

In a subsequent study, the effect of various concentrations of the specific beta-adrenergic receptor agonist l-isoproterenol on renin secretion and c-AMP content of renal cortical slices from sodium-replete rats was evaluated. The results can be seen in figure 2. The data from this experiment shows that at these various concentrations

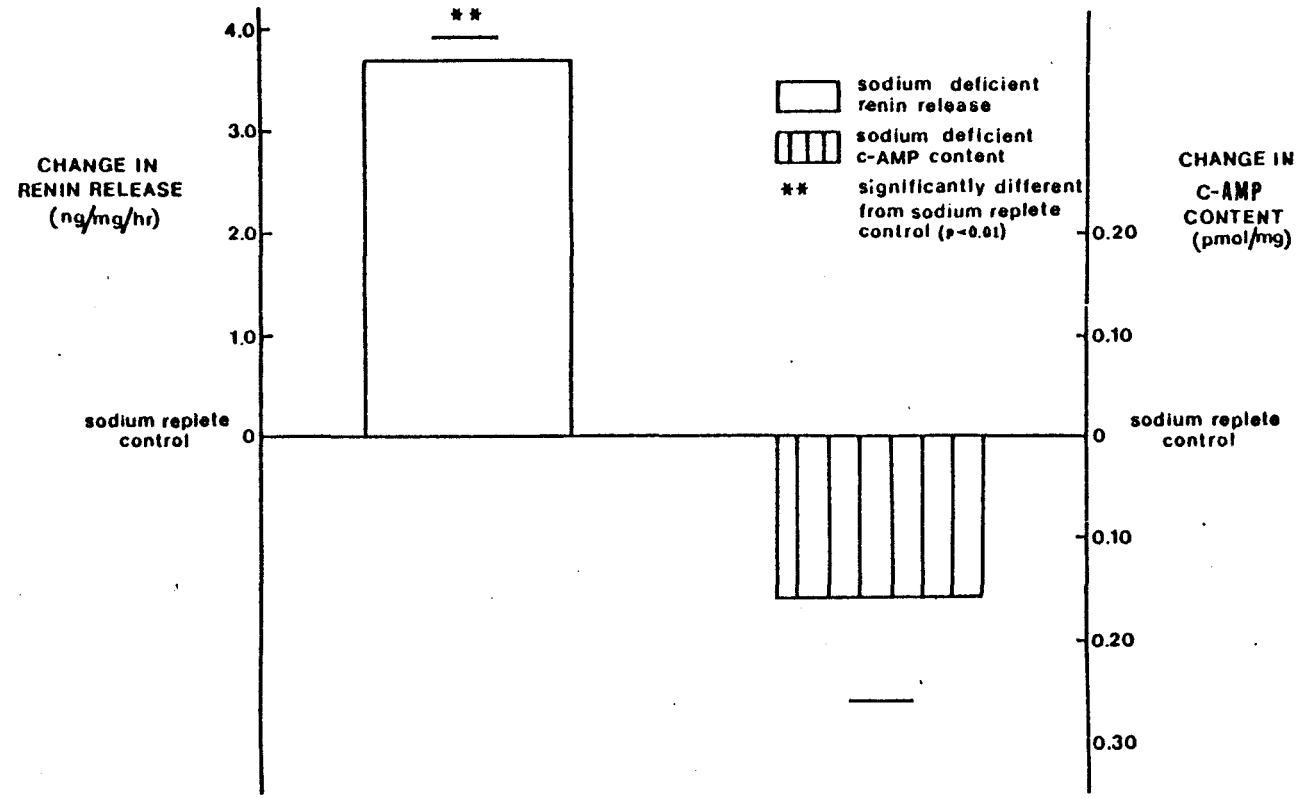

**
4.0
3.0
2.0
1.0
0
sodium replete control
CHANGE IN RENIN RELEASE (ng/mg/hr)
sodium deficient renin release
sodium deficient c-AMP content
** significantly different from sodium replete control (p<0.01)
0.20
0.10
0
0.10
0.20
0.30
sodium replete control
CHANGE IN C-AMP CONTENT (pmol/mg)

FIGURE 1

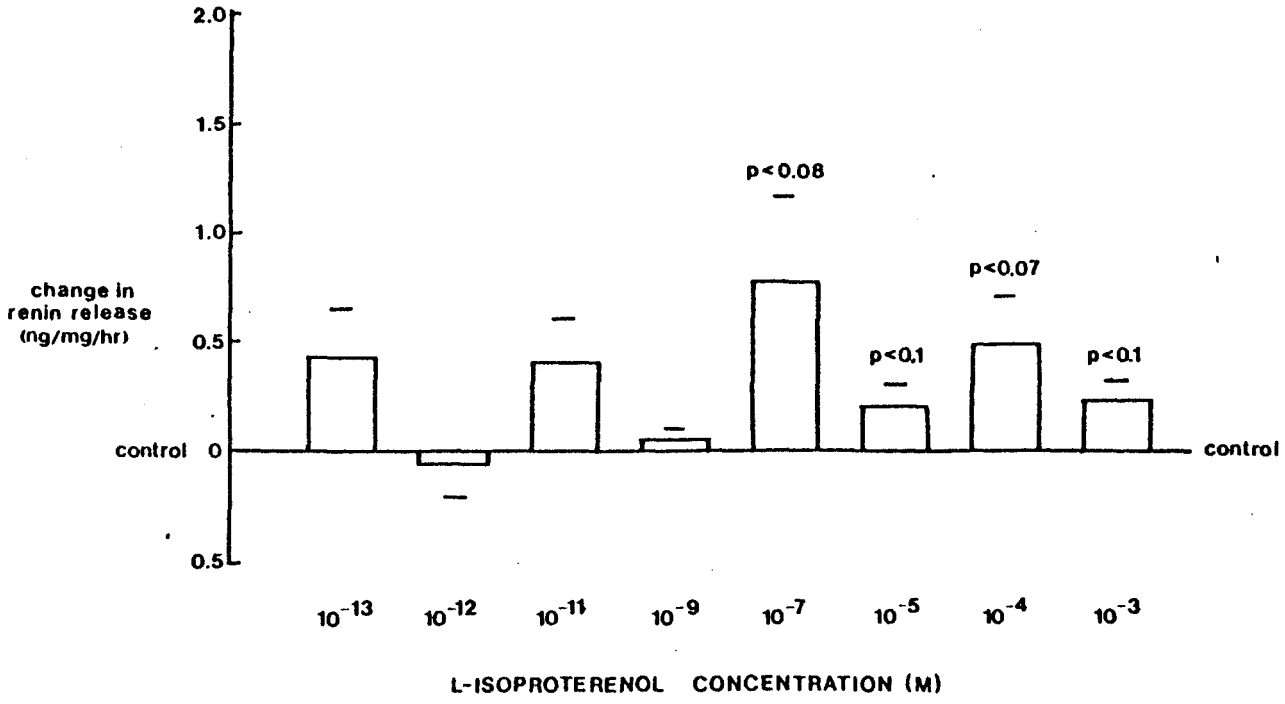

2.0
1.5
1.0
0.5
0
0.5
change in renin release (ng/mg/hr)
control
control
p<0.08
p<0.07
p<0.1
p<0.1
10^{-13}
10^{-12}
10^{-11}
10^{-9}
10^{-7}
10^{-5}
10^{-4}
10^{-3}
L-ISOPROTERENOL CONCENTRATION (M)

FIGURE 2

(10^{-13}M to 10^{-3}M), isoproterenol was ineffective in stimulating renin release from the incubated kidney slice tissue. However, it is also apparent from these data (figure 2) that as the concentration of isoproterenol was progressively increased, the mean renin secretory values of the isoproterenol-treated slices became increasingly greater than the mean values of the non-stimulated control slices. In the same study, changes in c-AMP content of the kidney slices in response to the same concentrations of isoproterenol (figure 3) were also not significantly different from controls, an observation which is consistent with the renin secretory responses shown in figure 2.

Significant changes in the parameters examined were observed in another study, when isoproterenol at various concentrations (10^{-13}M to 10^{-3}M) was added to kidney slices from sodium-deficient rats (figure 4). As shown in this figure, although isoproterenol doses of 10^{-9}M or lower were still ineffective in stimulating renin secretion, higher concentrations of this catecholamine (10^{-7}M, 10^{-5}M, 10^{-4}M, and 10^{-3}M) significantly stimulated renin release, with the highest mean renin release values seen at a concentration of 10^{-5}M). A statistical comparison among the four stimulatory

concentrations of isoproterenol indicated, however, that there were not significant differences between them in regard to stimulation of renin secretion. When their effects on renin release were compared to those exerted by lower isoproterenol concentrations (10^{-12}M to 10^{-9}M) a dose-response relationship was apparent. Thus, maximal stimulation of renin secretion was achieved with an isoproterenol dose as low as 10^{-7}M; higher doses were ineffective in further potentiating the stimulatory effect (figure 4).

When the renin release responses to added isoproterenol (figure 4) were compared with those exerted by the same concentrations of this catecholamine on tissue c-AMP content (figure 5), an apparent positive correlation between these two parameters was observed. These data show that at concentrations of 10^{-7}M, 10^{-5}M, and 10^{-3}M, isoproterenol significantly stimulated both renin release and the c-AMP content of renal slices from sodium deficient rats. At 10^{-4}M concentration, however, isoproterenol exerted a significant stimulatory effect only on renin release but not on c-AMP content. As expected, changes in c-AMP content of the isoproterenol-treated tissue slices were not significantly different from controls at isoproterenol concentrations of

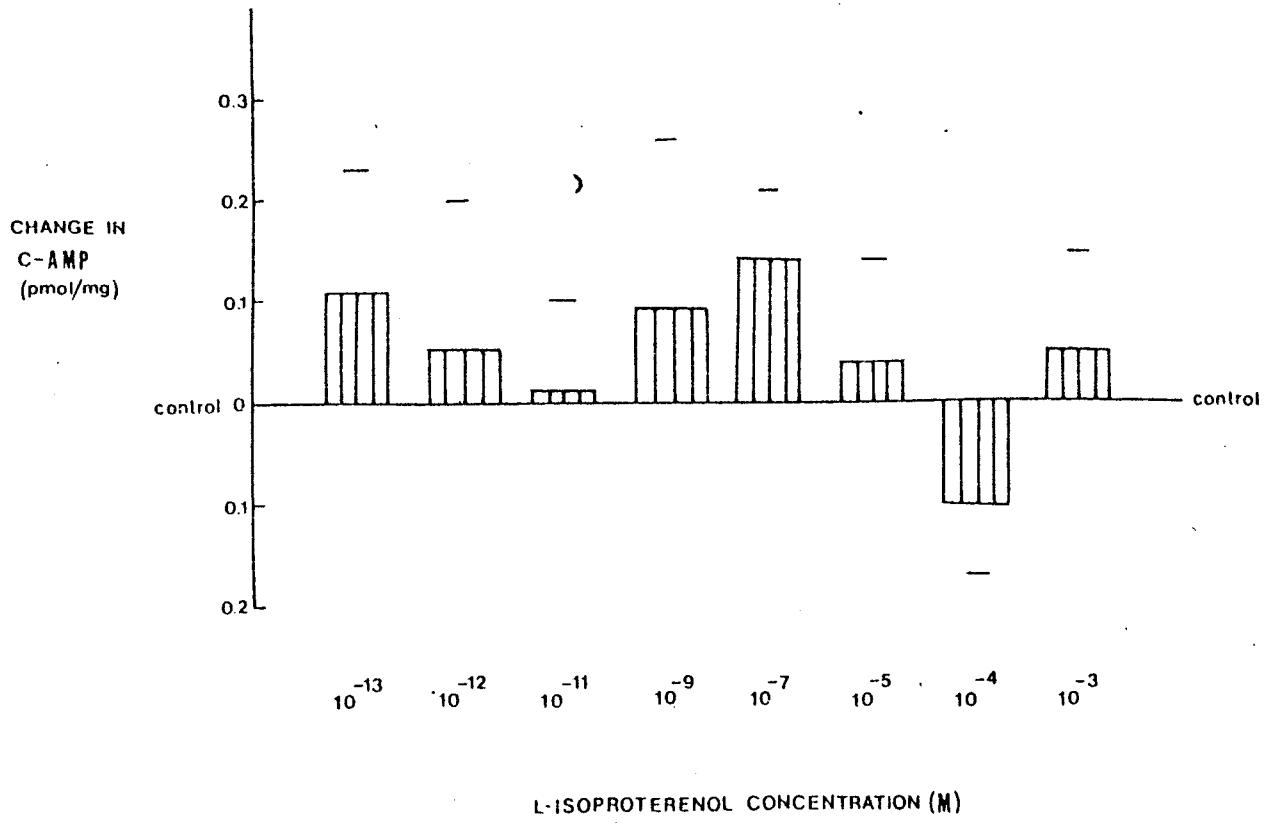

CHANGE IN
C-AMP
(pmol/mg)
0.3
0.2
0.1
control 0
0.1
0.2
control
10^{-13}
10^{-12}
10^{-11}
10^{-9}
10^{-7}
10^{-5}
10^{-4}
10^{-3}
L-ISOPROTERENOL CONCENTRATION (M)

FIGURE 3

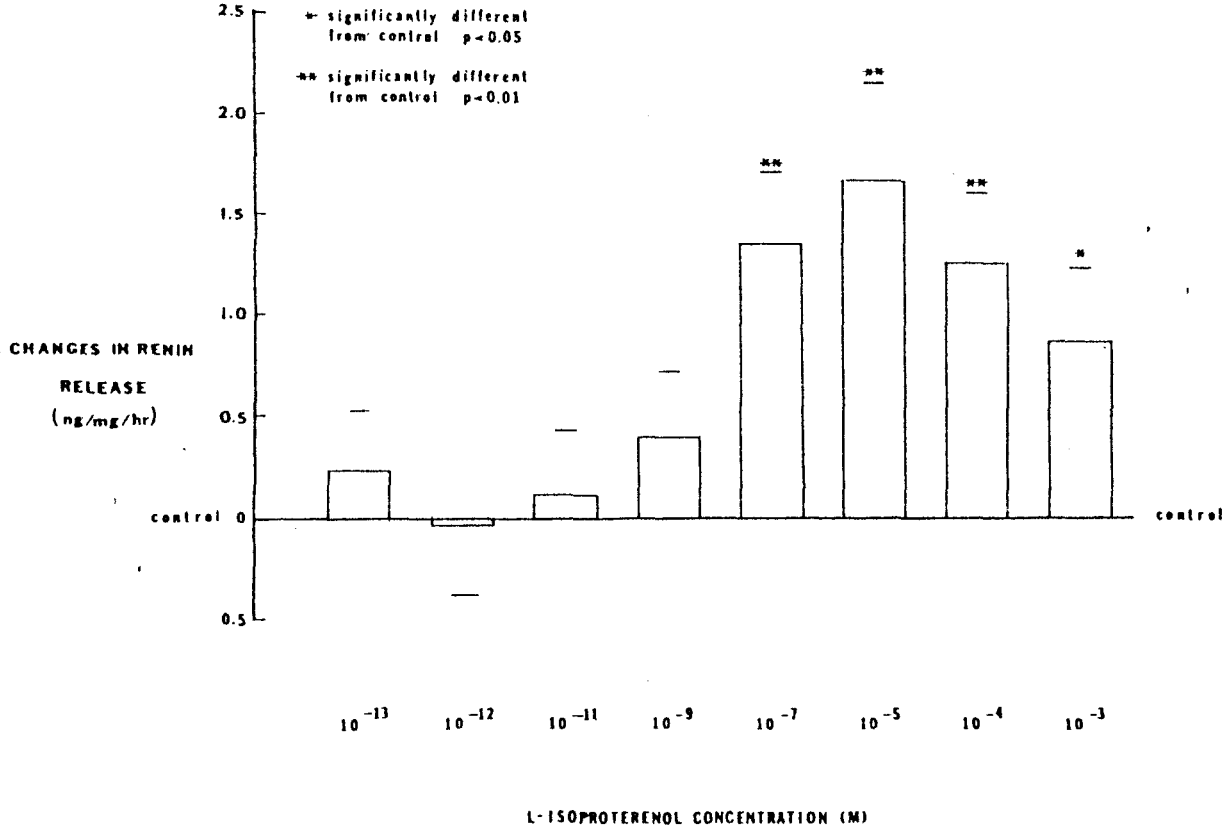
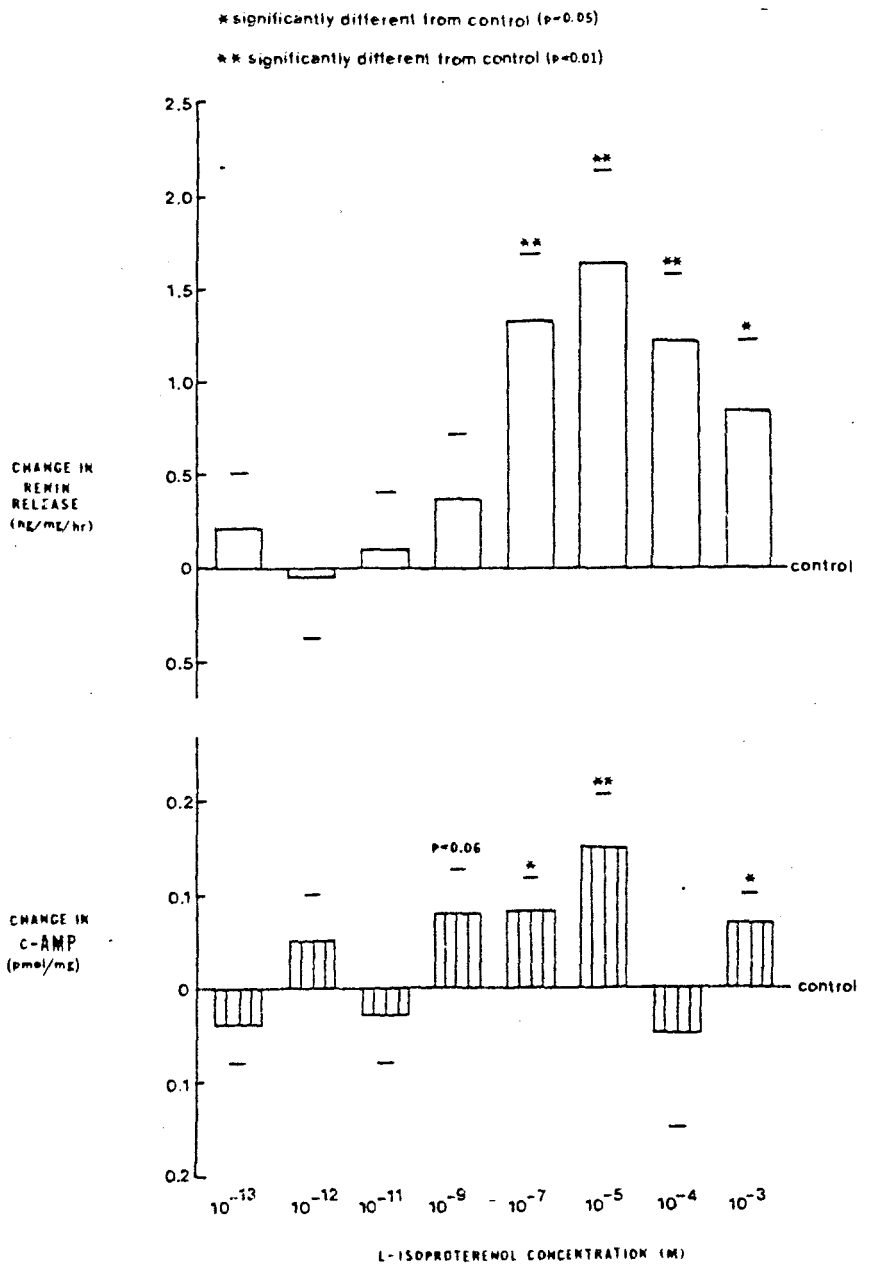

* significantly different from control p<0.05
** significantly different from control p<0.01
2.5
2.0
1.5
1.0
0.5
control 0
0.5
CHANGES IN RENIN RELEASE (ng/mg/hr)
control
10^{-13}
10^{-12}
10^{-11}
10^{-9}
10^{-7}
10^{-5}
10^{-4}
10^{-3}
L-ISOPROTERENOL CONCENTRATION (M)

FIGURE 4

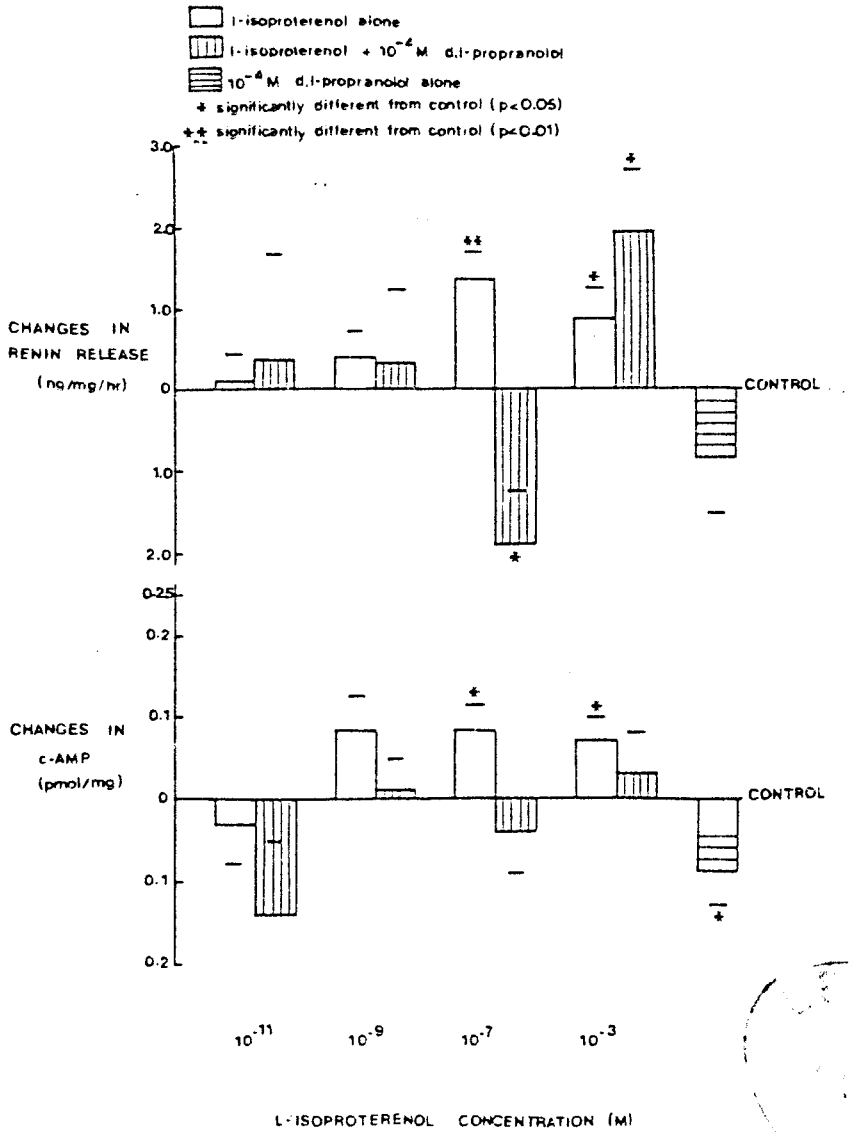
FIGURE 5


* significantly different from control (p<0.05)
** significantly different from control (p<0.01)
CHANGE IN RENIN RELEASE (ng/ml/hr)
2.5
2.0
1.5
1.0
0.5
0
0.5
control
**
**
**
*
CHANGE IN c-AMP (pmol/ml)
0.2
0.1
0
0.1
0.2
P=0.06
*
**
*
control
10^{-13}
10^{-12}
10^{-11}
10^{-9}
10^{-7}
10^{-5}
10^{-4}
10^{-3}
L-ISOPROTERENOL CONCENTRATION (M)

10^{-9}M or lower. This is consistent with the changes seen in renin secretion in response to the same doses of this catecholamine (figure 5).

Data from a subsequent study designed to further evaluate the extent of beta-adrenoreceptor control of renin release are depicted in figure 6. This figure shows the changes observed in renin release and c-AMP content of slices from sodium-deficient rats in response to four isoproterenol doses (10^{-11}M, 10^{-9}M, 10^{-7}M, and 10^{-3}M), added either alone or in conjunction with a 10^{-4}M concentration of the beta-adrenoreceptor blocking agent d,l-isoproterenol. As expected, when added together with two isoproterenol doses (10^{-11}M and 10^{-9}M) which by themselves were ineffective in stimulating either renin release or c-AMP content, propranolol did not affect the response observed with isoproterenol alone. Conversely, when propranolol was added in conjunction with a 10^{-7}M isoproterenol dose, it significantly inhibited the stimulatory effect of this concentration of isoproterenol on renin release and c-AMP content. When added together with a higher isoproterenol dose (10^{-3}M), however, propranolol at the concentration used (10^{-4}M) was unable to inhibit the significant stimulatory effect of this

FIGURE 6


l-isoproterenol alone
l-isoproterenol + 10⁻⁴M d,l-propranolol
10⁻⁴M d,l-propranolol alone
* significantly different from control (p<0.05)
** significantly different from control (p<0.01)
CHANGES IN RENIN RELEASE (ng/mg/hr)
3.0
2.0
1.0
0
1.0
2.0
CONTROL
CHANGES IN c-AMP (pmol/mg)
0.25
0.2
0.1
0
0.1
0.2
CONTROL
10⁻¹¹
10⁻⁹
10⁻⁷
10⁻³
L-ISOPROTERENOL CONCENTRATION (M)

catecholamine on renin secretion, although it did prevent the increase in c-AMP content seen with this dose of isoproterenol added alone. Surprisingly, addition of propranolol by itself to the slice preparation significantly decreased the c-AMP content of the tissue in relation to the non-stimulated control samples, although it did not significantly affect renin secretion as compared to control values.

Table 1 illustrates the results of an additional study designed to evaluate the effect of the alpha-adrenoreceptor blocking agent phentolamine (10^{-6}M), on the renin release and c-AMP content responses to four isoproterenol doses (10^{-11}M, 10^{-9}M, 10^{-7}M, and 10^{-3}M) in slices from sodium-deficient rats. Although when added alone to the slice preparation, phentolamine did not alter renin secretion or c-AMP content levels in relation to those of controls, it did partially prevent the stimulatory effect of 10^{-7}M and 10^{-3}M isoproterenol concentrations on renin release when added in conjunction with the catecholamine. However, the c-AMP content of slices treated with 10^{-7}M isoproterenol plus 10^{-6}M phentolamine remained significantly elevated in relation to controls, but it appeared no different from controls in response to 10^{-3}M isoproterenol

added in conjunction with phentolamine. It should be noted, however, that the mean renin secretion change levels of slices treated with 10^{-7}M and 10^{-3}M isoproterenol in conjunction with phentolamine, were equal to or greater than the statistically significant ones observed in response to the same doses of isoproterenol added alone, but the standard errors were larger in the former. From these data (Table 1), it is also clear that at no time phentolamine potentiated the effects of any of the isoproterenol doses on renin secretion or c-AMP content of the slice tissue, when added together with the catecholamine.

TABLE I: Effect of various doses of isoproterenol (10^{-11}M, 10^{-9}M, 10^{-7}M, 10^{-3}M) in the presence and absence of phentolamine (10^{-6}M) on renin secretion and c-AMP content of kidney slices from sodium-deficient rats.

	phen. 10^{-6}M	10^{-3}M iso.	10^{-3}M iso. 10^{-6}M phen.	10^{-7}M iso.	10^{-7}M iso. 10^{-6}M phen.	10^{-9}M iso.	10^{-9}M iso. 10^{-6}M phen.	10^{-11}M iso.	10^{-11}M iso. 10^{-6}M phen.
Change* ▲ in renin release (ng/mg/hr) from controls	$+.03\pm$** .59	$+.85\pm$ .37	$+2.23\pm$ 1.12	$+1.33\pm$ .36	$\pm.61\pm$ 2.59	$+.38\pm$ .33	$+.87\pm$ 1.34	$+.10\pm$ .32	$+1.86\pm$ 1.44
p=***	.96	.03	.10	.001	.82	.25	.55	.77	.25
n=	24.0	31.0	6.0	31	6.0	31.0	5.0	31.0	6.0
Change* ▲ in c-AMP (pMol/mg) from controls	$+.03\pm$** .05	$+.07\pm$ .03	$+.05\pm$ .05	$+.08\pm$ .04	$+.21\pm$ .06	$+.08\pm$ .04	$-.03\pm$ .08	$-.03\pm$ .05	$-.05\pm$ .07
p=***	.40	.04	.35	.04	.01	.06	.71	.46	.49
n=	24.0	31.0	6.0	31	6.0	31.0	5.0	31.0	6.0

* Difference in relation to control values.

** $\pm$ - Standard error.

*** Number of observations.

CHAPTER V

DISCUSSION

A renal cortical slice preparation of the type described in this study allows one to eliminate the various humoral and hemodynamic factors which are known to influence renin release in the intact animal. Thus, the effects of sympathetic stimuli on renin release may be considered to be exerted directly on the juxtaglomerular cells present in an in vitro kidney slice system (5, 7, 44). Furthermore, animals maintained on sodium-deficient diets have shown an increased basal resting renin release rate (12, 46), as compared to animals fed a sodium-replete diet. In addition, sodium-deficiency has been shown to potentiate the renin release responses to sympathetic stimuli (5), which makes a cortical slice preparation obtained from sodium-deficient rats an ideal tool for a clear evaluation of the effects of adrenergic agents on renin secretion.

The results obtained in an initial study (figure 1) confirm previous data (9, 12) which indicate that the resting

renin release rate in kidney slices of rats maintained on a sodium deficient diet is significantly higher than that seen in sodium-replete animals. These results (figure 1) also show that sodium-deficiency did not alter the c-AMP content levels of the tissue slices, suggesting that the increased resting renin secretory rates observed under low sodium conditions may not be mediated by changes in the intracellular concentration of this nucleotide.

In a subsequent study designed to examine the renin secretory responses to sympathetic stimuli in the sodium-replete state various concentrations (10^{-13}M to 10^{-3}M) of the specific beta-adrenoreceptor agonist l-isoproterenol added to kidney slices from sodium-replete rats were evaluated. These results (figure 2) indicate that although the mean renin release responses to increasing isoproterenol doses (10^{-7}M to 10^{-3}M) were consistently higher than control values, the increases were not of sufficient magnitude to be statistically significant. Similar results were observed in regard to tissue c-AMP changes in slices from sodium-replete rats in responses to the same concentrations of isoproterenol (figure 3).

The inability of the tissue slice preparation obtained from sodium-replete rats to respond to isoproterenol stimulation in this study is not surprising, since renin secretory mechanisms are very sensitive to sodium changes and these animals had ingested a diet providing over 2.2 mEq of sodium per day, which is about 12 times the amount of sodium recommended by the National Research Council for growing rats. Our results are supported by the work of Nash (24, 32) in which infusion of sodium chloride in live dogs resulted in a significant decrease in renin secretion. Thus, it appears that the increased sodium content of the diet fed to the animals used in this study is most likely responsible not only for the low levels of renin secretion observed in renal slices from these animals, but also for the lack of responses of the slice tissue to isoproterenol stimulation.

In another study, the effects of various concentrations (10^{-13}M to 10^{-3}M) of l-isoproterenol on renin release in kidney slices from sodium-deficient rats were evaluated (figure 4). As opposed to the results observed in tissue slices from sodium-replete rats upon addition of isoproterenol, four concentrations of this catecholamine

(10^{-7}M, 10^{-5}M, 10^{-4}M, and 10^{-3}M) significantly stimulated renin release in slices from sodium-deficient rats. At no time was inhibition of renin release by high isoproterenol doses seen in this study, as opposed to that seen with norepinephrine in other studies (7, 9). These data (figure 4) support previous in vivo (11, 12) and in vitro (7, 9) observations, suggesting a potentiation of the renin secretory responses in sympathetic stimuli in sodium-deficient animals. Furthermore, these results show a clear dose-response relationship which rules out leakage of renin from the cells as a contributing factor in the responses observed.

When the renin release responses to the four stimulatory concentrations of isoproterenol are compared to the c-AMP content changes seen in response to the same isoproterenol doses (figure 5), a clear correlation between these two parameters can be observed. These results provide strong support for the concept that stimulation of renin secretion by catecholamines is mediated by a beta-adrenoreceptor mechanism which utilizes intracellularly generated c-AMP as a second messenger of the catecholamine action. In addition, these data (figure 5) are in agreement with previous work (7, 9) which have shown a correlation between renin release

and c-AMP content changes in response to norepinephrine added to kidney slices from sodium-deficient rats.

To further extend these observations, the effect of the specific beta-adrenoreceptor blocking agent d,l-propranolol (10^{-4}M), added either alone or in conjunction with isoproterenol, on renin release and tissue c-AMP content of slices from sodium-deficient rats was examined (figure 6). These results support previous findings (1, 10, 42, 49) indicating that propranolol suppresses the stimulatory effect of catecholamines on renin secretion. This inhibition can clearly be seen in this study at an isoproterenol dose of 10^{-7}M, although a higher concentration of this catecholamine (10^{-3}M) appeared to be sufficiently potent to override the inhibitory effect of propranolol at the concentration used (10^{-4}M). These data (figure 6) also present evidence for the first time clearly linking the inhibitory effect of propranolol on renin secretion with a simultaneous inhibition of tissue c-AMP content, which strongly support the concept that catecholamine stimulation of renin secretion is mediated by a beta-adrenoreceptor complex most likely located on the membranes of the juxtaglomerular cells themselves.

Interestingly, propranolol (10^{-4}M) added alone to the tissue slice preparation (figure 6) significantly inhibited c-AMP content levels in relation to those seen in control (non-treated), slices, and mean renin secretory rates in response to this blocking agent also appeared decreased but not significantly. These observations suggest that endogenous catecholamines still present in the cortical slice tissue, contribute to the regulation of basal renin secretion in the sodium-deficient state.

The results obtained in an additional study involving the use of the specific alpha-adrenoreceptor blocking agent phentolamine are inconclusive (Table 1). Numerous studies (4, 7-9, 15) have shown that alpha-adrenoreceptor blockade potentiates the renin secretory responses to sympathetic stimuli. In our studies phentolamine did not potentiate but rather prevented the stimulatory effect of two isoproterenol doses (10^{-7}M and 10^{-3}M) on renin release. Its effect on tissue c-AMP content, however, was only partial since in the presence of phentolamine (10^{-6}M) plus isoproterenol (10^{-7}M), tissue c-AMP content levels remained significantly elevated. It should be pointed out, however, that although phentolamine appeared to prevent the stimulatory effect of 10^{-7}M

and 10^{-3}M isoproterenol on renin release, the mean renin secretory rates observed in the presence of this blocking agent concomitant with two different doses of isoproterenol were no different from those seen with isoproterenol added alone, but the standard errors were larger in the former. Thus, statistics notwithstanding, one could suggest that stimulation of renin secretion by isoproterenol with simultaneous addition of phentolamine was maintained to a certain degree, but that it certainly was not potentiated. Further studies utilizing additional doses of phentolamine are clearly needed to further evaluate the responses observed in our study at the concentration of phentolamine used.

In summary, the data presented in this thesis supports the view that sodium deficiency, in addition to significantly increasing basal renin release, potentiates the renin secretory responses to sympathetic stimulation in rat renal cortical slices. This increase in basal renin secretory levels as a result of sodium deficiency appears not to involve changes in tissue c-AMP content.

Furthermore, these results offer clear evidence for a beta-adrenoreceptor mechanism mediating the effect of isoproterenol on renin release, and strongly suggests that

this mediation is exerted through changes in intracellularly generated c-AMP.

Additionally, the effect of isoproterenol on renin release and c-AMP content appears to be dose-dependent, and at no time did higher doses of this agent cause inhibition of either parameter as suggested for large concentrations of norepinenephrine in previous studies. This observation is consistent with the view that alpha- receptors do not participate in mediating the effect of large concentrations of isoproterenol on renin secretion, a role which appears to be inhibitory in nature in regard to high norepinephrine doses.

If anything, our data pertaining to phentolamine would rather support the idea that alpha- adrenoreceptors partly mediate the stimulatory effect of isoproterenol on renin secretion instead of an inhibitory effect. Additional studies with this alpha- adrenergic blocker are needed before a more conclusive statement in this regard can be made.

Finally, the data obtained from these series of studies utilizing specific beta-adrenoreceptor agonist and antagonist agents, add strong support to the concept that the sympathetic nervous system directly regulates the secretion

of renin from the juxtaglomerular cells of the mammalian kidney through a receptor complex located on the membranes of these cells which utilizes c-AMP as the intracellular mediator.

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APPENDIX A

APPENDIX A

Renin Calculation

The concentration of renin in the incubated supernatant medium was calculated as follows:

$$\text{ng/mg/hr} = \frac{\text{picograms A-I from std. curve} \times 40 \times 2 \times (40, 20, \text{Or } 10) \times 2.5}{2 \times 1000 \times \text{mg wet tissue}}$$

where:

In numerator:

picograms A-I from std. curve = amount of unknown sample read from std. curve...

40 = 25 microliters of incubated sample diluted in one ml of nephrectomized plasma for generation...

2 = 1:1 dilution of generated sample with water prior to boiling...

40, 20, or 10 = whether 25, 50, or 100 microliters of generated-boiled sample were used for RIA...

2.5 = initial dilution in 2.5 ml of Robinson's buffer medium prior to 1-hr incubation.

where:

In denominator:

2 = hours of incubation at 37°C...

1000 = conversion of picograms to nanograms...

(mg wet tissue)= amount of incubated kidney slice tissue.

APPENDIX B

APPENDIX B

Legends

Figure 1. Changes in renin release and c-AMP content in renal cortical slices from sodium-deficient rats compared to rats maintained on a sodium-replete diet control. Length of high- or low-dietary intake was 2-3 weeks. The sodium-deficient renin data represents the mean change $\pm$ S.E. of 124 observations (number of slice pairs) compared to 20 observations for the sodium-replete data. The corresponding c-AMP data are based on 121 observations (sodium-deficient) compared to 18 observations (sodium-replete). The sodium-replete (control) renin release rate was 1.38 ± 0.32 ng/mg/hr and the c-AMP content was 0.64 ± 0.12 pMol/mg wet tissue, respectively. The incubation time was 60 minutes.

Figure 2. Effect of various concentrations of l-isoproterenol on renin release from renal cortical slices of sodium-replete rats. The data represent the mean renin release change $\pm$ S.E. of 10 observations

(number of slice pairs) for each isoproterenol concentration. The mean control (unstimulated) rate of renin release was 1.38 ± 0.3 ng/mg/hr during a 60 minute incubation period.

Figure 3. Effect of various concentrations of l-isoproterenol on the c-AMP content of renal cortical slices from sodium-replete rats. The data represent the mean c-AMP content change $\pm$ S.E. of 8-9 observations (number of slice pairs) for each isoproterenol concentration. The mean control (unstimulated) c-AMP content was 0.64 ± 0.1 pMol/mg wet tissue for a 60 minute incubation period.

Figure 4. Effect of various l-isoproterenol doses on renin release by renal cortical slices from sodium-deficient rats. The data represents the mean renin release change $\pm$ S.E. of 25-31 observations (number of slice pairs) for each isoproterenol dose. The mean control (unstimulated) rate of renin release was 4.76 ± 0.36 ng/mg/hr during a 60 minute incubation period.

Figure 5. Effect of various concentrations of l-isoproterenol on renin release and c-AMP content of kidney slices from sodium-deficient rats. The renin data

represents the mean renin release change $\pm$ S.E. of 25-31 observations (number of slice pairs) for each isoproterenol dose. The c-AMP data represents the mean content change $\pm$ S.E. of 25-33 observations (number of slice pairs) for each isoproterenol dose. The mean control (unstimulated) rate of renin release was 5.08 $\pm$ 0.23 ng/mg/hr. The mean control c-AMP content was 0.48 $\pm$ 0.1 pMol/mg wet tissue. The incubation time was 60 minutes.

Figure 6. Changes in renin release and c-AMP content in response to various l-isoproterenol concentrations, added either alone or together with 10^{-4}M d,l-isoproterenol to renal cortical slices of rats fed a sodium-deficient diet for 2-3 weeks. The renin data represents the mean renin release change $\pm$ S.E. of 25-31 observations (number of slice pairs) for each isoproterenol dose added alone, and 5-7 observations for each isoproterenol dose added together with d,l-propranolol. The c-AMP data represents the mean content change $\pm$ S.E. of 25-33 observations (number of slice pairs) for each isoproterenol dose added alone, and 6 observations for each isoproterenol dose added

together with d,l-propranolol. The mean control (unstimulated) rate of renin release was 5.08 $\pm$0.23 ng/mg/hr. The mean control c-AMP content was 0.43 $\pm$0.1 pMol/mg wet tissue. The incubation time was 60 minutes.

APPROVAL SHEET

The thesis sumbitted by Louis M. Lissuzzo
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The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

5/12/78
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