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NEUROENDOCRINE REGULATION OF RENIN SECRETION

by

Janice Helene Urban

A Dissertation Submitted to the Faculty of the Graduate School

of Loyola University of Chicago in Partial Fulfillment

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DEDICATION

This dissertation is dedicated to my husband, Keith McCrea, and my parents Dr. and Mrs. Henry and Therese Urban and my sister, Lynda for their constant moral support, encouragement and love.

VITA

The author, Janice Helene Urban, was born on May 15, 1960, in Chicago, Illinois. She completed her secondary education at Mother McAuley High School in Chicago, Illinois, in 1978.

In the Fall of 1978, Ms. Urban attended Saint Mary's College, Notre Dame, Indiana where she received her Bachelor of Science degree in Biology in May, 1982. She was Vice-president of the Biology Club and was a member of the Tri-Beta Biological Society.

In the summer of 1982, she was accepted as a graduate student in the Department of Pharmacology at Loyola University of Chicago Stritch School of Medicine, Maywood, Illinois, where she was awarded a Loyola University Basic Science Fellowship. Ms. Urban also served as Departmental Student Representative to the Faculty from 1985-1986. In the spring of 1986, she was awarded an Arthur J. Schmitt Dissertation Fellowship and a Sigma Xi Grant-in-Aid of Research. The author is a student member of the Society for Neuroscience. Ms. Urban has accepted a post-doctoral position in the laboratory of Dr. Daniel Dorsa in the Department of Pharmacology at the University of Washington, Seattle, Washington.

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CHAPTER I

INTRODUCTION

The renin-angiotensin-aldosterone system is involved in the regulation of blood volume and pressure (Fujii and Vatner, 1985; Joy and Lowe, 1970), and the maintenance of sodium balance (Parfrey et al., 1981; Davis and Freeman, 1976). Renin is the rate limiting enzyme in the formation of the vasopressor substance angiotensin II (ANG II). ANG II is the active component of this system and is involved in several physiological functions in addition to its vasoconstriction effect. The primary source of renin in the blood is the specialized juxtaglomerular cells located in the afferent arterioles of the kidney. The synthesis, storage and secretion of renin occurs in these juxtaglomerular cells (Taugner et al., 1984).

Renin secretion is regulated in part, by a renal baroreceptor that senses changes in renal perfusion pressure (Blaine et al., 1971). The release of renin from the kidney is also modulated by the amount of sodium that is reabsorbed across the specialized macula densa cells in the distal tubule (Fray, 1978; Itoh and Carretero, 1985). In addition, the renal nerves and circulating catecholamines increase renin secretion by stimulating renal beta-receptors. ANG II and vasopressin inhibit renin release (Bunag et al., 1967). Studies by De Vito et al.

(1971) provided evidence for the presence of a renin-releasing factor in the plasma of hypovolemic dogs. However, these results could not be replicated by Polomski et al (1974), and no further studies were reported.

Brain serotonergic neurons stimulate renin secretion. Administration of the serotonin (5-HT) releasers, PCA (p-chloroamphetamine) or fenfluramine, or the serotonin agonists MK-212 and quipazine produced dose-dependent increases in plasma renin activity (PRA; Lorens and Van de Kar, 1987; Van de Kar et al., 1985b; 1981). The effect of fenfluramine could be prevented by pretreatment with the 5-HT uptake inhibitors indalpine and fluoxetine or the 5-HT synthesis inhibitor PCPA (p-chlorophenylalanine; Van de Kar et al., 1985b). Injections of the 5-HT neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) into the dorsal raphe nucleus prevented the effect of PCA on renin secretion suggesting that brain 5-HT neurons in the dorsal raphe nucleus mediate the effect of PCA (Van de Kar et al., 1982). The dorsal raphe nucleus sends projections to the arcuate, anterolateral and the suprachiasmatic nuclei of the hypothalamus (Van de Kar and Lorens, 1979; Azmitia and Segal, 1978). Posterolateral deafferentation or mechanical ablation of the hypothalamus prevented the PCA-induced increase in PRA (Karteszi et al., 1982), suggesting that the dorsal raphe nucleus may send projections to the hypothalamus that are involved in the regulation of renin secretion.

The role of the sympathetic nervous system in mediating the PCA-induced increase in PRA has also been investigated. The serotonergic stimulation of renin secretion was not mediated by either the

sympathetic nervous system or adrenal catecholamines (Van de Kar and Richardson-Morton, 1986). Transection of the spinal cord proximal to the exit of the renal nerves did not prevent the effect of PCA on renin secretion. These studies suggest that the sympathetic nervous system is not involved in mediating the serotonergic stimulation of renin secretion.

Taken together, these results indicate that a serotonergic pathway originating in the dorsal raphe nucleus with nerve terminals in the hypothalamus regulate renin secretion. The exact location of the terminals has not been identified. It is possible that the paraventricular nucleus (PVN) may play a role in mediating the 5-HT-induced increase in renin secretion since studies by Gotoh et al. (1987) have demonstrated that electrolytic lesions of the PVN prevent the PCA-induced increase in PRA. Studies by Richardson Morton et al. (1986) and Gotoh et al. (1987) have also indicated that the PVN is involved in the stress-induced increase in PRA. Since the sympathetic nervous system was not shown to play a role in the PCA-induced increase in PRA, there should be another way for the brain to communicate with the kidneys. One possibility is that the hypothalamus releases a hormonal factor into the blood that circulates to the kidneys and increase renin secretion.

In order to test this hypothesis, a group of donor rats were treated with PCA or saline. Their plasma was collected and injected in a group of recipient rats. The PRA values in the recipient rats were increased after injection of the PCA-plasma, but injection of plasma from rats that were treated with saline did not alter PRA (Van de Kar

et al., 1982a). These data suggested that PCA may stimulate the release of a factor (renin-releasing factor; RRF) into the circulation that produces an increase in renin secretion in conscious rats. Pretreatment of the rats with the 5-HT synthesis inhibitor p-chlorophenylalanine (PCPA) did not alter the response of PRA to administration of the PCA-plasma. Depleting 5-HT stores with PCPA prevented any release of 5-HT by residual PCA molecules present in the plasma fractions.

The present studies were designed to investigate RRF in vitro using a kidney slice renin release bioassay. Studies were designed to determine the molecular weight of RRF and to characterize the nature of this substance is a peptide. Plasma from rats that received either injections of serotonin agonists or rats that were stressed were tested to determine if these stimuli could increase the plasma concentration of RRF. Other experiments were developed to study the distribution of RRF in the rat brain and attempt to identify where RRF cell bodies are located. In addition, hypothalamic explants were superfused in vitro with a high potassium solution to test if depolarization of the hypothalamus could stimulate the release of RRF.

Many neuroendocrine systems have a feedback system that regulates the synthesis and release of a hormone from the hypothalamus or pituitary gland. Once the factor reaches the target organ, the target increases the secretion of its hormone which acts as a message to the brain to halt the production of the hormone. To test for the possibility of a feedback loop in the regulation of the release of the RRF from the hypothalamus, the concentration of RRF in the hypothalami

of nephrectomized rats was compared with the RRF concentration in sham-operated rats. The results of this experiment suggest that there is a negative feedback loop from the kidney to the hypothalamus that regulates the release of RRF.

CHAPTER II

LITERATURE REVIEW

A. Overview

1. Renin-angiotensin enzyme cascade

The regulation of blood pressure in the body relies to a large extent on the integrity of the renin-angiotensin-aldosterone system (RAAS). Renin is the rate limiting enzyme in the formation of ANG II. The enzyme is synthesized, stored and released from the granular juxtaglomerular cells located in the afferent arterioles of the kidney (Taugner et al., 1986; Cantin et al., 1977).

The juxtaglomerular cells, afferent and efferent arterioles and distal tubule are referred to collectively as the juxtaglomerular apparatus (JGA). The JG cells are in close proximity to the macula densa, a specialized portion of the distal tubule that is sensitive to changes in sodium reabsorption across the tubular epithelium.

When released from the kidney, renin circulates in the blood and cleaves the leucine-leucine bond (a leucine-valine bond in humans) in the renin substrate, angiotensinogen, to liberate the decapeptide, angiotensin I (ANG I). Angiotensin converting enzyme (ACE) cleaves two amino acids from ANG I to form the octapeptide angiotensin II. Degradation of ANG II by the action of aminopeptidase A leads to the

formation of angiotensin III, while the action of other angiotensinases leads to inactive peptide fragments.

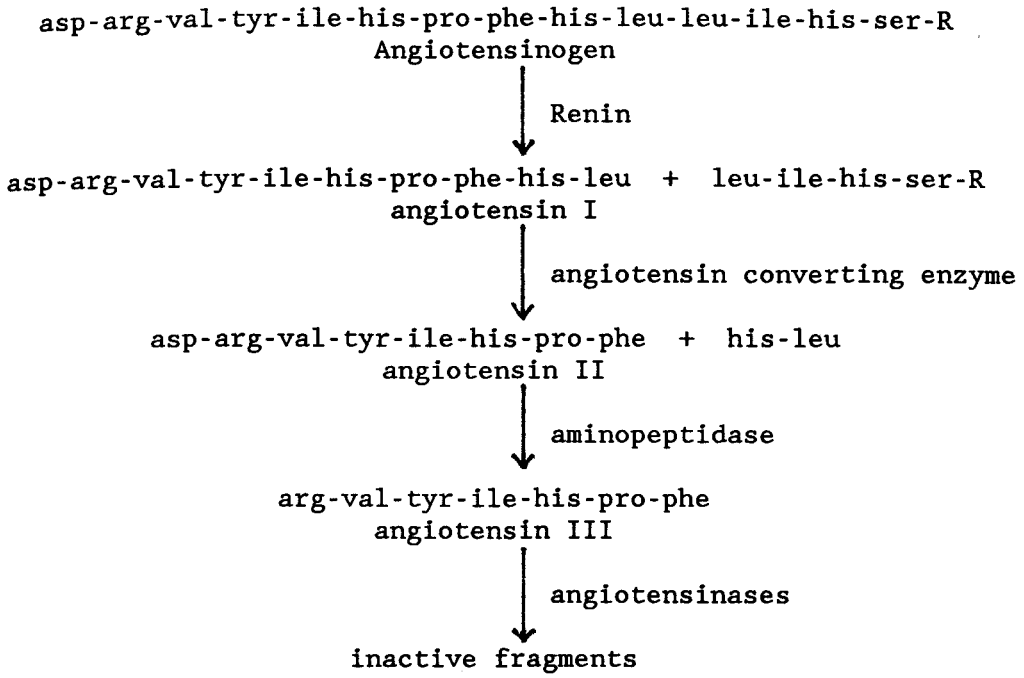


Figure 1. Diagrammatic representation of the renin-angiotensin system enzyme cascade.

2. Role of the angiotensins

The angiotensins (ANG I, ANG II and ANG III) exert a number of pharmacological actions, all of which are involved in maintaining blood pressure and plasma volume. ANG II is the most active peptide of the renin-angiotensin enzyme cascade and produces profound increases in blood pressure (Fujii and Vatner, 1985; Fagard et al., 1985; Ross and White, 1966). ANG II is a potent vasoconstrictor and contracts vascular smooth muscle directly by stimulating vascular receptors (Fujii and Vatner, 1985; Coruzzi et al., 1983). In addition to

increasing systemic blood pressure, ANG II acts directly on the heart to increase myocardial contractility (Koch-Weser, 1965). ANG I enhances the release of catecholamines from the adrenal medulla and sympathetic nerve terminals (Peach et al., 1971; Ross and White, 1966). In addition, ANG II also prevents the reuptake of norepinephrine by the sympathetic nerves (Khairallah, 1972), thus prolonging the effect of norepinephrine at the synapse. ANG I is equally effective as ANG II in stimulating the release of adrenal catecholamines but is less potent in producing vasoconstriction (Peach et al., 1971).

Another principal mechanism of action for ANG II is the stimulation of aldosterone synthesis and secretion from the zona glomerulosa of the adrenal cortex (Aguilera et al., 1980; Davis and Freeman, 1976). Aldosterone produces an increase in sodium and water reabsorption from the distal tubule (Biron et al., 1961) thus increasing extracellular fluid and volume. The heptapeptide, ANG III has been reported to be equally effective in inducing steroid synthesis in the adrenal cortex (Braley et al., 1983; Blair-West et al., 1980).

Circulating levels of ANG II are able to influence the release of pituitary hormones. Systemic ANG II facilitates the neuronal firing of neurohypophysial neurons of the paraventricular and supraoptic nuclei (Ferguson and Renaud, 1986). It has been demonstrated that the release of vasopressin and ACTH (adrenocorticotropin hormone) are stimulated by increased plasma levels of ANG II (Keller-Wood et al., 1986; Spinedi and Negro-Vilar, 1983; Ramsay et al., 1978). LH (luteinizing hormone) secretion is also stimulated by ANG II. However, this effect does not appear to be mediated by circulating intravenous ANG II but rather by

intracerebral injections of ANG II (Steele et al., 1983).

The subfornical organ (SFO), median eminence and area postrema are circumventricular organs of the brain. These brain regions are not located within the blood-brain barrier and therefore may be responsive to circulating factors. Immunoreactive staining for ANG II has been demonstrated in the SFO and area postrema (Gehlert et al., 1986; Lind et al., 1985). Destruction of the subfornical organ (SFO) prevents the increased neuronal firing of neurohypophysial neurons (Ferguson and Renaud, 1986). This finding suggests that ANG II may influence the firing rate and possibly the release of substances from neurohypophysial neurons by influencing SFO neuronal firing. The pressor response to ANG II and the ANG II-induced drinking response were also attenuated by lesions in the SFO (Lind et al., 1983). Joy and Lowe (1970) and Fink et al., (1987) demonstrated that ablation of the area postrema prevents the ANG II-induced pressor response. Therefore it appears that ANG II, by stimulating receptors in the circumventricular organs, can further influence the regulation of plasma volume through central nervous system pathways.

B. Historical Overview

Studies in the late 1800's, Tigersteadt and Bergmann (as reviewed by Brod, 1986) demonstrated that kidney extracts, when injected into bilaterally nephrectomized rabbits, produced a prolonged increase in blood pressure. This pressor material was found primarily in the renal cortex of the kidney whereas other tissue extracts did not produce an increase in blood pressure. This substance was called renin. Later,

in 1934, Goldblatt et al produced an increase in blood pressure by placing a clamp around, and occluding the renal arteries. The degree of hypertension appeared to be dependent on the tightness of the clamp around the renal artery. They concluded that the kidney may play a role in the development of hypertension. This increase in blood pressure was independent of the sympathetic nervous system or the adrenal medulla (Goldblatt et al., 1934). Tying off the renal veins prevented the hypertension from developing (Goldblatt et al., 1937). This indicated that the kidney releases a substance that is responsible for the development of hypertension.

The attention then turned to renin as a causative substance for hypertension. When renin was purified, it increased blood pressure in intact animals but had no vasopressor activity when it was infused into the isolated dog tail (Friedman et al., 1938). However, when blood was used to perfuse the dog tail preparation, the vasoconstrictor effect of the kidney extracts returned. This finding led to the theory that renin was not the vasoactive substance but it was the interaction of renin and renin-activator that led to the formation of the vasoactive product. The substrate (renin-activator) is present in the alpha-2 globulin fraction of plasma.

Two independent groups, Page and Helmer (1940) in the United States and Braun-Menendez (1940) in Argentina, studied the pressor effect of renin. They found that plasma (renin-activator) and renin when incubated together formed a heat stable and potent vasoconstrictor substance that produced an immediate and dose-dependent increase in blood pressure. Renin, when administered, had a latent period before

the vasopressor effect could be observed. It became evident that renin was the enzymatic catalyst in the formation of this peptide. Page and Helmer named this peptide angiotonin and Braun-Menendez referred to it as hypertensin. It was not until 1958 that the nomenclature was standardized when the two groups compromised and called the substance angiotensin. The renin-activator became known as angiotensinogen (renin-substrate; proangiotensin).

Studies were begun to isolate and purify angiotensin. However, upon purification it was discovered that dialyzing the solution containing angiotensin with sodium chloride instead of water, produced a pressor material in a different molecular weight range. This led to the discovery that there were two forms of angiotensin. The second peptide could be formed from angiotensin I by plasma supplemented with sodium chloride. However, if renin was incubated with plasma in the absence of chloride only angiotensin I was formed. Using isolated perfused kidneys, Skeggs et al. (1954) showed that ANG II was a more potent vasoconstrictor that ANG I. Therefore rapid conversion of ANG I to ANG II results in the pressor activity. These results suggested that an additional enzyme was required for the conversion of ANG I to ANG II. This newly found enzyme was named angiotensin converting enzyme (ACE). ACE circulated in the plasma and required the presence of chloride ions for its activity (Skeggs et al., 1956). A later study by Ng and Vane (1967) showed that as blood circulated through the lungs there was a stronger vasoconstriction produced than blood that circulated through other vascular beds. They therefore concluded that the highest concentration of this enzyme occurred in the lung.

The association of renin with the glomeruli was identified by Cook and Pickering (1959). They succeeded in separating the glomeruli from the rest of the kidney tissue by first injecting iron oxide into the glomerulus. The cortex was dissected and put through a sieve. A magnet was then used to separate the glomerular fragments from the tubules. Bioassay indicated that the vascular pole of the glomerulus contained more renin activity than the tubules. Similar results were reported by Bing and Wiberg (1958) who showed that destruction of the renal cortex reduces the renin content of the kidney. These data support earlier findings of Goormaghtigh (1939) who demonstrated that renin was localized in the arterioles of the juxtaglomerular apparatus. These were some of the first studies that reported the localization of renin at the glomerular area of the renal cortex.

These studies have indicated that there are two enzymatic steps needed to form ANG II from angiotensinogen. Since renin and angiotensin have been implicated in the pathogenesis of hypertension, many studies have focussed on the inhibition of this enzyme cascade as a possible treatment of hypertension. The development of substrate analogues (Plattner et al., 1986), converting enzyme inhibitors (Mento and Wilkes, 1987), renin antibodies (Dzau et al., 1980) and ANG II antagonists (Wilkes, 1984) are pharmacological approaches to the possible treatment of hypertension.

C. Angiotensinogen

Angiotensinogen is the substrate for renin and thus the precursor for ANG I, and ultimately ANG II. There is renin-substrate specificity

that allows the interaction of renin only with homologous substrate. For example, human renin will only react with human angiotensinogen; non-primate angiotensinogen will preferentially react with homologous substrate. It has been proposed that the specificity of the enzyme-substrate interaction may be due to the variation in the amino acid sequence between the human and non-primate forms (Tewksbury et al., 1981; Bouhnik et al., 1981).

Angiotensinogen is a glycoprotein that is primarily localized in the liver although other organs have been found to contain angiotensinogen. Recent studies using immunocytochemistry (Richoux et al., 1983) and Northern Blot analysis with complementary mRNA sequences for angiotensinogen (Ingelfinger et al., 1986) identified that the liver contains and synthesizes angiotensinogen. Clauser et al., (1983) demonstrated that angiotensinogen is released from liver slices and its productions can be prevented by treatment with cycloheximide, a protein synthesis inhibitor. Incubation of liver slices in the absence of oxygen also prevents the secretion of angiotensinogen indicating that the release is an active process.

Angiotensinogen circulates in the plasma in two forms which are referred to as Ao1 and Ao2 (Hilgenfeldt and Schott, 1987a; 1987b; Tewksbury, 1983). The dissimilarity in molecular weight is attributable to the difference in the number of carbohydrate residues present on Ao1 and Ao2. Hilgenfelt and Schott (1987) demonstrated that the two forms of angiotensinogen do not differ in their affinity for endogenous renin.

There are a variety of factors that can modulate the plasma

concentration of angiotensinogen. The levels of renin substrate can be decreased by diseases that affect liver function, such as cirrhosis (Schroeder et al., 1970; Ayers, 1967). Adrenalectomy (Carretero and Gross, 1967; Nasjletti and Masson, 1969; Reid, 1977) and hypophysectomy (Goodwin et al., 1970) also reduce the circulating levels of angiotensinogen. The decrease in angiotensinogen levels produced by adrenalectomy can be prevented by administration glucocorticoids (Reid, 1977; Nasjletti and Masson, 1969). Nasjletti and Masson, (1969) found that mineralocorticoids and sodium replacement also inhibited the effect of adrenalectomy. From these reports it appears that the pituitary adrenal-axis is necessary for maintaining plasma substrate levels. In addition to the pituitary-adrenal axis, the pituitary-thyroid axis may play a role in regulating the synthesis of angiotensinogen. Rats that have been thyroidectomized have a decreased plasma angiotensinogen concentration; this decrease is reversed by thyroid hormone replacement therapy (Bouhnik et al., 1981; Dzau and Herrmann, 1982). Rats that were made hyperthyroid by administration of l-thyroxine also had elevated levels of renin substrate (Dzau and Herrmann, 1982). Increased levels of angiotensinogen are also achieved by administration of ethinyl estradiol (Clauser et al., 1983; Krakoff and Eisenfeld, 1977). However, this treatment does not increase angiotensinogen levels in hypophysectomized animals. Dexamethasone, a glucocorticoid, also increases the production of angiotensinogen by the liver.

The release of angiotensinogen may be subject to a feedback control by the renin-angiotensin system. The final product of the

pathway, ANG II has been shown to stimulate the release of angiotensinogen (Herrmann et al., 1980; Reid, 1977). Captopril, a converting enzyme inhibitor, reduces circulating levels of ANG II, and also produces a decrease in the circulating levels of angiotensinogen (Radziwill et al., 1986; Hermann and Dzau, 1983).

D. Renin

1. Renin synthesis

Renin is a proteolytic enzyme with a molecular weight of approximately 36,000 to 40,000. Renin is primarily localized in and secreted from the granulated cells of the afferent arterioles (Lacasse et al., 1985; Taugner et al., 1982a; Taugner et al., 1979). Renin-like immunoreactivity has also been demonstrated in the cells of the efferent arteriole (Taugner et al., 1981) and along extended lengths of the afferent arteriole and interlobular artery (Taugner et al., 1979; Taugner et al., 1981). Renin-like immunoreactivity has also been described in tissues other than the kidney. Extrarenal sources of renin include blood vessels (Re et al., 1982), pituitary (Deschepper et al., 1986; Naruse et al., 1985), adrenal gland (Baba et al., 1982), testes (Deschepper et al., 1986) and heart (Dzau and Re, 1987). These studies were performed by using either immunocytochemical techniques or in situ hybridization using renin messenger RNA. These extrarenal renin sources are not identified in all species, only kidney renin is universally present in all species. Perhaps the richest source of renin is the submaxillary gland of the mouse (Menzie et al., 1978; Cohen et al., 1972). Purification and isolation studies for renin have

been performed using submaxillary renin. It is important to keep in mind that renal and submaxillary renin are not identical in structure; renal renin is glycosylated whereas submaxillary gland renin does not contain carbohydrate moieties (Kawamura et al., 1986). Studies have indicated that renal renin may be the major source of the circulating enzyme. Van de Kar and Richardson Morton (1986) have reported that after bilateral nephrectomy the plasma levels of renin are below the sensitivity limit of the radioimmunoassay (less than 10 pg) suggesting that renal renin is the major source of renin in the plasma.

Renin is synthesized from messenger RNA as a preprozymogen that is processed at the endoplasmic reticulum and converted to prorenin. This proenzyme (prorenin) is further processed in the rough endoplasmic reticulum and is packaged in a crystalline form in the golgi apparatus which then forms protogranules. The protogranules mature into dense secretory granules (Taugner et al., 1987; Lacasse et al., 1985) which secrete renin from the epithelial cells. It has long been believed that only the dense granules contain renin. Recently, the presence of immunoreactive renin vacuoles in the cytoplasm of these granule cells may suggest an alternative pathway for the packaging and release of renin (Lacasse et al., 1985; Taugner et al., 1984).

There has been some debate as to whether the dense granules contain and release active or inactive (prorenin) renin. The presence of prorenin has been identified in both plasma and kidney (Sealey et al., 1983; Morris and Johnston, 1976) and has been immunologically identified as inactive renin (Bouhnik et al., 1985). Kawamura et al. (1986) showed that isoelectric focusing of stored renin resulted in

several peaks of differing isoelectric points with the majority of renin activity present in one peak. Renin that was secreted from isolated glomeruli also exhibited different isoelectric points. However, there was a redistribution of renin activity between the different isoelectric points, and there were two peaks that contained a considerable amount of renin activity. Kawamura et al. (1986) therefore concluded that there may be some processing of renin occurring in the cytoplasm. Cathepsin B is an intracellular enzyme that has been cited to convert inactive to active renin (Takahashi et al., 1982). In other studies, Taugner et al. (1983) have found that mature secretory granule cells exhibit both renin-like and cathepsin B-like immunoreactivity. It is suggested that cathepsin B is involved in the activation of prorenin to active renin which probably occurs in the dense granules.

Studies using antisera raised against different determinants of the renin prosegment have indicated that prorenin is found predominantly in the protogranules of the epithelioid cells while there is very little staining in the mature granules (Taugner et al., 1987). Therefore it was concluded that this prosegment was cleaved off in the golgi complex. In contrast, labelling for immunoreactive renin increases from the protogranules to the mature granules (Taugner et al., 1987; Lacasse et al., 1985). It is therefore suggested that activation of renin from prorenin occurs in the protogranules. Altogether, these results indicate that there may be stores of inactive renin that are further processed to active renin and released into the circulation.

2. Renin release

Renin is released from the kidney primarily by exocytotic mechanisms. The granules have been observed to fuse with the plasma membrane of the juxtaglomerular cell and release their contents into the extracellular space (Taugner et al., 1986; Taugner, Buhrle and Nobiling, 1984). Other groups have reported invaginations of the plasma membrane into the interior of the epithelioid cell that come in close contact with the dense granules (Ryan et al., 1982; Peter, 1976). These invaginations of the plasma membrane contain material that appears to be similar to that present in the dense granules which suggests that these granules empty their contents into these invaginations.

It has been hypothesized that renin is released from the JG cells into the lymphatics and not directly into the blood. Lever and Peart (1962) first demonstrated renin activity in renal lymph. The highest renin activity was present in the renal venous plasma which correlated with increased levels in the lymph (Horky et al., 1971). Since injection of renin into the plasma produced an increase in blood pressure without altering the renin concentration in the lymphatics, Horky et al. (1971) hypothesized that renin was first secreted into the lymph and then diffused into the circulation. The relationship between renin in the plasma and renal lymph was illustrated by O'Morchoe et al. (1981). In response to injection of furosemide, renin concentration rose in the renal artery, renal vein and lymph. The increase in renin concentration was more immediate and marked in the renal lymph whereas a few minutes were required to reach peak renin levels in the plasma.

Therefore, renin may be released into the lymph where it then diffuses into the blood.

3. Metabolism of renin

Renin circulates in the plasma with a half-life of approximately 7.0 minutes (Kim et al., 1987). Other half-life values for renin have been reported to be between 3.0 and 15.0 minutes (Fiselier et al., 1984; Assaykeen et al., 1968). The clearance of renin from plasma follows a two component system with a rapid component ($t_{1/2}$ = 7.0 minutes) and a slow component with a $t_{1/2}$ of about 65 minutes (Kim et al., 1987).

Plasma renin values are dependent on the relative rates of renin release and the clearance from the plasma. A major portion of renin is inactivated by the liver (Heacox et al., 1967), however the kidney is also involved in eliminating renin from the plasma (Kim et al., 1987; Peters-Haefeli et al., 1971). Partial hepatectomy produced a decrease in the clearance of renin and increased the half-life of the slow phase (from 65 minutes to 94 minutes) while nephrectomy prolonged the half-life of the rapid phase (6 minutes to 10.5 minutes). The increased half-life suggests that both the kidney and the liver are involved in the catabolism of renin. Intravenous infusion of radiolabelled renin accumulated preferentially in the liver and kidney. Fifteen minutes after injection of renin, radioactive degradation products, with molecular weights lower than that for renin, were present in both the liver and kidney (Kim et al., 1987). Although the liver accumulated about 60% of the radiolabelled renin, the kidney accumulated only 11%.

These studies suggest that both the liver and kidney are responsible for the clearance of circulating renin with the liver being the more predominant.

A pure antibody against renin is not widely available for use in a direct radioimmunoassay (RIA) for renin. Renin is measured in the plasma primarily by RIA of generated ANG I. To prevent conversion of ANG I to ANG II, ACE inhibitors are added to the samples. Plasma renin activity (PRA) is the measure of the ability of plasma to generate ANG I, which depends on the amount of renin and renin substrate present in the plasma sample. Unless there is saturation of the enzyme with the substrate, variations in substrate levels may influence the amount of ANG I produced. Plasma renin concentration (PRC) is a measure of the ability of renin to generate ANG I in the presence of a saturating concentration of renin substrate. The normal plasma concentration of angiotensinogen is less than the Km required for renin to generate ANG I at maximum velocity. In order to measure renin concentration, the samples are saturated with renin substrate so that the reaction proceeds at V_{max}. Therefore any variation in substrate levels will not influence PRC values. PRC is a better indication of the amount of renin circulating in the plasma.

E. Regulation of renin secretion

1. Role of plasma electrolytes

1a. Macula densa

The macula densa region of the distal tubule is composed of columnar cells that are in contact with the afferent arteriole. The

basement membrane of the macula densa cells appears to fuse with the membranes of the afferent arteriole that surround the granular cells (Barajas and Powers, 1984). In addition, the macula densa cells have short extensions of cytoplasm that extend into the mesangial cell area of the JGA and the granule cells of the arterioles (Sottiurai and Malvin, 1982). These anatomical findings suggest that there may be a functional relationship between the cells of the macula densa and the renin containing cells of the afferent arteriole. It has been hypothesized that the macula densa is the sensor for changes in ionic concentrations of the tubular fluid that signals the granular cells to alter renin release.

1b. Sodium

Sodium has been shown to play a role in hypertension and the regulation of blood pressure (Parfrey et al., 1981; Dawson and Oparil, 1987; McCaa, 1982; Takata et al., 1986). In addition, renin secretion appears to be inversely regulated by distal tubular sodium concentration. In the study by Parfrey et al. (1981) it was observed that as patients were changed from a high to a low sodium diet their PRA values increased. In hypertensive patients, reduction of sodium led to lowered blood pressure. However, reduction in sodium levels did not alter blood pressure in normotensive patients. It appears that in hypertensive patients there is a greater sensitivity to changes in sodium. Rats that are supplemented with NaCl in their drinking water also have decreased PRA values and increased blood pressure (Pyykonen et al., 1986). It is conceivable that increased sodium may decrease

renin by stimulating the renal baroreceptor as a result of the increased blood pressure and extracellular fluid (ECF) volume. To test this hypothesis, Anderson et al. (1975) infused dextran solutions of different osmolarities or a Ringer's salt solution into sodium depleted dogs. PRA was decreased only in the group that received the Ringer's salt solution which restored the sodium levels. Replacement of extracellular fluid with the dextran solutions did not reduce PRA values. The plasma volume and mean arterial pressure were not significantly different between the dextran-infused and saline-infused groups. This suggests that renin secretion may be inversely proportional to plasma sodium concentration. A similar study was performed in salt-depleted humans (Tuck et al., 1975). When the subjects received saline infusions, PRA decreased, but there were no alterations in PRA after dextran infusion. The increase in ECF volume was similar in both the saline-infused and dextran-infused groups. If the renal baroreceptor contributed to the suppression of renin release, then all of the groups would be expected to have a similar decrease in PRA after restoration of plasma volume. These data support the proposed mechanism of sodium-induced suppression of renin release independent of changes in plasma volume.

One of the first studies that actually measured the amount of sodium at the macula densa area was performed by Churchill et al., (1978). The changes in sodium concentration in the distal tubule were correlated with alterations in renin secretion. Micropuncture sampling of the distal tubular fluid showed that the tubular sodium load was proportional to dietary sodium. In sodium-deprived rats, renin

secretion was increased when compared with the control group maintained on a standard diet. Renin secretion was decreased in the animals that were fed a high sodium diet. This study suggested that there was an inverse correlation between plasma renin activity and the sodium load in the distal tubule.

1c. High ceiling diuretics, sodium and renin secretion

The effect of sodium on renin secretion was also tested using the high ceiling diuretic ethacrynic acid and a thiazide diuretic, chlorothiazide (Cooke et al., 1970). Ethacrynic acid prevents sodium and chloride reabsorption by inhibiting the active sodium-chloride co-transporter in the ascending loop of Henle. The thiazides inhibit sodium reabsorption in the distal tubule downstream from the macula densa. It was observed that renin secretion was increased after administration of ethacrynic acid and persisted when the volume was restored. Chlorothiazide failed to produce an increase in renin secretion during volume restoration. The effects of these two diuretics implies that the effect of chlorothiazide on renin release is mediated by changes in plasma volume. The effect of ethacrynic acid appears to be mediated by the macula densa. The results of this study suggest that changes in sodium sensed by the macula densa cells in the early part of the distal tubule serve as a regulator of renin release. The finding with ethacrynic acid seems to contradict the results of other studies indicating that sodium ions decrease renin release (Parfrey et al., 1981; Fray, 1978; Churchill et al., 1978; Tuck et al., 1975; Anderson et al., 1975; Bunag et al., 1966a). However, another

study which used furosemide, a diuretic with actions similar to ethacrynic acid, to investigate the effect of salt and water loss on PRA (Vander and Carlson, 1969) supported the findings by Cooke et al. (1970). Small doses of furosemide produced a natriuresis and diuresis that was prevented by replacing the volume loss. Larger doses of furosemide produced significant increases in PRA in dogs that were not reversed by replenishing the salt and water losses. These results provided evidence that the loop diuretics may inhibit sodium transport at the macula densa cells, resulting in elevated PRA.

One proposed hypothesis by Meyer et al. (1968) was that furosemide might affect renin release by inhibiting sodium transport into the macula densa cells. This would account for the inability of the increased sodium load to the macula densa after furosemide to decrease renin release. Meyer et al. (1968) noticed that furosemide produced a large increase in renin release in volume-replete rabbits. They concluded that since the loss of volume did not increase renin there may be a change in the delivery of sodium to the cells of the macula densa. This theory was tested in 1982 by Sottiurai and Malvin. They correlated intracellular sodium concentration in the macula densa cells with changes in PRA that occurred as a result of altering sodium intake. In sodium-depleted states, there was the expected rise in PRA but without any changes in the intracellular content of sodium in the macula densa cells. Infusion of saline reduced PRA but did not alter the intracellular concentration of sodium. The authors concluded that intracellular sodium in the macula densa cells is not affected by changes in dietary sodium and furthermore, that this is not the

mechanism by which the macula densa regulates renin secretion from the afferent arteriole.

Itoh and Carretero (1985) have observed that the macula densa is necessary for the regulation of renin release from isolated afferent arteriole preparations. They noted that in vitro afferent arteriole preparations had a higher rate of renin release than afferent arterioles with an intact macula densa. When these arterioles are incubated with furosemide, in doses that correspond to those used in vivo studies, only the arterioles with the macula densa were stimulated to increase renin release. This implies a role for the macula densa in the furosemide-induced increase in renin release that is independent of the baroreceptor effect. Since this was an in vitro study, it appears that furosemide may directly affect the macula densa.

Upon histological analysis of the macula densa cells Sottiurai and Malvin (1982) noted that the intercellular spaces between the cells were dilated after perfusion. This finding was also observed by Kaissling and Kitz (1982). In addition, they noted that when rats received either mannitol infusion (osmotic diuretic) or furosemide, the intercellular spaces between the macula densa cells were narrower than those of control animals. From a functional perspective, the closing of these spaces might prevent sodium from gaining access to the JG cells and therefore sodium would not be able to inhibit renin secretion. In hypervolemic conditions the intercellular spaces were dilated. Kaissling and Kitz (1982) did not attribute these findings as artifacts of the tissue processing since the spacing between the epithelial cells of the tubules were not altered under the different

test conditions. Whether the treatment effects on the macula densa cell spaces reflect a true occurrence in vivo remains to be determined. However, these findings are interesting and may suggest a mechanism of action for the macula densa in transducing the signal from the ionic concentration of the tubular lumen to the renin secreting cells.

1d. Potassium

In addition to sodium, another cation, potassium, is thought to regulate renin secretion. The first study on potassium-regulated renin release was done by Vander (1970). In normal dogs, infusion of potassium chloride into the renal artery produced a decrease in PRA without altering renal plasma flow or blood pressure. Infusion of potassium increased the urinary excretion of both sodium and potassium from the perfused kidney. The decrease in PRA could have been due to a decrease in either sodium or potassium reabsorption that would have resulted in an increase of sodium delivery to the distal tubule/macula densa. The possibility that potassium exerts its effect on the JGA was not ruled out. Shade et al. (1972) demonstrated that potassium produces a decrease in renin secretion that is dependent on an intact renal system and glomerular filtration rate. In the non-filtering kidney, potassium was without effect on renin secretion (Shade et al., 1972). Kirchner and Mueller (1982) tested the potassium-induced inhibition of renin secretion using salts other than chloride. PRA was measured after infusion of potassium chloride, bicarbonate, nitrate and acetate in sodium deficient rats. After each infusion, PRA levels decreased about 50% from the control values. Arterial pressure, plasma

volume and sodium balance were similar between the control and treatment groups. In conscious rats maintained on a sodium-deficient, high potassium diet, the increase in potassium levels blocked the rise in PRA after sodium depletion. In 1976, Kotchen et al. found similar results using a low sodium chloride diet supplemented with potassium bicarbonate. If potassium chloride was used instead of potassium bicarbonate, not only was there a blockade of the sodium-induced increase in PRA but a further suppression of PRA was observed.

1e. Chloride

Since potassium chloride produced a greater suppression of PRA, Kotchen et al. (1976) suggested a role for chloride in addition to potassium in regulating renin release. This spurred interest in chloride as a new modulator for renin secretion. Not only did potassium chloride inhibit renin secretion when compared with potassium bicarbonate but sodium chloride inhibited renin release when compared with sodium bicarbonate (Kotchen et al., 1976). Furthermore, Kotchen et al. (1978) and Kirchner et al. (1978) demonstrated that both sodium chloride and sodium bromide decreased PRA while sodium infusion as an acetate, nitrate or carbonate salt did not alter renin release. It has been postulated that bromide is handled by the kidney in a similar manner to chloride, and either of these ions can influence renin secretion. Kotchen et al. (1978) tested the role of chloride by infusing choline chloride versus the bicarbonate salt. Again, only the chloride salt produced a suppression of renin release. These changes occurred independently of variations in sodium or potassium balance

(Kotchen et al., 1978) or blood pressure or volume (Kirchner et al., 1978).

Rostand et al. (1985) measured the quantities of chloride in the tubules and correlated the concentration of chloride with changes in renin release in the perfused rat kidney. Substituting sodium nitrate and thiocyanate salts for chloride reduced the amount of chloride in the kidney and also produced an elevation of renin release. Analysis of the data revealed a negative correlation between chloride and renin activity. Therefore, it is possible that chloride, in addition to the cations, may be important in regulating renin release from the juxtaglomerular cells.

1f. Calcium

Calcium plays an important role in secretory and smooth muscle cells. It provides a signal for the release of neurotransmitters and for muscle contraction. However, with respect to renin release, there may be an inverse relationship between renin secretion and the intracellular concentration of calcium. In 1974, Kotchen et al. infused calcium chloride into the renal artery of dogs and observed a fall in renin release that was accompanied by increased calcium levels in the blood. There was no alteration of renal blood flow or of arterial pressure. An increase in sodium excretion was observed while the plasma concentration of sodium was unaffected by calcium treatment. Addition of calcium directly to kidney cortical cells produced a dose-dependent suppression of renin release whereas sodium did not alter the spontaneous release of renin (O'Dea et al., 1984). Kotchen et al.

(1974) examined the effect of calcium on the generation of ANG II from renin. Infusion of calcium did not interfere with the renin-substrate interaction or with angiotensinase activity. It was therefore concluded that calcium inhibited renin release by the macula densa or by a direct effect on the juxtaglomerular cells.

Recent studies investigating the role of calcium in regulating renin release have used pharmacological agents to alter calcium concentrations. BAY K 8644 is a calcium channel agonist that activates the slow inward calcium channel. It has been shown that BAY K 8644 vasoconstricts smooth muscle and reduces renal blood flow, dose-dependently (Dietz, 1986). Infusion of the agonist into perfused kidney (Dietz, 1986) or addition to cortical slices (Matsumura et al., 1985) does not alter basal levels of renin release. However, when BAY K 8644 was added with 15 mM potassium there was a significant decrease in renin release from the slices (Matsumura et al., 1985; May and Peart, 1986). The dose of potassium was reported to be below the threshold level for depolarization of the cells. The effect of BAY K 8644 was reversed by addition of nifedipine, a calcium channel antagonist. Other investigators have indicated that addition of calcium channel antagonists elevate renin release from kidney slices by decreasing the amount of intracellular calcium (Henrich and Campbell, 1986; Antonipillai and Horton, 1985).

The hypothesis that renin secretion is modulated by stores of intracellular calcium has not been defined. However, calmodulin, an intracellular protein that has multiple binding sites for calcium, has been implicated in controlling renin release (Park et al., 1986; Fray

and Park, 1986; Fray et al., 1983). Addition of calmodulin antagonists such as trifluoperazine (Fray et al., 1983), calmidazolinium (Fray and Park, 1986) or W-7 (Shinyama et al., 1987) produced dose-dependent increases in renin release when added to the kidney. Trifluoperazine also increased the basal levels of renin secretion from isolated renal cortical cells (Fray et al., 1983). Infusion of W-7 into the renal artery of rats did not alter mean arterial pressure, renal blood flow or urine flow. The control compound, W-5, which does not have calmodulin antagonist actions, also did not affect these parameters. W-7 produced dose-related increases in PRA and renin secretion rate whereas W-5 did not produce these changes in renin release (Shinyama et al., 1987). These data suggest a role for calmodulin and intracellular calcium levels in the juxtaglomerular cells in modulating renin release.

Some of the stimuli that inhibit renin secretion require the presence of extracellular calcium. Vanadate (Churchill and Churchill, 1980), ouabain (Cruz-Soto et al., 1984), potassium (Park et al., 1986) and high renal perfusion pressure (Fray and Park, 1986) are dependent on calcium in order to mediate their effect. For example, ANG II suppressed renin release when added directly to the kidney via the renal artery (Bunag et al., 1967). Removal or lowering of the concentration of calcium in the perfusate medium prevented the effect of ANG II on renin release (May and Peart, 1986; Antonipillai and Horton, 1985). In addition, the ANG II-induced stimulation of aldosterone production from glomerulosa cells was enhanced after addition of BAY K 8644 (Hausdorff et al., 1986). These findings

suggest that calcium is necessary in order for ANG II to exert an action, and that inhibition of renin secretion by ANG II is dependent on a calcium mediated mechanism. Dietz (1986) demonstrated that verapamil, another calcium channel antagonist prevented the decrease in renin secretion produced by increasing perfusion pressure. This supported an earlier proposal by Fray (1980) stating that calcium influx is needed for elevated perfusion pressure to decrease renin release. Overall, the evidence implies that calcium suppresses renin release and may be the mechanism by which other factors (ANG II and increased perfusion pressure) inhibit renin release.

2. Volume receptors

The early study by Goldblatt et al. (1934) demonstrated that graded constriction of the renal arteries induced hypertension in dogs. This was believed to be due to the presence of a circulating vasopressor substance. In 1959, Tobian et al. observed that there was an inverse relationship between the granulation of juxtaglomerular cells and perfusion pressure. He therefore proposed the theory that the juxtaglomerular cells act as baroreceptors, changing the rate of renin release as determined by changes in arterial pressure. Imagawa et al. (1984) showed that as arterial pressure is gradually increased by suprarenal aortic constriction, PRA decreases. These studies suggest that changes in perfusion pressure may be a stimulus for renin release.

2a. Intrarenal baroreceptor

The role of an intrarenal baroreceptor has been studied using the non-filtering kidney model (Blaine et al., 1971). This model was developed to obstruct glomerular filtration and prevent changes in sodium delivery to the macula densa from altering renin release (Blaine et al., 1970). Reduction of plasma volume by hemorrhage or reduction of renal blood flow by partial suprarenal aortic constriction produced significant increases in renin secretion (Blaine et al., 1970; 1971; Blaine and Davis, 1971). Renal denervation or adrenalectomy did not diminish the renin response to decreased blood flow. Since glomerular filtration ceased, the authors deduced that the change in renin release was not dependent on the macula densa and was likely due to the presence of an intrarenal baroreceptor.

The role of an intrarenal baroreceptor has also been studied using an isolated perfused kidney (Fray, 1976). Increased perfusion pressure suppressed renin release from the isolated kidney. Alteration of the sodium concentration in the perfusion medium did not affect the renin response to increased perfusion pressure. These findings support the hypothesis of Blaine et al. (1971) that the macula densa may not be involved in mediating the changes in renin secretion produced by increased arterial pressure. Kaloyanides et al. (1973) demonstrated that the increase in renin secretion produced by ureteral occlusion was prevented by increasing renal arterial pressure.

Results have also accumulated suggesting that the juxtaglomerular cells are sensitive to changes in arterial stretch. Vasodilation produced by papaverine decreased renin release from the isolated

perfused kidney (Fray, 1976). Perfusion of the kidney with either phenylephrine or methoxamine, two α agonists that produce vasoconstriction, increased renin activity. The increase in renin secretion produced by phenylephrine was blocked by co-administration of papaverine or by increased perfusion pressure. Since the juxtaglomerular cells are modified smooth muscle cells, it is likely that they may depolarize in response to stretch (similar to the response seen with increased plasma volume) or increased perfusion pressure. Depolarization may influence the membrane permeability to certain ions (Fray, 1976). Later, Fray (1980) demonstrated that the decrease in renin release produced by increased perfusion pressure was mediated by calcium. Intracellular recordings from renin-containing cells show that depolarization of the juxtaglomerular cells is produced by stimuli that inhibit renin release and may be mediated by an increase in calcium influx (Buhrle et al., 1985).

2b. Cardiac mechanoreceptors

In addition to the intrarenal baroreceptor, cardiac mechanoreceptors also are involved in the maintenance of renin secretion. Increased left and right atrial pressure produced by inflation of arterial balloons produces a decrease in plasma renin activity (Brennan et al., 1971). Kaufman (1987) on the otherhand, demonstrated that increased right atrial stretch did not alter plasma renin activity or the isoproterenol-induced increase in PRA in conscious rats. Other studies have supported the results of Brennan et al. (1971) indicating that the mechanoreceptors in the right atrium

influence renin release (Sanchez et al., 1987; Julius et al., 1983). In human studies, lower body compression produced by inflation of a cuff around the legs, caused a significant increase in right arterial pressure and a suppression of PRA (Sanchez et al., 1987; Julius et al., 1983). Tilting the subject upwards promoted gravitational pooling of blood and also increased PRA. The effect of tilting on renin was counteracted by lower body compression. Inversely, decreasing right atrial pressure by induced pooling of blood in the extremities, results in an increase in PRA (Julius et al., 1983). These findings suggest that the cardiopulmonary mechanoreceptors play an important role in the integration of postural changes and renin release.

3. Autonomic nervous system

3a. Role of the sympathetic nervous system

The juxtaglomerular apparatus of the kidney was analyzed for the presence of adrenergic nerve terminals. Using electron microscopy, Barajas and Muller (1973) documented the presence of dense core varicosities in the vicinity of the JGA that are generally associated with adrenergic neurons. These nerve terminal regions synapsed on the cells of the afferent and efferent arteriole as well as on the glomerulus and the proximal and distal tubules (Buhrle et al., 1985; Barajas and Muller, 1973). Using horseradish peroxidase labelling techniques, the origin of the renal nerves has been identified in the celiac and nodose ganglion (Gattone et al., 1986) and there is innervation extending from the dorsal root ganglia in the spinal cord segments T10-L1 (Ciriello and Calaresu, 1983; Kuo et al., 1982).

Typically, stimulation of the renal nerves results in a renal vasoconstriction, increased renin release and an increase in sodium reabsorption (DiBona, 1985). The increase in renin release occurs at stimulation frequencies that do not alter renal hemodynamics (Osborn et al., 1981). Renal denervation produces a decrease in basal renin release (Grandjean et al., 1978) and a decrease in the amount of sodium reabsorbed from the tubules (Bello-Reuss et al., 1975; Fernandez-Repollet et al., 1985). The antinatriuresis produced by renal nerve stimulation can be prevented by pretreatment with the α antagonist phentolamine. The beta blockers, propranolol and atenolol reduce the renal nerve-induced increase in renin secretion (Osborn et al., 1983).

It has been hypothesized that the increase in renin release resulting from sodium depletion is partially dependent on intact renal nerves. Denervation reduced the response of natriuresis to volume expansion in sodium-depleted rats. There was no difference in sodium excretion in rats that were maintained on a regular sodium diet. These results suggest that renal nerve stimulation plays a larger role in maintaining sodium excretion in sodium-deficient than in control states (DiBona and Sawin, 1985).

Renal sympathetic nerve activity has been shown to influence the onset of hypertension by changing renal arterial pressure, sodium and water balance and renin release. Winternitz and Oparil (1982) demonstrated that renal denervation decreases blood pressure and increases the renal excretion of sodium in spontaneously hypertensive rats. Renal denervation delays the development of hypertension in renovascular hypertension (Vari et al., 1987) and in genetic forms of

hypertension (Kline et al., 1980; Dietz et al., 1978).

3b. Beta receptors

$3b_1$. Renal beta receptors

Infusion of either epinephrine or norepinephrine into dogs with maintained arterial pressure produces the same response with respect to renin release and renal function as renal nerve stimulation (Vander, 1965). Renin release increases and the renal plasma flow and sodium excretion decreases. Vander (1965) proposed that stimulation of the renal nerves produced an increase in renin secretion that occurred secondarily to changes in sodium excretion. Studies using kidney slices have demonstrated that adminstration of the beta receptor agonist, isoproterenol, produces increases in renin release that are prevented by pretreatment with d,l-propranolol (Weinberger et al., 1975; Vandongen et al., 1973). Addition of norepinephrine and epinephrine also produce increases in renin release that are prevented by addition of d,l-propranolol (Weinberger et al., 1975). These effects are due to stimulation of beta receptors since pretreatment with either phenoxybenzamine, an alpha-antagonist or d-propranolol, a membrane stabilizing form of the beta antagonist propranolol, did not prevent the catecholamine-induced stimulation of renin secretion.

Beta-adrenergic receptor subtypes were localized in rat kidney slices by autoradiography using the non-selective B antagonist, iodocyanopindolol, in the presence of the selective antagonists for the B_1 (betaxolol) or B_2 (zinterol) receptors (Healy et al., 1985). The results from this study identified the renal cortex as having the

highest concentration of renal beta-receptors. B_1 binding occurs primarily on the afferent arterioles and on the glomeruli; B_2 binding is predominantly on the renal tubules of the medulla. Functionally, the B_1 adrenoceptor has been associated with renin release from the kidney. Himori et al. (1979) demonstrated that atenolol, a selective B_1 antagonist, attenuates the increase in renin secretion produced by isoproterenol. A B_2 antagonist, IPS-339, was not as effective in reducing the isoproterenol-induced increase in renin release. Stimulation of renal nerves at a low frequency (0.5 Hz for 0.5 ms) produces an increase in renin release without changing renal blood flow, sodium excretion or arterial pressure (Osborn et al., 1985; 1981). Pretreatment with atenolol blocked the renin response to renal nerve stimulation. In contrast, blockade of B_2 receptors did not modify the increase in renin secretion. These studies suggest that the sympathetic nervous system increases renin release by stimulating B_1 receptors on the JG cells.

$3b_2$. Extrarenal beta receptors

Recently, data have accumulated indicating that an extrarenal beta receptor regulates renin secretion independently of the renal beta receptors. In 1972, Reid et al. infused the beta adrenergic receptor agonist, isoproterenol, into the femoral vein of dogs and noticed increases in PRA and renin secretion rate in both innervated and denervated kidneys. Renal perfusion pressure was held constant by adjusting an aortic clamp so there was no influence of renal perfusion pressure on the renin response to isoproterenol. No changes in

glomerular filtration, renal plasma flow or electrolyte excretion in these animals were detected. In contrast, infusion of isoproterenol into the renal artery at similar doses produced no change in PRA or renin secretion rate. These results do not support the involvement of intrarenal beta receptors in the regulation of renin secretion, and indicate that beta adrenergic stimulation is mediated by an extrarenal mechanism.

In 1979, Johnson et al. reproduced the findings of Reid et al. (1972) with isoproterenol and tested the effect of epinephrine on renin secretion infused both intravenously and intrarenally. Similarly, epinephrine produced increases in PRA when infused intravenously but not intrarenally. This increase in renin secretion was independent of renal nerves, changes in perfusion pressure and prostaglandins (Johnson et al., 1979a). Propranolol blocked the effect of epinephrine. Further experiments were designed to identify the location of these extrarenal beta-receptors. Infusion of epinephrine into splanchnic circulation (Johnson, 1983) did not produce an increase in PRA as did intravenous infusion. Johnson (1982) also tested the possibility that the beta receptors were located in the cerebral vasculature by infusing epinephrine into the carotid arteries. At higher doses epinephrine produced an increase in PRA when infused into the carotid arteries and this dose also increased the circulating levels of epinephrine. At slightly lower doses there was no change in PRA. Epinephrine also produced increases in PRA in adrenalectomized animals, indicating that the extrarenal beta receptors are not located in the adrenal glands (Johnson, 1985). At this point Johnson established a role for

extrarenal beta receptors but did not address whether there was an interaction between renal and extrarenal receptors. A submaximal dose of epinephrine was infused intravenously and into the renal artery. The renin secretion rate was measured in both kidneys and the values were not different from one another (Johnson, 1984). It was concluded that there is no interaction between beta receptors and that renin secretion is regulated only by extrarenal beta receptors. Contrary to these findings by Johnson (1979; 1982; 1984; 1985) are the data that we have obtained in our laboratory (Urban and Van de Kar, 1986). We have demonstrated that addition of the beta agonist, isoproterenol, to rat kidney cortical slices produces significant increase in renin release from the kidney slices. The dose of isoproterenol that we used in the renin release bioassay, is more concentrated than that used by Johnson. Therefore, it may be likely that the beta receptors at the kidney level require a higher concentration of isoproterenol in order to produce an effect whereas extrarenal beta receptors may be more sensitive to lower doses.

3c. Parasympathetic nervous system

Cholinergic innervation of the kidney has been described by Barajas (1979). He observed that labelling for acetylcholinesterase followed the distribution pattern of catecholamine fluorescence. However, when the animals were treated with 6-hydroxydopamine (6-OHDA), a drug that destroys catecholaminergic nerve terminals, both norepinephrine and acetylcholine staining disappeared from the kidney. This suggested that the acetylcholinesterase and catecholamine-

containing neurons may in fact be the same neurons. Addition of acetylcholine directly to kidney slices did not produce a significant change in renin release (De Vito et al., 1970). Schrier et al. (1975) studied the effects of vagotomy on renin secretion in dogs. Vagotomy produced a suppression of renin secretion. However, in hypophysectomized dogs the suppression of renin secretion after vagotomy was prevented. This result indicates that inhibition of renin secretion is due to release of a pituitary hormone and not to a direct innervation of the kidney by the parasympathetic fibers. The authors attributed this effect to an enhanced release of vasopressin after vagotomy. Therefore, it appears that cholinergic neurons do not contribute to the innervation of the kidney or to the regulation of renin release.

4. Other factors that regulate renin secretion

4a. Vasopressin

Vasopressin (AVP) is a potent vasoconstrictor and antidiuretic hormone. In addition, vasopressin inhibits renin secretion. However, whether this inhibition occurs by direct action of AVP on the juxtaglomerular cells or secondarily due to AVP-induced changes in blood pressure has not been determined.

Vander (1968) showed that infusion of AVP produced a decrease in basal renin release. However, Vander noted that the excretion of sodium was increased and proposed that AVP may inhibit renin by acting on the macula densa. Another study (Shade et al., 1973) using the non-filtering kidney model, also demonstrated that AVP suppresses renin

release after intrarenal infusion. They reported that the doses of AVP used did not alter mean arterial pressure or renal blood flow. Furthermore, since this study was performed in a non-filtering kidney, the decrease in renin release occurred without a functioning macula densa, suggesting a direct effect of AVP on the juxtaglomerular cells. Addition of AVP to rat renal cortical slices produced a significant decrease in renin release (Park et al., 1981). This is further indication that AVP acts directly on the juxtaglomerular cells.

In addition to a direct effect on the kidney, AVP decreases renin release secondarily to changes in blood pressure (Schwartz and Reid, 1986). The inhibitory effect of AVP on renin release has been studied using analogues for AVP that are selective for either antidiuresis (V_2 receptor) or vasoconstriction (V_1 receptor). Infusion of the antidiuretic agonist, DDAVP (d-arg-vasopressin), does not modify PRA or arterial pressure. Pretreatment with a V_1 receptor antagonist [$(CH_2)_5$Tyr(Me)AVP], prevented the AVP-induced increase in blood pressure and the decrease in PRA (Schwartz and Reid, 1986). These studies indicate that AVP reflexly inhibits renin secretion through increased blood pressure. However, whether AVP primarily suppresses renin release by this mechanism or by a direct action on the kidney has yet to be established.

4b. Adenosine

Adenosine has recently been shown to inhibit the release of renin from the kidney. Churchill and Churchill (1985) have described the presence of two adenosine receptors (A_1 and A_2) that differentially

regulate renin secretion. Both receptors are coupled to adenylate cyclase, but the A_1 receptor inhibits while the A_2 receptor stimulates adenylate cyclase. Stimulation of the A_1 receptor produces suppression of renin release while activation of the A_2 receptor increases renin release from kidney slices. These effects of adenosine on renin release are anatagonized by calcium channel inhibitors and are probably mediated by changes in intracellular calcium (Churchill and Churchill, 1985).

Adenosine produces vasoconstriction when injected into the renal artery (Osswald, 1984). Other studies have indicated that adenosine can also alter renal nerve activity. Stimulation of the renal sympathetic nerves at different frequencies produces vasoconstriction that is blocked, dose-dependently, by adenosine (Ekas et al., 1981). However, Arend et al. (1984) have demonstrated that the decrease in renin release produced by adenosine was not influenced by changes in renal blood flow or blood pressure. Administration of adenosine prevents the increase in renin release produced by prostacyclin (PGI_2) and norepinephrine, suggesting that adenosine acts directly on the juxtaglomerular cell to inhibit renin release (Deray et al., 1987).

4c. Prostaglandins

Administration of the prostaglandin precursor, arachidonic acid, produces an increase in renin release from isolated glomeruli (Beierwaltes et al., 1982). Superfusion of the glomeruli with prostaglandins PGE_1, PGE_2 or thromboxane had no effect on renin release (Beierwaltes et al., 1982). However, if the rapid breakdown of PGI_2

was prevented, thereby increasing the concentration of PGI_2, or if the pH of the perfusate was increased which favors stability of the prostaglandins, renin release was stimulated. Inhibition of PGI_2 synthesis also prevented the arachidonic acid-induced increase of renin release (Beierwaltes et al., 1982). The increase in renin release produced by PGI_2 has also been described by Kirchner (1985) and Henrich and Campbell (1984).

Prostaglandins have been shown to mediate the increase in renin release in response to sodium depletion. Indomethacin, a prostaglandin synthesis inhibitor, reduces renin release in the sodium depleted dog. However, when the animals are maintained on a regular sodium diet, indomethacin does not affect the basal levels of PRA (Deforrest et al., 1980). The role of prostaglandins in mediating the response of PRA to changes in renal arterial pressure is more controversial. In dogs with either a denervated non-filtering kidney or an intact filtering kidney, suprarenal aortic constriction produces an increase in PRA. This increase in PRA is not blunted by treatment with indomethacin (Freeman et al., 1982) or meclofenamate (Villarreal et al., 1984). However, the decreases in renal perfusion pressure in these experiments were below the autoregulatory range (80 mmHg). Imagawa et al. (1985) produced an increase in renin release after suprarenal aortic constriction without going below the autoregulatory range. After treatment with indomethacin, the renin response to aortic constriction was abolished. It has been suggested that the difference in the prostaglandin response to suprarenal aortic constriction following reductions in perfusion pressure within the autoregulatory range may be dependent on renal

prostaglandins (Freeman et al., 1984).

5. Feedback regulation of renin release

As has been discussed, the renin-angiotensin system partially controls aldosterone secretion which maintains sodium and water balance. The mechanisms involved in renin secretion can also be described in terms of a feedback control system. The primary signals that stimulate the renin-angiotensin system are a decrease in plasma volume and blood pressure. The decrease in blood pressure not only activates the stretch receptor but also the macula densa by decreasing glomerular pressure and sodium delivery to the distal tubule.

ANG II is the end product of the renin-angiotensin system and plays an important role in the feedback regulation of renin secretion. ANG II has been shown to inhibit renin release from the kidney (Vander and Geelhoed, 1965). This is referred to as the short feedback loop. Receptors for ANG II have been localized on the glomerulus (Bianchi et al., 1986). However, it is possible that stimulation of ANG II glomerular receptors would affect renin release as a result of changes that would occur as a consequence of alterations in the glomerular capillary pressure. Furthermore, ANG II constricts the efferent renal arterioles which increases the pressure in the capillaries, resulting in enhanced sodium and water reabsorption (Hall, 1986). The increased sodium and water reabsorption would lead to an increased plasma volume and concomitant decrease in renin release. ANG II stimulates the production of aldosterone from the adrenal gland (Aguilera et al., 1980; Davis and Freeman, 1976). Aldosterone acts at the renal distal

tubule to increase the reabsorption of sodium and water from the tubular lumen. This restores plasma volume and blood pressure, thus completing a long feedback loop.

F. Central nervous system regulation of renin secretion

1. Role of the central nervous system

There has been increasing evidence suggesting a role for central nervous system pathways in regulating renin secretion. The effect of electrical stimulation of several brain sites have been electrically stimulated on PRA have been studied. Richardson et al. (1974) compared the effects of ventrolateral medulla (VLM) stimulation on renin release from control and denervated kidneys. They observed that denervation prevented the increase in renin release following stimulation of the VML in cats. Propranolol also blocked this increase. These studies suggest that the renal sympathetic nerves are involved in mediating the increase in renin secretion after stimulation of the VLM. Passo et al. (1971) stimulated the dorsal medulla and similarly demonstrated that destruction of the renal nerves attenuated the PRA response to excitation of the dorsal medulla. In this study (Passo et al., 1971), plasma epinephrine levels were elevated, implicating the involvement of the sympathetic nervous system and adrenal catecholamines.

Stimulation of the dorsal periaqueductal grey (PAG) in the mesencephalon produced a pressor response that was associated with an increase in renin activity. Renal denervation prevented the effect of electrical stimulation of the PAG on blood pressure and renin secretion (Ueda et al., 1967).

The fastigial nucleus of the cerebellum has been implicated in regulating autonomic functions, namely, elevating arterial pressure and heart rate. This has been referred to as the fastigial pressor response and requires an intact sympathetic nervous system (Del Bo et al., 1983). Koyama et al. (1980) stimulated the fastigial nucleus and observed a pressor response accompanied by a significant increase in PRA. This increase was reduced by transection of the superior cerebellar peduncles (SCP). The major output from the fastigial nucleus is to the pontine and medullary areas via the SCP. The authors therefore concluded that the pressor response to fastigial stimulation may be mediated by these cardioregulatory centers through the sympathetic nervous system.

An increase in renin activity was observed after electrical stimulation of the lateral hypothalamus (Zanchetti and Stella, 1975). This was associated with a transient constriction of the renal artery. However, the elevation in PRA was maintained beyond the vascular response and was prevented by destruction of the renal nerves. These data are supported in part by Frankel et al. (1976) who also observed increases in PRA after stimulation of the lateral hypothalamus. On the other hand Zehr and Feigl (1973) observed a suppression of renin release in dogs after stimulation of a site in the lateral hypothalamus. This site was postulated to be a sympathetic inhibitory site since both blood pressure and renin levels decreased. No histology was presented to identify the sites of stimulation, so a comparison with those areas stimulated by Zanchetti and Stella (1975) could not be made. Stimulation of the posterior hypothalamus

(supramammillary region) produced an increase in PRA and blood pressure (Natcheff et al., 1977). This effect was also dependent on an intact sympathetic nervous system. Electrical stimulation of the paraventricular nucleus of the hypothalamus (PVN) produced dose-dependent increases in renin release with increased stimulation frequency (Porter, 1986). Blood pressure was not affected by stimulation of the PVN. These data suggest a role for the PVN in increasing renin release independently of changes in blood pressure. The role of the renal nerves in mediating this effect has not yet been reported.

2. Stress-induced renin secretion

Stress is implicated in the pathogenesis of hypertension and cardiac disease. A variety of stress paradigms produce increases in blood pressure and the circulating levels of different hormones including corticosterone, prolactin and PRA. One of the first studies on the effect of stress on renin secretion was conducted by Leenen and Shapiro (1974), who subjected rats to different periods of intermittent foot shock. PRA was increased in rats 15 minutes after they received the shock. However, after the rats were exposed to this stimulus for over 2 hours, there was an apparent adjustment to the shock since there was no difference in PRA between the shock and control groups. Paris et al. (1987) reported that intermittent foot shock for 2, 12 and 22 minutes produced increases in PRA. In addition, PRA was increased by 20 minutes of forced swimming in deep cold water, 20 minute immobilization and by both a 3 and 12 minute conditioned fear (CER)

paradigm. Jindra et al. (1980) also observed increases in PRA in rats that were immobilized for 20 minutes or received repeated immobilization of 150 minutes daily for 34 days. Exposure to a novel environment (open field) or to the presence of a hungry cat produced elevations in PRA (Clamage et al., 1976). The effect of novel environment on PRA could be attenuated by pretreatment with propranolol. Blair et al. (1976) reported that baboons that were subjected to an avoidance operant conditioning paradigm had elevated PRA values. The baboons were taught to sit in a chair and their task was to push a lever in order to avoid an electric shock. This study suggested that a psychological stimulus could evoke a stress response as measured by an increase in PRA.

There are a variety of stress models that produce increases in PRA. The stress paradigm used in this laboratory consisted of a 3 or 10 minute conditioned emotional or fear response paradigm. The rats are placed in a chamber on three consecutive days and after either a 3 or 10 minute period, they receive a footshock. On the fourth day the rats are placed in the chamber and do not receive any shock. At this point they are anticipating the shock, which simulates anxiety or fear. The effect of CER on PRA can be attenuated, but not completely prevented by pretreatment with the beta receptor blocker propranolol (1.0 mg/kg i.p.; Van de Kar et al., 1984). Administration of the same dose of propranolol to rats that were subjected to restraint stress did not affect the increase in PRA (Sigg et al., 1978); however, the increase in PRA was diminished with higher doses of propranolol. Adrenal medullectomy combined with chemical sympathectomy (peripheral

injections of 6-OHDA) did not prevent the stress-induced increase in PRA (Richardson Morton et al., unpublished observations). With the restraint stress paradigm, demedullectomy did not alter the stress-induced increase in PRA but renal denervation did reduce the observed increase in PRA (Sigg et al., 1978). These conflicting results might suggest that different neural pathways are involved in mediating the stress-induced increase in PRA.

Pharmacological studies have indicated that peripheral administration of the benzodiazepines, chlordiazepoxide and midazolam, which enhance GABA neurotransmission, did not alter the renin response to the CER paradigm (Van de Kar et al., 1984b). Naloxone, an opiate antagonist or diisopropyl fluorophosphate (DFP) an acetylcholinesterase inhibitor, also did not prevent the stress-induced rise in PRA or alter basal levels of renin release. Therefore, these results imply that neither the cholinergic, GABAergic or opiate systems are involved in regulating renin release in response to the CER (stress) paradigm.

Electrolytic lesions in the dorsal raphe nucleus (DRN) prevented the stress-induced increase in renin secretion with both the 3 and 10 minute CER paradigms (Van de Kar et al., 1984a; Richardson-Morton et al., 1986). However, this effect was not due to disruption of serotonergic fibers since 5,7-DHT injections into the DRN and pretreatment of rats with the 5-HT_2 antagonist, LY53857 did not prevent the effect of stress on renin secretion (Lorens et al., 1986). Gotoh et al. (1987) and Richardson-Morton et al. (1986) showed that electrolytic lesions in the PVN prevented the increase in renin secretion in rats subjected to either immobilization or CER,

respectively. Selective destruction of the cell bodies in the PVN with ibotenic acid, also prevented the increase in PRA (Richardson-Morton et al., 1986). In order to test the possible involvement of catecholamines in regulating the stress-induced release of renin, 6-OHDA was injected into the PVN and was found to attenuate the stress (CER) response on PRA (Richardson Morton et al., 1987).

3. Role of serotonin (5-HT) on renin release

3a. Effects of peripheral administration of 5-HT

Administration of 5-HT, or the serotonin precursor, 5-hydroxytryptophan (5-HTP) to rats produced increases in water intake (Kikta et al., 1981) that are mediated by the renin-angiotensin system (Meyer et al., 1974; Kikta et al., 1983). Pretreatment with the serotonin receptor antagonist, methysergide, prevented the dipsogenic effect of 5-HT and 5-HTP. In addition, rats that received either the beta blocker, propranolol (Meyer et al., 1974; Kikta et al., 1983) or the angiotensin converting enzyme inhibitor captopril (Kikta et al., 1983) had an attenuated drinking response to 5-HT. These results suggest that the renin-angiotensin system mediates the 5-HT-induced dipsogenesis. However, these studies did not examine whether this was a central effect of 5-HT nor did they postulate a mechanism for 5-HT stimulation of renin release. A study by Bunag et al. (1966b) showed that infusion of serotonin into the renal artery did not directly stimulate renin release from the kidney. Data presented in this study (Table 9) demonstrate that addition of 5-HT to kidney slices does not directly increase renin release.

Barney et al. (1981) and Meyer et al. (1974) demonstrated that injection of 5-HT either subcutaneously or intramuscularly to rats, produces increases in PRA. The effect of 5-HT on PRA is prevented by pretreatment with metergoline, a 5-HT antagonist. Treatment with propranolol or camphidonium, a ganglionic blocker, attenuated the 5-HT induced increase in PRA (Meyer et al., 1974) indicating that both 5-HT and the sympathetic nervous system are involved in regulating renin release. Zimmermann and Ganong (1980) administered two 5-HT precursors, 5-HTP and l-tryptophan to anesthetized dogs and observed increases in PRA that were not due to changes in blood pressure. The elevation of PRA due to the 5-HT precursors was prevented by infusion of the central aromatic amino acid decarboxylase inhibitor, benserazide, but not by carbidopa, a decarboxylase inhibitor that does not cross the blood brain barrier. Pretreatment with metergoline or renal denervation also prevented the 5-HT-induced increase in PRA. Earlier studies by Epstein and Hamilton (1977) showed that in humans, the 5-HT antagonist, cyproheptadine, inhibited the furosemide-induced increase in PRA. Other studies by Modlinger et al. (1979) showed that oral administration of the 5-HT precursor l-tryptophan to humans, produced an increase in renin secretion that was prevented by pretreatment with cyproheptadine. These were the first studies that suggested a role for brain 5-HT in stimulating renin release.

3b. Role of brain 5-HT neurons

Administration of the 5-HT releaser, p-chloroamphetamine (PCA) or the 5-HT agonist quipazine, produces dose-dependent increases in PRA

(Van de Kar et al., 1981). The effect of PCA is prevented by pretreatment with p-chlorophenylalanine (PCPA) a drug that inhibits 5-HT synthesis. Fenfluramine, another 5-HT releaser, also produces dose-dependent increases in PRA that are prevented by pretreatment with PCPA or the 5-HT reuptake inhibitors, fluoxetine or indalpine (Van de Kar et al., 1985b). The effect of a submaximal dose of fenfluramine on renin secretion was enhanced after pretreatment with l-tryptophan. Administration of the 5-HT_2 antagonist LY53857, prevents the increase in PRA and PRC produced by fenfluramine and the 5-HT agonist MK-212 (Lorens and Van de Kar, 1987). LY53857 alone did not alter PRA or PRC. The 5-HT_{1a} agonist 8-OH-DPAT (8-hydroxy-2-[di-N-propylamino]tetralin) did not produce any change in either PRA or PRC. Ipsapirone, another 5-HT_{1a} agonist, produced increases in PRA and PRC only at higher doses (Lorens and Van de Kar, 1987). Administration of buspirone, a non-benzodiazepine anxiolytic that also has 5-HT_{1a} agonist activity produces a decrease in renin secretion at relatively low doses (1.0 mg/kg i.p.; Van de Kar et al., 1985c). These findings suggest that stimulation of 5-HT_2 receptors enhances renin release whereas the 5-HT_1 receptors may not have a role in the control of renin secretion.

Injection of the 5-HT neurotoxin 5,7-DHT (5,7-dihydroxytryptamine) into the dorsal raphe nucleus, a serotonergic cell group in the midbrain, prevented the PCA-induced increase in PRA. Injections of 5,7-DHT into the median raphe nucleus were without effect on renin secretion (Van de Kar et al., 1982b). Both of these lesion treatments significantly reduced brain 5-HT content. These were the first definitive findings for a role of brain serotonin neurons in the

regulation of renin secretion. Destruction of the mediobasal hypothalamus, either destroying or sparing the median eminence, prevents the increase in PRA produced by PCA (Karteszi et al., 1982). Posterolateral knife cuts through the mammillary bodies destroyed neuronal inputs to and from the hypothalamus, also blocked the increase in PRA produced by PCA. However, anterolateral deafferentation did not reduce the PRA response to PCA. Hypophysectomy did not prevent the PCA-induced increase in PRA 4 days after surgery (Karteszi et al., 1982). Since the dorsal raphe nucleus is known to send projections to the hypothalamus it is likely that a serotonergic pathway stimulates renin secretion by some structure within the hypothalamus. Recently, Gotoh et al. (1987) reported that electrolytic lesions of the paraventricular nucleus (PVN) in the hypothalamus prevent the PCA-induced increase in PRA.

In the study by Zimmermann and Ganong (1980), renal denervation prevents the increase in PRA produced by 5-HTP or tryptophan. To investigate the role of the sympathetic nervous system in mediating the PCA-induced increase in PRA, beta blockers were administered before PCA. Alper and Ganong (1984) showed that pretreatment with the beta-receptor antagonists propranolol and sotalol prevented the increase in PRA produced by PCA. These results were confirmed by Van de Kar and Richardson-Morton (1986). Both the selective $beta_1$ receptor antagonist, atenolol, and the non-selective beta antagonist, sotalol, completely prevented the effect of PCA. However, more conclusive studies indicated that the sympathetic nervous system was not involved. Treatment of rats with the catecholamine blocker, bretylium tosylate,

or the cholinergic muscarinic antagonist, methyl atropine did not alter the PCA-induced increase in PRA (Van de Kar and Richardson-Morton, 1986). Transection of the spinal cord proximal to the exit of the renal nerves, between the T_1 or T_2 vertebrae, did not modify the renin response to PCA. In addition, the effect of adrenal medullectomy combined with peripheral sympathectomy was also tested on renin secretion. The adrenal medulla was removed from rats and one week later they received weekly injections of 6-hydroxydopamine (6-OHDA) for 4 weeks. This treatment decreased the renal content of norepinephrine to levels that were below the sensitivity limit of the assay (Van de Kar and Richardson-Morton, 1986). Administration of PCA produced significant increases in PRA in both the sham-operated rats and the rats that were sympathectomized. These results indicate that the sympathetic nervous system does not mediate the PCA-induced increase in renin release. These results appear to contradict the data obtained with the beta blockers. However, it should be considered that beta blockers have been shown to cross the blood brain barrier and compete with 5-HT for receptor sites. Propranolol, for example, is known to interact at the 5-HT receptor (Middlemiss et al., 1977) and has been shown to decrease the rate of 5-HT synthesis in the hypothalamus, midbrain and frontal cortex (Giarcovich and Enero, 1984). Therefore, it is possible that the beta blockers may be acting centrally to influence serotonergic transmission and that a central site of action for these beta blockers cannot be discounted.

G. Role of humoral factor in regulating renin release

The existence of an extrarenal humoral factor was presented by DeVito et al. in 1971. Plasma was collected from bilaterally nephrectomized dogs that were made hypotensive by controlled hemorrhage. When the plasma from hypovolemic dogs was injected into unanesthetized control dogs, there was a significant increase in PRA. This elevation of PRA was not observed after injection of plasma from normotensive dogs. Polomski et al. (1974) could not reproduce these findings. No further studies on this factor were performed.

Since the effect of PCA on renin secretion is not mediated by either the parasympathetic or the sympathetic nervous systems, another possibility is that the hypothalamus may release a factor into the circulation that can stimulate renin release from the kidney. A study by Van de Kar et al. (1982a) tested the hypothesis that PCA induces the release of a factor into the blood. A group of rats were nephrectomized and received either saline or PCA 24 hours after surgery. Their plasma was collected and administered at different times to recipient rats. Administration of the plasma from PCA-treated rats produced a significant increase in PRA at 30 minutes after injection. These results suggest that brain serotonin stimulates renin secretion by releasing a factor into the blood. Further transfusion experiments have indicated that the renin-releasing factor is heat stable and is present in the plasma of PCA-treated rats within the molecular weight range of 500-10,000.

CHAPTER III

MATERIALS AND METHODS

A. Animals

Male Sprague-Dawley rats (150-300 g) were purchased from Sasco-King Animal Laboratories (Oregon, WI) and were housed, two per cage, in a temperature (22°C), light (12:12 light/dark cycle) and humidity (45% - 55%) controlled room. Rat chow (Wayne Lab Blox, Allied Mills Inc., Chicago,IL) and water were available *ad libitum*.

B. Analytical methods

1. *In vitro* renin release kidney slice bioassay for measurement of RRF activity

A rat (150-200 g) was perfused under halothane anesthesia, with cold saline through the left cardiac ventricle. The abdomen was opened to expose the kidneys which were removed and immediately placed in cold saline (0.9% NaCl). The kidneys were palpated to remove excess blood and decapsulated. The poles (2-3 mm) were dissected and removed from the kidneys, and the kidney was cut in half through the papilla, glued to a 1-inch square piece of Plexiglas, and mounted on an Oxford Model G vibratome (10° angle, settings of 0 for vibration and speed). The kidney was always maintained in cold saline, and saline ice cubes were

added during the slicing to maintain the temperature at 4°C. Coronal slices (400 μm) were cut, and the first slice was discarded. Once the slice was cut, it was placed on ice in a petri dish containing cold saline, and the medullary tissue was excised from each slice with a scalpel. The renal medulla does not contain renin and the highest density of renin secreting cells is in the outer cortex. Therefore, using coronal slices of cortical tissue ensures that the slices were homogenous with respect to renin content (Katz and Malvin, 1982a; Jones et al., 1979). Each slice was cut in half and randomly placed, two kidney slice halves per vial, in 10.0 ml vials that were siliconized (Prosil-28; Scientific Products, Specialty Chemicals, Gainesville, FL). Two ml of a Krebs-Ringer buffer were added to each vial. The Krebs-Ringer solution has a composition of 118.0 mM NaCl, 1.22 mM KH_2PO_4, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10.0 mM glucose, and 25.0 mM $NaHCO_3$ and was used throughout the experiments. The vials were placed in a water bath and the kidney slices were incubated at 37°C. Each vial received its own supply of a 95% O_2-5% CO_2 gas mixture via a needle (20-gauge) that was attached to plastic tubing (Scientific Products, 3/32 in. O.D., 1/32 in. I.D.) and inserted into plastic snap-on caps which fit snugly onto the vials. This allowed proper oxygenation of the vials without bubbling the medium. Cho and Malvin (1979) reported that bubbling of the incubation medium in which the kidney slices incubated inactivates renin at the air-water interface. This would result in low and variable values for renin release and the assay for renin would not indicate real changes in renin release over time. A few small holes in the caps relieved the pressure inside the vials.

The other end of the tubing was attached to a 12 place pipette manifold which was fitted to the hose that supplied the 95% O_2-5% CO_2 gas. This system, when set up with 4-5 manifolds, could oxygenate a total of 48-60 vials.

The slices were placed in the vials with 2.0 ml of Krebs-Ringer buffer for a 30 minute preincubation period. After 30 minutes, a 0.2 ml sample was withdrawn from the vials for the determination of renin concentration. The vials were refilled with 2.0 ml of Krebs-Ringer solution and test solutions (plasma fractions, plasma samples, drugs or brain and peripheral tissue extracts) were added to the vials.

As a routine for each kidney slice bioassay, six vials received the vehicle (usually saline or Krebs-Ringer buffer) and another six vials received a dose of isoproterenol (10^{-6}M - 10^{-5}M). This served as two controls for the bioassay: the vehicle was a control of basal levels of renin release and isoproterenol demonstrated maximal stimulus-induced renin release or the upper limit of the bioassay.

The vials incubated for 1 hr; 0.2 ml was saved for renin assay, and the Krebs-Ringer solution was decanted and saved for measurement of pH and, on occasion, lactate dehydrogenase (LDH) activity. LDH is a cytosollic enzyme that is used as an indicator of cell lysis. The LDH assay were performed to ensure that the increases observed in renin release from the kidney slices were due to the applied stimulus and not due to lysis of the kidney cells. A final 2.0 ml was added for a 30 minute postincubation period and a final 0.2 ml sample was taken for determination of renin concentration. The slices in each vial were blotted dry and weighed. All samples were stored at -40°C until

determination of renin concentration.

For each bioassay, in addition to the incubation values, samples were also measured for renin release during the 30 minute pre-incubation and post-incubation periods. These were used as quality controls for each kidney slice vial. After each incubation, the post-incubation value for renin release should have returned back to the levels seen in the pre-incubation. This indicated: (1) if the increase seen during the incubation period was truly due to the applied stimulus and (2) whether the kidney cells were dying. Using the comparisons of the pre-incubation and post-incubation periods along with the measurement of the pH and LDH enabled checks on the bioassay that would validate the viability of the kidney slices.

2. Specificity of the bioassay for RRF

2a. Test for non-specific activation of renin by plasma fractions

Krebs-Ringer samples (2.0 ml) were preincubated with kidney slices for 1 hour. The kidney slices were removed and the Krebs-Ringer was pooled. Samples (0.2 ml) of either saline, saline-plasma (M.W. = 5,000-10,000) or PCA-plasma (M.W. = 5,000-10,000) fractions were added to 2.0 ml of the Krebs-Ringer solution. These samples incubated at 37°C for 1 hour and afterwards, 0.2 ml aliquots were taken and saved for the determination of renin concentration (Method section B part 3b).

2b. Test for non-specific generation of angiotensin I from renin substrate by plasma fractions and brain extracts

The samples that were used in the previous studies (plasma

fractions and brain extracts) were tested for possible renin-like activity that would result in the non-specific generation of angiotensin I in the incubation for the radioimmunoassay. A volume of 2.0 μl of either rat hypothalamic, cerebellar, and pituitary extracts, or the saline-plasma and PCA-plasma fractions (M.W. = 5,000-10,000) were added to 0.2 ml of the Krebs-Ringer solution containing PMSF, 8-HQ, phosphate buffer and nephrectomized plasma, which corresponded to the dilution of these test substances in the kidney slice bioassay. The samples incubated for 1 hour at 37°C, and the incubation was terminated by addition of 0.2 ml of distilled water and immersion of the samples in a boiling water bath. The samples were then assayed for ANG I.

3. Determination of renin concentration and plasma renin activity

Renin activity of the kidney slices samples and plasma is measured by radioimmunoassay for generated ANG I according to the method of Haber et al. (1969) and Stockigt et al. (1971).

3a. Preparation of renin substrate (angiotensinogen)

Renin substrate (angiotensinogen) was obtained from the plasma of nephrectomized, male Sprague-Dawley rats (retired breeders, 450-500 g). Removal of the kidneys (the major source of renin) prevented the enzymatic reaction between renin and angiotensinogen in the plasma and allowed the plasma concentration of substrate to increase (Radziwill et al., 1986).

Approximately 40 animals were used for the preparation of renin substrate. The rats were anesthetized with halothane (Burns, Glenview,

IL) and care was taken to avoid any undue stress to the rats. The kidneys were palpated and a dorsal incision was made through the skin at the kidney level. The muscles of the back were teased apart by blunt dissection and the kidneys were removed through the openings in the body wall. The renal artery and vein were ligated and the kidneys were removed. The ligatures were placed back into the body cavity and the incision was sutured closed. Each rat then received an injection of dexamethasone (0.2 mg/rat, s.c.; Sigma, St. Louis, MO). Dexamethasone treatment increases the amount of angiotensinogen in the plasma (Reid, 1977).

The rats were decapitated 24 hours after surgery. The trunk blood was collected in a beaker containing 0.3 M ethylene diamine tetraacetate (EDTA, pH 7.4; approximately 0.5 ml per rat). The blood was centrifuged at 1500 x g, and the plasma was collected and stored at -40°C.

3b. Determination of renin concentration in the Krebs-Ringer medium after incubation with kidney slices and in plasma.

Renin concentration was measured by determining the amount of angiotensin I (ANG I) generated by incubating the sample (0.2 ml Krebs-Ringer from kidney slices or plasma) for 1 hour with a saturating concentration of renin substrate. Renin substrate was 0.1 ml of plasma from nephrectomized rats that received an injection of dexamethasone (0.2 mg/rat, s.c.) 24 hours before sacrifice.

The converting enzyme inhibitors 8-hydroxyquinoline (8-HQ, 10% suspension in 0.3 M EDTA; final concentration: 0.86 mM; Mallinckrodt,

St. Louis, MO) and phenylmethylsulfonyl fluoride (PMSF, 5 g in 150 ml ethanol; final concentration: 2.3 mM; Sigma, St. Louis, MO) were added (5 μl each) to the samples to prevent the degradation of ANG I to ANG II. PMSF inhibits the angiotensin converting enzyme by sulfonating the active site of the enzyme (Fahrney and Gold, 1962). 8-HQ prevents the action of converting enzyme by chelating divalent metals (Phillips, 1956) which are necessary for its activity (Fitz, Boyd and Peart, 1971). A volume of 0.1 ml of sodium phosphate buffer (0.5 M, pH 6.0) was added to the samples to maintain the pH at 6.0-6.5 during the incubation. This pH range has been determined by us to be optimal for renin activity. The samples incubated at 37°C for 1 hour. The reaction was halted by addition of 1.6 ml cold distilled water to the Krebs-Ringer samples, or 0.2 ml of cold distilled water to the plasma samples, and subsequent immersion of the samples in a boiling water bath for 3 minutes.

3c. Plasma renin activity

Plasma renin activity was measured by radioimmunoassay of ANG I generated in the plasma sample after a 3 hour incubation. The converting enzyme inhibitors, 8-HQ and PMSF were added (25 μl and 20 μl) to 1.0 ml of plasma at the same final concentration (0.86 mM 8-HQ and 2.3 mM PMSF). Sodium phosphate buffer (0.5 ml; 0.5 M pH 6.0) was added to the plasma samples to reduce the pH of the samples to 6.0-6.5. The samples were incubated for 3 hours at 37°C to generate ANG I from endogenous angiotensinogen (renin substrate). The incubation was stopped by addition of 0.5 ml distilled water and immersion of the

samples in a boiling water bath for 3 minutes.

3d. Radioimmunoassay for generated angiotensin I (ANG I)

After the incubation, the samples (renin concentration, plasma renin activity and plasma renin concentration) were centrifuged (13,000 x g for 10 minutes; Fisher microcentrifuge Model M235A) and aliquots of the supernatant (20 μl and 50 μl) were diluted to a final volume of 0.5 ml with Tris-HCl (pH 8.0) and added to tubes (polystyrene, 12 x 75) with a 0.3 ml solution of an ANG I antibody in a Tris buffer (0.1 M pH 8.0 containing 0.1% gelatin; Sigma, St.Louis, MO). The final volume is 0.8 ml. The ANG I antisera (Reid #3 and Brownfield T1351) were a gift from Dr. M.S. Brownfield, University of Wisconsin. These ANG I antisera were used at dilutions of 1:100,000 (Reid #3) or 1:40,000 (T1351) with 35% binding and a sensitivity limit of 10 pg per tube. After a 24-48 hour incubation period at 4^{o}C with the antiserum, an ^{125}I labelled ANG I tracer was added to the tubes (10,000 counts per minute per tube) for an overnight incubation at 4^{o}C. On the following day, 0.5 ml of a charcoal-dextran (T500; Pharmacia, Piscataway, NJ) suspension and 1.5 ml of cold distilled water were added to the tubes. The tubes were then centrifuged at 2000 x g for 15 minutes. This separated the unbound ^{125}I-ANG I from the antibody-bound tracer in the supernatant which was decanted. The charcoal pellets, containing the free ^{125}I-ANG I, were counted on a Micromedic gamma counter. The data were reduced by RIA_AID computer program (Robert Maciel Associates, Inc., Arlington, MA). Intra-assay variability was 4.6% and interassay variability was 11.9%. The values for renin release from the kidney

slices are reported as ng ANG I generated/hr. These values were then divided by the weight of the kidney slices to obtain ng ANG I/mg kidney/hr. PRA and PRC values were reported as ng ANG I/ml/3 hr and ng ANG I/ml/hr, respectively.

ANG I (Beckman, Arlington Heights, IL) was radiolabelled with ^{125}I (New England Nuclear, North Bellerica, MA) using the chloramine T (Sigma, St. Louis, MO) method of iodination (Greenwood, Hunter and Glover, 1963). Phosphate buffer (0.05 M; 0.02 ml), 12 μl ANG I (0.5 μg/μl) and 20 μl chloramine T (3.5 mg/ml phosphate buffer) were added to the vial containing the ^{125}I and mixed for 3 seconds. This was immediately followed by addition of 25 μl of sodium metabisulfite (4.5 mg/ml phosphate buffer; J.T. Baker Chemical Co., Phillipsburg, NJ) to halt the reaction. Radiolabelled ANG I was first eluted with 0.1 N acetic acid on a Bio-Rad AG 1-X4 anion exchange resin (Bio-Rad Laboratories; Richmond, CA) column that was made from a siliconized disposable pasteur pipette (5 3/4"; Scientific Products) with a small amount of glass wool placed in the tip to support the anion exchange resin. Fractions (12 drops per tube) were collected from the column and the radioactivity in each tube was measured. The radioactive peak fractions eluted off this column (usually tube numbers 4-5) were then placed on a Sephadex G15 column (K15/90, 90 x 1.5 cm; Pharmacia, Piscataway, NJ) and eluted with 0.05 N acetic acid containing 0.1% bovine serum albumin (BSA; Sigma, St. Louis, MO). Fractions (2.0 ml) were collected off the Sephadex column and measured for radioactivity. The tubes that contained the radioactive ANG I (approximately five tubes, numbers 35-39) were pooled and stored at -10°C.

C. In vivo studies of the renin-releasing factor

1. The effect of a serotonin releaser, PCA and serotonin agonists (MK-212 and TFMPP) on PRA, PRC and plasma RRF concentration

PCA is a drug that exerts its action by inducing the release of serotonin from the nerve endings (Sanders-Bush et al., 1975). Administration of PCA has been shown to increase the plasma concentration of RRF. To test if direct activation of serotonin receptors would induce the release of RRF into the blood, the serotonin agonists MK-212 [6-chloro-2-phenyl-(1-piperazinyl)-pyrazine] and TFMPP (m-trimethylphenyl piperazine) were administered to rats and their plasma was collected and assayed for RRF concentration, PRC and PRA.

PCA (10 mg/kg, i.p.) was administered 1 hour before sacrifice. The serotonin agonists, MK-212 (Merck, Sharp and Dohme, Rahway, N.J.) and TFMPP (m-trifluoromethyl-phenylpiperazine; Aldrich Chemical CO. Inc.; Milwaukee, WI) were administered, at a dose of 10 mg/kg i.p, to rats 30 minutes before sacrifice. Saline and the drugs were administered at a volume of 2.0 ml/kg i.p. The rats were decapitated and their plasma was collected in chilled centrifuge tubes containing 20 units of heparin. The samples were centrifuged at 1,500 x g for 30 minutes. The plasma from each rat was divided into 3 aliquots. One aliquot of 1.0 ml was saved for the determination of plasma renin activity and the second aliquot (0.2 ml) was saved for the determination of plasma renin concentration (PRC). The third aliquot (2.0 ml), was saved for the determination of plasma RRF concentration. It was diluted with 2.0 ml distilled water, placed in a boiling water bath (to denature renin) for 20 minutes and centrifuged at 13,000 x g

for 20 minutes. The supernatant was lyophilized to dryness. The dry peptide residues were resuspended in 0.25 ml saline and 0.2 ml of the resuspended plasma was added to vials containing 2.0 ml Krebs-Ringer and kidney slices to test for renin-releasing activity as described above (section B part 1b).

2. Effect of a stressor on PRA and plasma RRF concentration

2a. Description of the conditioned emotional response (stress) paradigm

Rats were subjected to a conditioned emotional response (CER), or fear paradigm. The CER was performed in a rectangular chamber (49 cm long x 23 cm wide x 28 cm high) with a grid floor composed of stainless steel rods (7.6 mm diameter) spaced 1.3 cm apart. The front wall of the chamber was constructed from clear Plexiglas. The remaining walls and ceiling of the chamber were made of white Plexiglas. Illumination was provided by a fluorescent lamp (20 W) mounted on the outside of the rear wall. The chamber was located in a sound attenuated room 7.5 meters from the animal quarters. Scrambled constant current shock was delivered through the grid floor by a Grayson-Stadler shock generator. The rats were carried to the stress room in a plastic cage that was identical to their home cage. Three minutes following their placement in the chamber, the experimental animals received an inescapable foot shock (1.0 mA DC for 10 seconds). Immediately thereafter, the rats were returned to their home cage. This procedure was repeated once a day for three consecutive days. Control rats were treated the same, except that shock was not administered at any time. By the third day,

it was quite apparent that the stressed rats had learned that placement in the chamber would be followed by a shock. In contrast to control animals, the stressed rats defecated, urinated and alternated between freezing and jumping behaviors. On the fourth day, the rats were placed in the chamber for three minutes. Instead of receiving the shock, they were removed and immediately sacrificed by decapitation in a room located 3.0 meters from the stress room.

2b. Preparation of plasma from stressed and unstressed rats

Blood from the decapitated rats was collected into centrifuge tubes containing 0.5 ml of a 0.3 M EDTA (ethylene diamine tetraacetate, pH 7.4; Sigma, St. Louis, MO) solution. The plasma was divided: 1.0 ml for determination of PRA and 0.2 ml for determination of PRC. The third aliquot (2.0 ml), saved for determination of plasma RRF concentration, was diluted with 2.0 ml distilled water, placed in a boiling water bath (to denature renin) for 20 minutes and centrifuged at 13,000 x g (Fisher microcentrifuge Model 235A) for 20 minutes. The supernatant was lyophilized to dryness. The dry peptide residues were resuspended in 0.25 ml saline and 0.2 ml of the resuspended plasma was added to vials containing 2.0 ml Krebs-Ringer and kidney slices to test for renin-releasing activity as described above.

3. Role of disulfide bonds in RRF: In vivo study with cysteamine and PCA

3a. In vivo study with PCA and cysteamine pretreatment

It is possible that since the RRF is a peptide, it may be contain

disulfide bonds that stabilize the molecule and maintain its active conformation. Administration of cysteamine (2-aminoethanethiol) has been shown to destroy somatostatin (Palkovits, 1982) and prolactin (Sagar et al., 1985) immunoreactivity by reducing the disulfide bonds present in these molecules. To determine if RRF contained disulfide bonds, cysteamine was administered 3 hours before PCA. The rationale behind this experiment was if RRF contained disulfide bonds, cysteamine would reduce the disulfide bonds and render RRF inactive and prevent the PCA-induced increase in plasma renin activity.

Male rats were injected with cysteamine (300 mg/kg; Aldrich Chemical Co, Inc., Milwaukee, WI) or saline (2.0 ml/kg), subcutaneously (s.c.), 4 hours prior to sacrifice. This was followed 3 hours later, by injection of PCA (10 mg/kg) or saline at a volume of 2.0 ml/kg i.p. The rats were decapitated 1 hour after PCA injection, and trunk blood was collected in centrifuge tubes containing 0.5 ml of 0.3 M EDTA (pH 7.4). The plasma was stored at -40°C until PRA and PRC were determined.

3b. Lack of an effect of cysteamine in the renin assay

The generation of ANG I from angiotensinogen has been found to be increased when the sulfhydryl reagents, cysteamine or dithiothreitol, were added during the incubation period (Poisner and Hong, 1977; Funae, Sasaki and Yamamoto, 1979). To ensure that the values for PRA in this experiment were not influenced by treatment with cysteamine, control plasma pools were incubated with and without cysteamine to test for interference in the assay.

Cysteamine was added to plasma samples for a final concentration of 3.8×10^{-3} M. This value was calculated to be the hypothetical distribution of cysteamine per body weight (allowing 70% for body water) after mixing. High and normal PRA samples (1.0 ml) were prepared with 8-HQ and PMSF (25 μl and 20 μl; final concentration 0.86 mM 8-HQ and 2.3 mM PMSF) and sodium phosphate buffer (0.5 ml of a 0.5 M solution, pH 6.0). In addition, 0.01 ml of cysteamine (0.38 M) was added to the samples for incubation for 3 hours at 37°C. The assay for generated ANG I was performed as previously described (Method section B part 3).

4. Role of parathyroid hormone (PTH) as the mediator of the PCA-induced increase in renin release (a possible RRF)

Parathyroid hormone (PTH) was investigated as a possible renin-releasing factor. It has been shown that PTH produces increases in plasma renin activity (Powell et al., 1978; Smith et al., 1979) and the molecular weight of PTH is approximately 9,000 which is similar to the molecular weight of RRF. To test the involvement of PTH in PCA-induced increase in PRA, parathyroidectomized rats were injected with PCA to test if removal of the parathyroid gland prevented the increase in PRA.

Thyroparathyroidectomized and sham-operated rats were purchased from Kurt Johnson Laboratories (Bridgeview, IL). Rats were anesthetized with ether and a ventral incision was made on the ventral surface of the neck and the muscles were teased apart. The thyroid and parathyroid glands were removed and the wound was sutured. The thyroparathyroidectomized rats received daily injections of tri-

iodothyronine (T_3; 5 μg/kg s.c., dissolved in 1.0 M NaOH and saline; Calbiochem-Behring Corp., La Jolla, CA) after surgery and their water was supplemented with 2% calcium lactate and 10% glucose. Rat chow was available ad libitum. Sham operated animals underwent the same surgical procedure only without removal of the parathyroid and thyroid glands. They received daily vehicle injections. Rat chow and tap water were available ad libitum. On the fourth day after surgery, the rats received either an injection of PCA (10 mg/kg, i.p.) or saline 1 hour before sacrifice. The rats were decapitated and trunk blood was collected into centrifuge tubes containing 0.3 M EDTA (pH 7.4). Plasma was stored at -40°C until determination of PRA.

5. A dose-response study for possible renin-releasing activity of neurophysin II (NPII)

Neurophysin II (NPII; bovine), the vasopressin associated neurophysin, was tested as a possible renin-releasing factor. Male Sprague-Dawley rats received intraperitoneal injections of neurophysin II (0.1, 0.5, 2.0, 10.0 or 20.0 μg/kg; Sigma, St. Louis, MO). The peptide was dissolved in 0.9% saline and administered at a volume of 2.0 ml/kg i.p., 20 minutes before sacrifice. The rats were sacrificed, and trunk blood was collected in centrifuge tubes containing 0.5 ml of 0.3 M EDTA (pH 7.4). The plasma was stored at -40°C until PRA and PRC were determined.

D. In vitro characterization of RRF

1. Preparation of PCA-plasma and saline-plasma fractions with molecular weights of 1,000-5,000; 5,000-10,000 and 10,000-20,000

To determine the approximate molecular weight range of RRF, plasma from either PCA-treated or saline-treated rats was pooled and filtered to obtain plasma fractions of different molecular weights. These fractions were tested for renin-releasing activity in the renin release bioassay using kidney slices.

In each of the following experiments, approximately 40 rats were nephrectomized under halothane (Burns; Glenview, IL) anesthesia 20 hours before administration of p-chloroamphetamine (PCA; Regis, Morton Grove, IL) or saline. PCA (12 mg/kg, i.p.) and saline were administered at a volume of 2.0 ml/kg, 1 hour before decapitation. The blood was collected in centrifuge tubes containing 20 units of heparin (Upjohn; Kalamazoo, MI) and was centrifuged in a refrigerated centrifuge (1,500 x g) for 30 minutes at 4°C. The plasma was pooled into either PCA-plasma (plasma from rats that were injected with PCA) or saline-plasma (plasma from rats treated with saline) pools (of approximately 150 ml each) depending on which treatment the animal received. Ultrafiltration of the plasma was performed in a low pressure stirred cell (Nuclepore, Pleasanton,CA) at 4°C.

For this fractionation procedure, the PCA-plasma and saline-plasma pools were placed in a boiling water bath for 20 minutes, and centrifuged at 13,000 x g for 20 minutes. Ultrafiltration of the supernatant of the plasma pools was performed at 4°C in a low pressure stirred cell first with a low adsorption membrane with a molecular

weight cut-off of 20,000. The filtered fraction containing solutes with molecular weights below 20,000 was refiltered through a membrane with a molecular weight cutoff of 10,000. The latter fraction was then refiltered through a membrane with a molecular weight cutoff of 5,000. The remaining fraction containing solutes with molecular weights below 5,000 was refiltered through a membrane with a molecular weight cutoff of 1,000. The material that was retained on the respective membranes was resuspended in 3-4 ml saline. Thus, 3 separate fractions were obtained, one that contained solutes in the molecular weight range of 1,000-5,000, one that contained solutes with a molecular weight range of 5,000-10,000 and the last fraction that contained solutes with molecular weights of 10,000-20,000. All the fractions were stored at -40°C.

The PCA-plasma and saline-plasma fractions (M.W.=1,000-5,000; 5,000-10,000 and 10,000-20,000) were added to the kidney slices (non-bubbled method) at a volume of 0.2 ml for the 1 hour incubation period to test for renin-releasing activity.

2. Evaluation of possible renin releasing activity of 5-HT and PCA <u>in vitro</u>

Since PCA, a 5-HT releaser, was used to release RRF, it was also possible that the plasma that was tested for renin releasing activity contained some residual PCA molecules. In addition, since PCA releases 5-HT, the 5-HT content in the plasma could also have been increased. PCA and 5-HT were added to the kidney slice bioassay to test if these drugs could have altered renin release. Fenfluramine (another 5-HT

releaser) and saline, were also added to kidney slices to test for renin-releasing activity. The drugs PCA (M.W. 206), 5-HT (M.W. 176; Sigma, St. Louis, MO) and fenfluramine (M.W. 249; A.H. Robins, Richmond, VA) were dissolved in saline for an initial concentration of 10^{-4}M. A volume of 0.02 ml of each drug was added to the kidney slices for the incubation period. The final dilution of the drug in the vials was 10^{-6}M; this corresponded to the calculated distribution of the drug in body water. This calculation was performed by estimating the concentration of the drug in body water (which was assumed to be 70% body weight). This value was then divided by the molecular weight of the drug to obtain the concentration of the substance within the animal.

3. Incubation of the PCA-plasma and saline-plasma fractions with Pronase

In order to test whether or not RRF is a peptide, a fraction of PCA-plasma and a fraction of saline-plasma (M.W. = 5,000-10,000) were incubated with pronase, a mixture of non-specific proteases (Narahashi, 1970). If RRF were a peptide or a protein, then incubation with pronase should destroy its renin-releasing activity.

Pronase E (a non-specific protease, type XIV, isolated from Streptomyces griseus that has an activity of 4 units per mg; Sigma, St. Louis, MO) was added to the PCA-plasma fraction that had renin-releasing activity (the fraction containing solutes with molecular weights between 5,000-10,000) and to the equivalent fraction from saline-treated rats. Pronase was dissolved in a 0.1 M borate buffer pH

7.5 containing 5 mM $CaCl_2$, at a concentration of 20 μg/200 μl and was added to a volume of 1.6 ml of PCA-plasma and saline-plasma fractions (M.W. = 5,000-10,000). The borate buffer vehicle that was used to dissolve pronase was added at the same volume (0.2 ml) to an equal aliquot (1.6 ml) of the PCA-plasma and saline-plasma fractions (M.W. = 5,000-10,000). All of the samples were incubated at 37°C for 4 hours. After incubation, all aliquots were placed in a boiling water bath for 20 minutes (to denature the pronase) and centrifuged at 13,000 x g for 30 minutes. The plasma fractions were added to the kidney slices at a volume of 0.2 ml per vial to test for renin-releasing activity.

E. Studies of RRF in rat brain

1. Dissection technique

Rats were sacrificed by decapitation and the brains were removed immediately and placed on a cold glass plate kept on ice for dissection. The brains were dissected by the procedures outlined by Glowinski and Iversen (1966). The pituitary gland was removed from the sella turcica with the aid of a spatula. The cerebral cortices were gently dissected apart and removed from the rest of the brain. The hippocampus, caudate-putamen and amygdala were removed from the cortical tissue. The hippocampal formation was easily removed from the cortex by blunt dissection with a spatula. The amygdala was cut from the cortex at a site rostral to the hippocampus. The corpus callosum and lateral ventricle formed the boundaries for the caudate-putamen which was carefully dissected with a curved scissors.

The hypothalamus was delineated rostrally by the optic chiasm and

caudally by the mammillary bodies. A transverse cut made at the level of the mammillary bodies separated the hypothalamus from the midbrain. The anterior commissure (located at the level of the optic chiasm) was the horizontal reference for the separation of the thalamus from the hypothalamus.

The midbrain was separated from the pons by a diagonal knife cut just caudal to the inferior colliculi. The cerebellum was removed from the remaining brain tissue by blunt dissection and severing of the cerebellar peduncles. The pons was separated from the medulla by a cut caudal to the transverse fibers of the pons. After dissection, the brain parts were wrapped in aluminum foil and placed on dry ice. The samples were stored at -70°C.

2. Extraction of the peptide from rat brain

2a. Comparison of different extraction media from whole brain tissue

Whole rat brains were cut sagittally and each half was homogenized (1 g/10 ml) with either: (1) cold 0.1 N hydrochloric acid, (2) hot 0.1 N hydrochloric acid, (3) cold 0.1 N hydrochloric acid/absolute ethanol mixture (20:80 vol:vol), (4) cold 0.1 N perchloric acid, (5) hot 0.1 N perchloric acid or (6) boiling distilled water. The tissue was homogenized with a Potter-Elvehjem tissue grinder (Scientific Products, McGaw Park, IL), and centrifuged for 30 minutes at 18,000 x g. The supernatant was transferred to polypropylene tubes, lyophilized and stored at -70°C until they were tested for renin-releasing activity. The samples were reconstituted in 2.0 ml of Krebs-Ringer buffer and the pH of the samples was readjusted to 7.0-7.4 with 1.0 M NaOH before

addition to the kidney slices. The extract (0.20 ml) was added to the kidney slices for the 1 hour incubation period.

2b. Comparison of extraction with cold 0.1 N perchloric acid and boiling distilled water from rat hypothalamus

The extraction media that yielded the best results with whole brain tissue were the 0.1 N cold perchloric acid and boiling distilled water. Hypothalamic tissue was homogenized with either 0.1 N perchloric acid or boiling distilled water to test which medium was more effective in extracting RRF from hypothalamic tissue. Rat hypothalami (6 hypothalami, approximately 0.11 g) were homogenized in either cold 0.1 N perchloric acid or boiling distilled water in a 1 g/10 ml ratio with a pyrex glass tissue homogenizer. The homogenates were centrifuged for 30 minutes at 18,000 x g. The supernatants were transferred to polypropylene tubes, lyophilized and stored at -70°C until they were tested for renin-releasing activity. These samples were resuspended in 0.1 ml of Krebs-Ringer buffer, the pH was readjusted (pH 7.0-7.4 with 1.0 M NaOH) and 0.02 ml was added to the kidney slices during the incubation period.

3. Preparation of hypothalamic, cerebellar and pituitary extracts

Hypothalamic tissue was dissected from 10 rat brains immediately after decapitation and stored at -70°C. The brain was placed, dorsal surface down, and the hypothalamus was excised from the ventral surface with fine curved scissors. The rostral border was the decussation of the optic tracts and the decussation of the anterior commissure. The

caudal border was the mammillary bodies. The pituitary was removed from the sella turcica and the cerebellum was removed from the brainstem. A volume of 10 ml/g of boiling water was added to the hypothalamic (0.97 g), cerebellar (0.85 g) or pituitary (0.74 g) tissue. The tissue was homogenized with a glass tissue grinder, placed in a boiling water bath for 20 minutes and centrifuged at 13,000 x g for 30 minutes. An aliquot of 4.5 ml of the supernatant from each tissue was lyophilized to dryness and resuspended in 0.2 ml of saline. A volume of 0.02 ml was added to the kidney slices to test for renin-releasing activity.

4. Dose response of hypothalamic tissue extracts

Rat hypothalamic tissue (5.8 g, approximately 280 hypothalami) was homogenized in boiling distilled water (1 g/ml) with a pyrex tissue grinder. The homogenate was placed in a boiling water bath for 20 minutes and centrifuged at 13,000 x g for 30 minutes. The supernatant was collected, 0.4 ml of the extract was saved for serial dilutions and 1.6 ml of the supernatant was lyophilized (Savant Speed-Vac). The straight, non-lyophilized, hypothalamic extract was serially diluted with saline (1:15, 1:10, 1:5, 1:2 and 1:1) to obtain the equivalent volume of the content of 0.07, 0.1, 0.2, 0.5 and 1 hypothalamus when added (0.2 ml) to the kidney slices, respectively. The lyophilized pellet was resuspended in 0.4 ml of 0.9% saline and diluted (1:2 and 1:1) to yield extracts that were equivalent to the content of 2 and 4 hypothalami. Aliquots (0.02 ml) of the hypothalamic extracts were added to the kidney slices for the 1 hour incubation.

5. Distribution of RRF in rat brain

Rat brains were dissected as described in section E part 1. The brain areas that were analyzed for renin-releasing activity were the pituitary gland, pons, thalamus, caudate-putamen, hippocampus, midbrain, amygdala, hypothalamus, cerebral cortex, medulla oblongata and cerebellum. The brain tissues were homogenized in boiling distilled water (1 g/ml) with a glass tissue grinder. Afterwards, the homogenates were placed in a boiling water bath for 20 minutes and then centrifuged at 13,000 x g for 30 minutes. The extracts were added to the kidney slices at a volume of 0.02 ml for the incubation period. This volume corresponded to the equivalent of 1 hypothalamus using the 1 g/ml dilution. Standardizing the dilution and volume of the different extracts allowed direct comparison of the renin-releasing activity of the different brain areas.

6. Distribution of RRF in colchicine-treated rat brains

In order to identify the cell bodies of origin of RRF, rats were treated with colchicine, a drug that inhibits axonal transport of materials from the cell body, to prevent the movement of RRF from the cell body. The results from the renin release bioassay of the colchicine-treated brain parts would indicate where the cell bodies that contain RRF were located. After colchicine treatment, the areas that have high renin-releasing activity would indicate RRF contained in cell bodies.

6a. Animals

Rats were prepared for stereotaxic surgery by anesthetizing them with pentobarbital (50 mg/kg i.p.; Butler Co., Columbus, OH) and pretreating them with methyl atropine bromide (0.4 mg/kg i.m.; Regis Chemical Co., Morton Grove, IL) which minimized respiratory tract secretions. After surgery, the rats received an injection of ampicillin (50 mg/kg, i.m.; Sigma, St. Louis) to prevent infection. Care was taken to avoid undue discomfort to the animals.

6b. Intracerebroventricular injections of colchicine

The rats were secured in a Kopf stereotaxic apparatus with the incisor bar 5.0 mm below the intra-aural line. The injection needle was placed bilaterally, 1.4 mm lateral to the midsagittal suture, 0.5 mm caudal from bregma and 4.5 mm ventral from the skull surface. Colchicine (Sigma, St. Louis) was administered bilaterally (150 μg/20 μl saline; 10 μl into each side over a 10 minute period) into the lateral cerebral ventricles. The needle was left in place for 5 minutes to prevent dorsal diffusion of the drug.

The rats were decapitated 48 hours after injection of colchicine and the brains were dissected as indicated in section E part 1. The brain parts were homogenized in boiling distilled water and centrifuged. The supernatant (0.02 ml) was added to the kidney slices to test for renin-releasing activity.

6c. Verification that colchicine does not interfere in the renin release bioassay

In a test that preceded the determination of RRF in the brains of colchicine-treated rats, colchicine was added to the kidney slices to test whether colchicine would alter renin release. Saline or colchicine was added to three test substances saline, isoproterenol (10^{-5}M) or cerebellar extract, (homogenized as 1 mg/ml in boiling distilled water). The concentration of colchicine was calculated as the approximate dilution of colchicine in the treated rat brains [150 μg colchicine into one brain (1.80 g) homogenized in 1.8 ml boiling distilled water, which corresponded to 0.083 mg colchicine/ml of sample]. This calculation did not take into account any diffusion of colchicine from the brain. The samples were added to the kidney slices at a volume of 0.02 ml for the incubation period.

F. Distribution of RRF in peripheral tissues

After decapitation, the adrenal gland, spleen, liver, kidney and skeletal muscle (from the abdomen) were dissected from normal rats. The tissues were homogenized (1 g/ml) with boiling distilled water in glass tissue homogenizers. The homogenates were placed in a boiling water bath for 20 minutes and centrifuged at 13,000 x g for 30 minutes. The supernatant of each extract (0.02 ml) was added to the kidney slices for the incubation period.

G. Effect of nephrectomy on the hypothalamic content of RRF

Rats were bilaterally nephrectomized as previously described in the Method section B part 3a. Another group of control or sham-operated rats were treated identically except that their kidneys were not removed. Twenty-four hours after surgery the rats were decapitated. The brains were quickly removed and the hypothalami were dissected and stored at -70°C. On the day of the kidney slice bioassay, the hypothalami were individually homogenized 1 g/ml in boiling distilled water, placed in a boiling water bath and centrifuged (13,000 x g) for 20 minutes. Each hypothalamic extract (20 μl) was tested individually in the RRF bioassay.

H. Stimulation of RRF from hypothalamic neurons

Superfusion of hypothalamic explants was performed to determine whether neurons in the hypothalamus could release RRF in response to depolarization. The method used for the superfusion of rat hypothalamus was adapted from that used by Gallardo and Ramirez (1977).

1. Preparation of hypothalamic explants

Rats (175-200 g) were decapitated and the hypothalami were quickly dissected and placed in a petri dish containing cold Krebs-Ringer buffer that was bubbled with 95% O_2-5% CO_2. The Krebs-Ringer superfusion buffer used for this study had a composition of 117 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 11.5 mM glucose and 25 mM $NaHCO_3$ (final pH 7.4). The rostral and caudal boundaries for the hypothalamus were the optic chiasm and the

mammillary bodies, respectively. The anterior commissure was used to define the dorsal limit of the hypothalamus.

In some cases rat hypothalamo-hypophyseal explants were used, in others, the hypothalamic explants were used without the hypophysis. The brain was removed from the caudal approach and the pituitary was carefully removed away from the sella while still attached to the hypothalamus. The hypothalamus was then dissected from the rest of the brain and the hypothalamo-hypophyseal explant was placed in cold oxygenated Krebs-Ringer. A total of four explants were placed in each superfusion chamber.

2. Superfusion of rat hypothalamic explants

The superfusion chambers (0.5 ml) were constructed from 3.0 ml plastic disposable syringes that were cut in half. The hypothalamic tissue was placed at the bottom of the chamber and rested on a few strands of glass wool. Superfusion buffer entered the chamber from the bottom through a hypodermic needle (18 gauge) that was attached to the syringe (superfusion chamber; refer to Figure 2). The needle was inserted into rubber stoppers on a Plexiglas stand and the apparatus (stand with superfusion chambers) was placed in a water bath maintained at 37^{o}C. The buffer was continuously delivered into the chamber by a Gilson peristaltic pump (Gilson Medical Electronics, Inc., Middleton, WI) at a flow rate of 0.08 ml/minute. A PE90 tubing (Becton Dickinson and Co., Parsippany, N.J.) led the buffer through a silastic manifold tubing (0.065 inch I.D.) and another length of PE90 tubing connected the silastic tubing to the needle on the chamber. The Krebs-Ringer

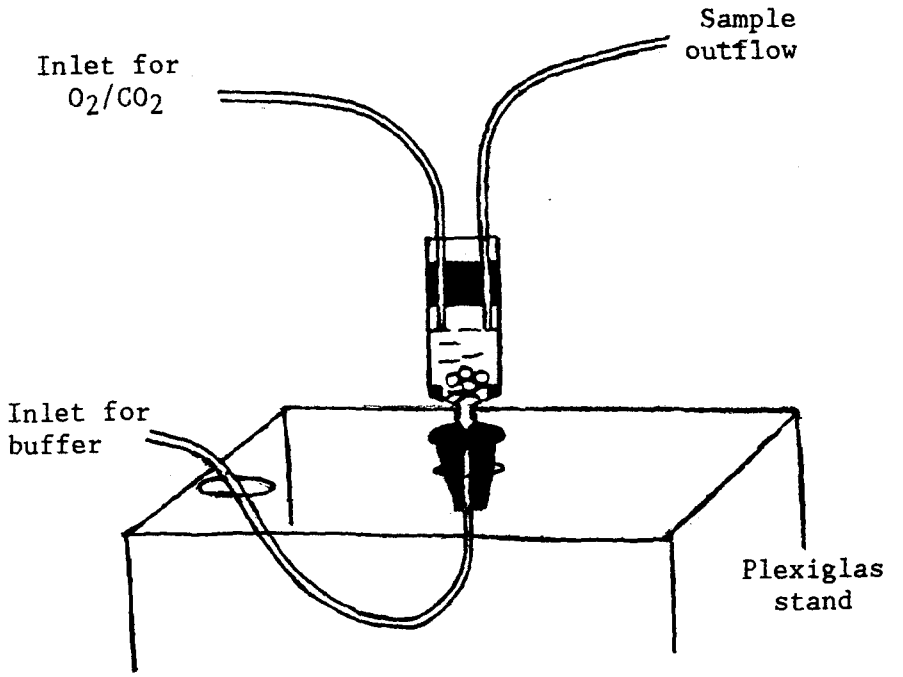


Figure 2. Illustration of superfusion chamber.

buffer entered the chamber from the bottom and filled the chamber as it filtered up through the tissue. The chamber was closed with a rubber stopper containing two 20 gauge needles; one was fitted with PE90 tubing that allowed the inflow of the 95% O_2 - 5% CO_2 mixture and the other needle was fitted with tubing (PE90) that served as the outlet for the liquid (sample) and oxygen. As the gas entered the chamber, it formed bubbles that expelled the liquid sample through the tubing. This also kept the volume of the chamber constant at 0.5 ml.

After a 30 minute equilibration period, samples were collected over a 30 minute control period into polypropylene tubes that were maintained on ice. To test for the potassium stimulated release of RRF from the hypothalamus, the Krebs-Ringer buffer was replaced with a high potassium Krebs-Ringer buffer containing 60 mM KCl. The composition of the high potassium Krebs-Ringer buffer was 61.7 mM NaCl, 60 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 11.5 mM glucose and 25.0 mM $NaHCO_3$ (final pH 7.4). The sodium concentration of the buffer was reduced to maintain the proper osmolarity. Samples were collected for 30 minutes into polypropylene tubes that were kept on ice. For both the control and experimental (high potassium) periods, the pH of the buffer was monitored to confirm that the pH did not fluctuate over time.

3. Preparation of superfusate samples

After the samples were collected (approximately 3 ml), they were filtered and concentrated by ultrafiltration on a micropartition system (MPS I, Amicon, Danvers, MA) through a membrane that had a molecular

weight cutoff of 500. The samples that were collected with the high potassium buffer were further washed with 0.9% saline to remove the excess potassium ions. The final volume for all the samples after concentration on the filter was 0.3 ml. This corresponded to approximately a 10 fold increase in concentration. A volume of 0.2 ml was added to the kidney slices to test for the renin-releasing activity of the hypothalamic superfusates.

I. Statistical analysis of the data

1. Representation of the data

The data were represented as mean ± S.E.M. (standard error of the mean). The sample mean was the average of the numbers in the experimental group (n). The standard error of the mean (S.E.M.) was reported along with the mean to establish a confidence interval for the population mean. The S.E.M. was calculated from a formula relating the standard deviation and the number of samples in the test group (n).

2. Statistical tests

2a. Student's t-test

The Student's t-test was used to compare two means either from the same sample population (paired t-test) or from different sample populations (unpaired t-test). As a rule for the analysis of these data, the significance levels used for the t-test and other tests (ANOVA and Duncan's new multiple range test) were 5% and 1%. The t-tests used were all two-tailed. Two-tailed t-tests are indicated when looking for a difference between the means ($mean_1$ not equal to

mean$_2$) rather than testing the probability that one mean is greater than the other (one-tailed t-test).

2b. Analysis of variance

The analysis of variance (ANOVA) was designed to compare several groups (means) after a treatment or to compare the same group using different treatments. The one way ANOVA was used to compare the effect of different treatments between relatively homogenous, random groups. The F value is calculated and defined as the ratio of two independent estimates of the variance (Steel and Torrie, 1960). The F value is compared to values in a table for the level of significance. A significant F value indicates that there is less than 5% or 1% that two or more means are not different between the treatment groups. However, the ANOVA does not show which means are significantly different from one another.

The two way ANOVA is used when there are two or more ways of grouping the data (Steel and Torrie, 1960). The individuals in the experiment are grouped into blocks according to characteristics. For example, animals that are subject to surgery before receiving a drug treatment would comprise one block. The units within the block should be treated identically, minimizing any variations in techniques or conditions. This assures that the differences observed will be due to the effects of treatment. For a two way ANOVA, three F values are obtained: one indicates if there is a treatment effect; another indicates a difference between the blocks and the third indicates if there is an interaction between the treatment and block effects.

2c. Duncan's new multiple range test

The Duncan's new multiple range test was applied in addition to the ANOVA to determine which means are statistically different (Duncan, 1955). Generally, tests of comparison should be performed only when the F values are significant, however, a Duncan's multiple range test can be performed without a significant F value. The Duncan's also has a protection level within the computation of statistic that is determined by the degrees of freedom. The protection guards against the possibility of finding a false positive (Type I error; rejecting the Null hypothesis when it is true). Other multiple range or comparison tests have a protection level that is the same for all sample sizes. The data necessary to perform a multiple range test are the mean squared error obtained from the ANOVA, the number of samples in the group (n) and a value that is obtained from a table of significant ranges. The value obtained from the table is dependent on the degrees of freedom, the level of significance and the number of means being tested. The desired range is then multiplied by the standard error of the mean (obtained by taking the square root of the mean squared error divided by the number of samples) to make a shortest range of significance. If the distance between two means is greater than the shortest range of significance then the difference between the means is significant.

CHAPTER IV

RESULTS

A. Development of a renin release bioassay

Table 1 compares the values for renin release after a 30 minute incubation between the bubbled and unbubbled kidney slice methods. The unbubbled method yields renin values that were higher and more consistent (5.0 ± 0.6 to 7.5 ± 0.5 ng ANG I/mg kidney/hr) than those obtained with the bubbled method (0.23 ± 0.04 to 1.90 ± 0.30 ng ANG I/mg kidney/hr).

The values for pH, kidney slices weight and renin release in one experiment are presented in Table 2. All these values were measured after a 30 minute incubation period. The kidney slice weights were within a small range of distribution with a mean of 18.3 ± 0.5 mg. The pH values of the incubation medium of the 24 vials ranged from 7.4 to 7.5 with a mean of 7.45 ± 0.01. Both the values for pH and kidney slice weight were within a close range indicating that the method of oxygenation and method of slicing the kidneys (vibratome) were reliable. The values for renin release from the slices ranged from 3.4 to 11.1 ng ANG I/mg kidney tissue/hr with a mean value of 5.9 ± 0.37 ng ANG I/mg kidney/hr. This is a much smaller range than that observed with the bubbled kidney slice method. The small range of distribution

TABLE 1 Comparison of interexperimental variation of renin release (ng ANG I/mg kidney/hr) from kidney slices between the bubbled and improved unbubbled *in vitro* methods.

Experiment	Bubbled Method	Experiment	Unbubbled Method
1	1.90 ± 0.30 (24)	5	7.2 ± 0.6 (24)
2	1.80 ± 0.15 (24)	6	5.0 ± 0.6 (24)
3	0.23 ± 0.04 (24)	7	7.5 ± 0.5 (24)
4	0.53 ± 0.07 (24)*	8	6.4 ± 0.4 (24)
		9	6.7 ± 0.4 (24)
		10	5.9 ± 0.4 (24)
		11	7.1 ± 0.4 (36)

* This value for renin release (experiment 4) was obtained from kidney slices that were cut on a vibratome, but were incubated in Krebs-Ringer that was bubbled with the 95% O_2-5% CO_2 gas. The kidney slices used in experiments 1,2 and 3 were cut using a hand-held razor.

TABLE 2 Intraexperimental variability for renin release from kidney slices during incubation with Krebs-Ringer Bicarbonate solution for 30 minutes.

Vial	pH	Weight of the slices (mg)	Renin release (ng ANG I/mg kidney/hr)
1	7.4	17.9	4.9
2	7.5	19.0	6.7
3	7.6	16.0	6.2
4	7.4	16.3	3.6
5	7.4	20.1	5.0
6	7.6	18.1	3.3
7	7.5	19.8	3.4
8	7.4	15.6	4.4
9	7.5	19.6	6.5
10	7.4	14.2	8.7
11	7.5	17.2	5.1
12	7.4	19.7	5.7
13	7.4	17.2	5.2
14	7.5	16.9	5.7
15	7.5	16.6	7.0
16	7.5	17.3	6.0
17	7.5	22.0	6.1
18	7.4	22.8	5.8
19	7.5	22.0	6.6
20	7.5	18.5	8.4
21	7.4	15.3	3.4
22	7.4	20.2	7.6
23	7.4	14.1	11.1
24	7.5	22.8	6.2
Mean ± S.E.M	7.45 ± 0.01	18.3 ± 0.5	5.9 ± 0.4

of renin release, both intraexperimentally and interexperimentally, is is an indicator of the reliability of the new kidney slice method.

Addition of isoproterenol produced a significant increase (Student's unpaired t-test, two-tailed, $t=2.460$, $df=14$, $p < 0.05$) in renin release from the kidney slices (Table 3). As can be seen in the following experiments, the values for renin release in the slices that received either saline or isoproterenol are reproducible between experiments.

B. Check for specificity in the bioassay for RRF

1. Test for non-specific activation of renin by plasma fractions (Table 4)

This control experiment was performed to verify that the effect of the PCA-plasma fraction (M.W. 5,000-10,000) was not due to non-specific activation of inactive renin that could have been released by the kidney slices. Kidney slices were removed from the Krebs-Ringer medium after a 1 hour incubation, and the Krebs-Ringer solutions were pooled and then divided into 2.0 ml aliquots. Saline, saline-plasma (M.W. 5,000-10,000) or PCA-plasma (M.W. 5,000-10,000) samples were added to the Krebs-Ringer aliquots for a 1 hour incubation at 37°C. The concentration of renin was measured as the ability to generate ANG I from a saturating concentration of renin substrate. As can be seen from Table 4, there was no activation of renin by the plasma fractions.

TABLE 3 Effect of saline or isoproterenol (10^{-7}M) on renin release from kidney slices using the unbubbled method.

	Renin release (ng ANG I/mg kidney/hr)
Saline (n=10)	10.6 ± 0.9
Isoproterenol 10^{-7}M (n=6)	19.7 ± 3.4*

Data represent mean ± S.E.M.
* Significant difference from the saline group, $p < 0.05$ (Student's unpaired t-test, two tailed: t=2.460, df=14).

TABLE 4 Test for non-selective activation of renin in the incubation medium (Krebs-Ringer) by the fractions (M.W. = 5,000-10,000) of plasma from saline-treated or PCA-treated rats.

Fraction added	Renin activity (ng ANG I/ml/hr)
PCA-plasma fraction (M.W. 5,000-10,000)	231.2 ± 19.0
Saline-plasma fraction (M.W. 5,000-10,000)	276.0 ± 8.8
Saline	238.6 ± 22.8

Each data point represent mean ± S.E.M. of 6 determinations.

2. Test for non-specific generation of angiotensin I from renin substrate by plasma and brain fractions (Table 5)

Another control experiment was performed to verify that the saline-plasma and PCA-plasma fractions (M.W. 5,000-10,000) and the different brain extracts did not have renin-like activity that would have resulted in the non-specific generation of ANG I. Rat hypothalamic, cerebellar, and pituitary extracts and the PCA-plasma and saline-plasma fractions (M.W. 5,000-10,000) were added to the Krebs-Ringer buffer and incubated with renin substrate for 1 hour. As can be seen in Table 5, the values for the amount of generated ANG I were below the sensitivity limit of the assay indicating that the effect of the test substances on renin release from the kidney slices was not due to non-specific generation of ANG I in the bioassay. The normal rat plasma was tested at the same concentration as the other substances and produced detectable amounts of ANG I.

C. In vivo studies of the renin-releasing factor

1. The effect of the serotonin releaser, PCA and serotonin agonists (MK-212 and TFMPP) on PRA, PRC and plasma RRF concentration (Table 6)

The 5-HT releaser PCA (10 mg/kg, i.p.) and two 5-HT agonists, MK-212 (10 mg/kg, i.p.) and TFMPP (10 mg/kg, i.p.) were administered to rats to test if stimulation of serotonin receptors would increase PRA, PRC and the concentration of RRF in the blood. PCA, TFMPP and MK-212 all significantly increased plasma renin activity [ANOVA: $F(3,36)=22.741$, $p < 0.001$; Duncan's multiple range test: shortest range of significance between means = 9.6, $p < 0.01$] and plasma renin

TABLE 5 Test for non-specific generation of angiotensin I from angiotensinogen (renin substrate) by hypothalamic, cerebellar, pituitary and plasma extracts and fractions.

Substance added	ng ANG I generated/ml/hr
Rat hypothalamic extract	N.D.
Rat cerebellar extract	N.D.
Rat pituitary extract	N.D.
PCA-plasma fraction (M.W. 5,000-10,000)	N.D.
Saline-plasma fraction (M.W. 5,000-10,000)	N.D.
Unfractionated normal rat plasma	3.75 ± 0.15

Each point represents mean ± S.E.M. of 6 determinations. The extracts and fractions were incubated with renin substrate (20 μl/2.0 ml) without renin in the medium to test if these extracts possessed renin-like activity.

TABLE 6 The effect of saline or PCA, MK-212 or TFMPP (10 mg/kg i.p.) on plasma renin activity (PRA), plasma renin concentration (PRC) and plasma concentration of renin-releasing factor (RRF) in conscious rats.

Treatment	PRA (ng ANG I/ml/3hr)	PRC (ng ANG I/ml/hr)	RRF (ng ANG I/mg kidney/hr)
saline (n=12)	3.5 ± 0.4	5.1 ± 0.5	12.0 ± 0.8
PCA (n=11)	20.1 ± 2.2**	23.5 ± 3.4**	18.9 ± 1.5*
MK-212 (n=11)	40.7 ± 3.8**†	56.3 ± 11.7**†	19.3 ± 3.0*
TFMPP (n=6)	17.2 ± 2.5**	21.4 ± 3.4**	18.8 ± 2.8*

Data represent mean ± S.E.M. The number of rats (n) in each group is represented in parentheses.
One way ANOVA for: PRA; $F(3,36)=22.741$, $p < 0.001$.
PRC; $F(3.36)=12.394$, $p < 0.001$.
RRF; $F(3,36)= 3.147$, $p < 0.05$.
Duncan's new multiple range test:
* Significant difference from saline group, $p < 0.05$.
** Significant difference from saline group, $p < 0.01$.
† Significant difference from all other groups, $p < 0.01$.

concentration [ANOVA: $F(3,36)=12.394$, $p < 0.001$; Duncan's multiple range test: shortest range of significance between means = 13.5, $p < 0.05$; = 18.0, $p < 0.01$] when compared with the saline control group. The PRA and PRC values in the group that received MK-212 were significantly elevated over the other treatments (PCA and TFMPP) groups (Duncan's multiple range test: $p < 0.01$). The plasma concentration of RRF was significantly increased in the rats that received either PCA, MK-212 or TFMPP [ANOVA: $F(3,36)=3.147$, $p < 0.05$; Duncan's multiple range test: shortest range of significance between means = 6.4, $p < 0.05$]. There were no significant differences between any of the drug-treated groups for RRF activity. These results indicate that stimulation of 5-HT receptors can increase PRA, PRC and the plasma concentration of RRF.

2. Effect of stress on PRA and plasma RRF concentration (Table 7)

It has been shown that rats that were stressed using a conditioned emotional response (CER) or fear paradigm had significantly elevated levels of PRA (Van de Kar et al, 1985; Richardson-Morton et al, 1987). Therefore, the plasma from stressed rats was tested in the kidney slices bioassay to determine whether stress could be a physiological stimulus that would increase the plasma concentration of RRF. Table 7 shows the effect of stress on plasma renin activity (PRA) and on the plasma concentration of RRF. Rats that were subjected to the CER paradigm had significant increases in PRA (Student's t-test, two-tailed; $t=3.380$, $df=19$, $p < 0.01$) and also in the plasma concentration of RRF (Student's unpaired t-test, two-tailed; $t=2.711$, $df=19$, $p <$

TABLE 7 The effect of stress (conditioned emotional response or fear paradigm) on plasma renin activity (PRA) and the plasma concentration of renin-releasing factor (RRF).

	PRA (ng ANG I/ml/3 hr)	RRF (ng ANG I/mg kidney/hr)
control (n=9)	4.5 ± 0.8	12.7 ± 1.1
stress (n=12)	29.1 ± 6.2**	16.0 ± 0.7*

Data represent mean ± S.E.M.
** Significant difference from corresponding saline control group, $p < 0.01$, (Student unpaired t-test, two-tailed; t=3.380, df=19).
* Significant difference from corresponding saline control, $p < 0.02$ (Student unpaired t-test, two-tailed; t=2.711, df=19).

0.02). These results indicate that stress can act as a physiological stimulus to induce the release of RRF into the blood.

3. Presence of disulfide bonds in RRF: Study with cysteamine and PCA (Table 8a and 8b)

Since RRF was characterized as a heat stable peptide, it is possible that this peptide contains disulfide bonds that stabilize the molecule and maintain the conformation for its renin-releasing activity. Therefore, destruction of these disulfide bonds with cysteamine, a drug that reduces disulfide bonds, might change the conformational change of RRF and destroy its renin-releasing activity. To test this hypothesis, rats were pretreated with cysteamine (300 mg/kg, s.c.) before administration of PCA (10 mg/kg, i.p.). Tables 8a and 8b show the effect of cysteamine and PCA and cysteamine on plasma renin activity (PRA) and plasma renin concentration (PRC), respectively. Cysteamine did not increase in resting PRA values [Two way ANOVA: $F(1,20)=3.345$, $p > 0.05$]. Administration of PCA significantly increased PRA [Two way ANOVA: $F(1,20)=17.016$, $p < 0.001$; Duncan's multiple range test: shortest range of significance between means = 6.9, $p < 0.05$]. The two way ANOVA indicates that there was no interaction between cysteamine and PCA [Two way ANOVA: $F(1,20)=0.0371$, $p > 0.1$]. The cysteamine-PCA group was not different from the saline-PCA group. PCA did not produce an increase in PRA after cysteamine when compared with its cysteamine-saline control group.

The effects of cysteamine and PCA on plasma renin concentration (PRC) were very similar to the data obtained for PRA. Cysteamine

TABLE 8a Effect of pretreatment with cysteamine (300 mg/kg s.c.) on the p-chloroamphetamine (PCA; 10 mg/kg i.p.)-induced increase in plasma renin activity (PRA)

	Plasma renin activity (ng ANG I/ml/3 hr)	
	saline	cysteamine
saline	6.0 ± 1.1	11.8 ± 2.6
PCA	13.5 ± 1.8*	16.9 ± 3.0

Data represent mean ± S.E.M. n=8.
Two way ANOVA: Factor A (cysteamine): $F(1,20)=3.345$, $p > 0.05$, N.S.
Factor B (PCA): $F(1,20)= 17.016$, $p < 0.001$.
Factor A x B: $F(1,20)= 0.0371$, $p > 0.10$, N.S.
Duncan's new multiple range test:
Significant difference from saline-saline control group,
* $p < 0.05$, shortest range of significance between means = 6.9.

TABLE 8b Effect of pretreatment with cysteamine (300 mg/kg s.c.) on the p-chloroamphetamine (PCA)-induced increase in plasma renin concentration (PRC)

	Plasma renin concentration (ng ANG I/ml/hr)	
	saline	cysteamine
saline	7.3 ± 1.2	14.6 ± 4.5
PCA	22.7 ± 3.8**	21.5 ± 3.5

Data represent mean ± S.E.M. n=8.
Two way ANOVA: Factor A (cysteamine): $F(1,20)= 0.585$, $p > 0.100$, N.S.
Factor B (PCA): $F(1,20)= 19.811$, $p < 0.001$.
Factor A x B: $F(1,20)= 1.325$, $p > 0.100$, N.S.
Duncan's new multiple range test:
Significant difference from saline-saline control group,
** $p < 0.01$, shortest range of significance between means = 11.9.

treatment did not increase PRC when compared with the saline-saline control [Two way ANOVA: $F(1,20)=0.585$, $p > 0.100$]. PCA produced a significant increase in PRC [Two way ANOVA: $F(1,20)=19.811$, $p < 0.001$]. Treatment with cysteamine did not block the effect of PCA [Two way ANOVA: $F(1,20)=1.325$, $p > 0.100$]. For both PRA and PRC, the values for the cysteamine-saline and cysteamine-PCA groups were not statistically different from each other. However, PCA did not produced a significant increase in the cysteamine-PCA group when compared with its corresponding control. There is no difference between the saline-PCA and cysteamine-PCA groups.

The generation of ANG I from angiotensinogen was reported to be increased when disulfide reducing agents, such as cysteamine, were added to the incubation media (Poisner and Hong, 1977). To test for the possible interaction of cysteamine in the renin assay, control plasma pools were incubated with cysteamine, at a dose that was calculated as the theoretical distribution within body water. Cysteamine did not modify the amount of ANG I generated in either the normal [Student's t-test (unpaired), two-tailed: $t=0.7612$, $df=10$, $p > 0.05$] or high [Student's t-test (unpaired), two-tailed: $t=0.4155$, $df=10$, $p > 0.50$] plasma renin activity pools (Table 9).

These results indicate that cysteamine was not capable of preventing the PCA-induced increase in renin secretion. Furthermore, the concentration of cysteamine used in this experiment did not influence the generation of ANG I from the renin substrate and thus did not bias the results.

TABLE 9 Lack of cysteamine interference in the plasma renin activity assay.

	Plasma renin activity (ng ANG I/ml/3 hr)	
	saline	cysteamine
normal plasma renin activity	9.3 ± 0.9	10.2 ± 0.8
high plasma renin activity	23.7 ± 1.6	23.0 ± 0.7

Data represent mean ± S.E.M. n=5 in each group.
Student's unpaired t-test (two-tailed):
for normal PRA: $t=0.7612$, $df=10$, $p > 0.50$, N.S.
for high PRA: $t=0.4155$, $df=10$, $p > 0.50$, N.S.

4. Test for parathyroid hormone (PTH) as the mediator of the PCA-induced increase in renin release (Table 10)

Several laboratories have demonstrated that parathyroid hormone (PTH) increases PRA (Powell et al., 1978; Smith et al., 1979). Therefore, PTH was tested as a possible renin-releasing factor. PCA was administered to thyroparathyroidectomized rats to test if removal of the parathyroid gland would prevent the PCA-induced increase in plasma renin activity.

Basal levels of plasma renin activity were unaffected by parathyroidectomy [Two way ANOVA: $F(1,28)=2.154$, $p > 0.100$]. Administration of PCA produced a significant increase in plasma renin activity in the sham-operated rats [Two way ANOVA: $F(1,28)=22.774$, $p < 0.001$; Duncan's multiple range test: shortest range of significance between means = 33.2, $p < 0.05$]. Parathyroidectomy did not prevent the PCA-induced increase in plasma renin activity [Two way ANOVA: $F(1,28)=1.736$, $p > 0.100$; Duncan's multiple range test: shortest range of significance between means = 44.9, $p < 0.01$]. The sham-PCA and parathyroidectomy-PCA groups were not significantly different from each other. These results suggest that the parathyroid hormone is not the RRF.

TABLE 10 Effect of p-chloroamphetamine (PCA; 10 mg/kg i.p.) on plasma renin activity in sham and thyroparathyroidectomized rats.

	Plasma renin activity (ng ANG I/ml/3 hr)	
	saline	PCA
Sham-operated	15.2 ± 2.6	52.3 ± 9.5*
Parathyroidectomized	16.8 ± 1.7	82.2 ± 19.0**

Data represent mean ± S.E.M. n = 8.
Two way ANOVA:
Factor A (PCA): $F(1,28)=22.774$, $p < 0.001$.
Factor B (parathyroidectomy): $F(1,28)=2.154$, $p > 0.1$, N.S.
Factor A x B: $F(1,28)=1.736$, $p > 0.1$, N.S.
Duncan's new multiple range test:
Significant difference from corresponding saline group,
* $p < 0.05$, shortest range of significance between means = 33.2.
** $p < 0.01$, shortest range of significance between means = 44.9.

5. Test for possible renin-releasing activity of neurophysin II (Table 11)

Lesions in the paraventricular nucleus (PVN) prevent both the PCA-induced (Gotoh et al., 1987) and the stress-induced increases (Richardson Morton et al., 1986; Gotoh et al., 1987) in plasma renin activity. Neurophysin II (NPII), is synthesized in the PVN, released from the neural lobe, and has a molecular weight of approximately 10,000. There is no known physiological action for the neurophysins and therefore, NPII was tested as a possible renin-releasing factor. Intraperitoneal injection of increasing doses of neurophysin II did not alter PRA [One way ANOVA: $F(5,39)=0.2291$, $p > 0.100$] or PRC [One way ANOVA: $F(5,39)=0.4959$, $p > 0.100$] levels indicating that neurophysin II does not have any renin-releasing activity and is probably not the RRF.

D. Summary of data using unbubbled kidney slice method

1. Effect of saline-plasma or PCA-plasma fractions (M.W. 1,000-5,000; 5,000-10,000 and 10,000-20,000) on renin release from kidney slices (Table 12)

Table 12 shows the effect of the PCA-plasma and saline-plasma fractions (M.W. 1,000-5,000; 5,000-10,000 and 10,000-20,000) on renin release from the kidney slices. All of the fractions exhibited some renin-releasing activity. The PCA-plasma fraction containing substances with molecular weights 5,000-10,000 produced a three-fold increase in renin release when compared with any of the other plasma fractions; this increase was significant [ANOVA: $F(6,55)=10.48$, $p < 0.001$; Duncan's multiple range test: shortest range of significance

TABLE 11 Failure of increasing doses of neurophysin II (bovine) to alter plasma renin activity (PRA) and plasma renin concentration (PRC) in conscious rats.

Dose of Neurophysin II (μg/kg, i.p.)	PRA (ng ANG I/ml/3 hr)	PRC (ng ANG I/ml/hr)
saline	7.9 ± 0.9	10.2 ± 0.6
0.1	9.0 ± 1.0	11.9 ± 1.6
0.5	9.1 ± 1.1	11.0 ± 1.2
2.0	9.1 ± 1.0	12.4 ± 1.2
10.0	8.9 ± 0.7	11.0 ± 1.0
20.0	8.2 ± 1.2	10.2 ± 1.6

Data represent mean ± S.E.M. n=8 per group.
One way ANOVA: PRA; $F(5,39) = 0.229$, $p > 0.1$, N.S.
PRC; $F(5,39) = 0.495$, $p > 0.1$, N.S.

TABLE 12 The effect of saline or plasma fractions from saline-treated or PCA-treated (10 mg/kg i.p) nephrectomized rats on renin release from kidney slices.

PCA-plasma fractions	Renin Release (ng ANG I/mg kidney/hr)
M.W. = 1,000-5,000 (10)	12.0 ± 1.8
M.W. = 5,000-10,000 (17)	32.0 ± 4.1*
M.W. = 10,000-20,000 (6)	12.3 ± 1.6
Saline-plasma fractions	
M.W. = 1,000-5,000 (5)	11.3 ± 1.5
M.W. = 5,000-10,000 (10)	9.5 ± 1.3
M.W. = 10,000-20,000 (5)	10.9 ± 1.9
Saline (9)	6.2 ± 1.0

Data represent mean ± S.E.M., n is represented in parentheses.
One way ANOVA $F(6,55)=10.48$, $p < 0.001$.
Duncan's new multiple range test:
* Significant difference from all other groups, $p < 0.01$; shortest range of significance between means = 18.0.

between means = 18.0, $p < 0.01$]. Lactate dehydrogenase was also measured in the incubation medium and was determined to be 0.034 ± 0.006 μmol pyruvate/ml/min for the saline-plasma group (M.W. 5,000-10,000) and 0.032 ± 0.006 μmol pyruvate/ml/min for the PCA-plasma group (M.W. 5,000-10,000). The LDH values were lower than those reported in the literature for kidney slices (Fray et al., 1983), and indicate that the increase in renin release from the group that received the PCA-plasma fraction (M.W. 5,000-10,000) was not due to cell lysis.

2. Test for possible renin-releasing activity of 5-HT or PCA (Table 13)

Since the PCA-plasma produced increases in renin release from the kidney slices it could be suggested that either 5-HT or PCA molecules in the plasma fractions could increase renin release from the slices. Therefore, 5-HT, PCA and fenfluramine, another 5-HT releaser were tested for renin-releasing activity. None of these drugs were effective in stimulating renin release from the kidney slices [ANOVA: $F(3,11)=0.18$, $p > 0.1$]. These results suggest that the increase in renin release seen with PCA-plasma was not due to either PCA or 5-HT molecules.

3. Incubation of the saline-plasma and PCA-plasma fraction (M.W. 5,000-10,000) with pronase (Table 14)

To determine if RRF is a peptide, the PCA-plasma and saline-plasma fractions that contained renin-releasing activity (M.W. 5,000-10,000) were incubated with pronase and the samples were added to the kidney slices. Incubation of the PCA-plasma fraction (M.W. 5,000-

TABLE 13 Inability of 5-HT (10^{-6}M), p-chloroamphetamine (10^{-6}M) and fenfluramine (10^{-6}M) to increase renin release from kidney slices.

Test substance	Renin release (ng ANG I/mg kidney/hr)
Saline (4)	9.4 ± 1.0
10^{-6}M 5-HT (4)	8.8 ± 1.2
10^{-6}M PCA (3)	8.6 ± 1.1
10^{-6}M Fenfluramine (4)	9.5 ± 0.4

Data represent mean ± S.E.M.. n is represented in parentheses.
One way ANOVA $F(3,11)=0.18$, $p > 0.100$, N.S.

TABLE 14 Ability of pronase to destroy the renin-releasing activity of the PCA-plasma fraction (M.W. 5,000-10,000).

Fraction added	n	Renin release (ng ANG I/mg kidney/hr)
Vehicle-treated PCA-plasma fraction	9	15.7 ± 1.2*
Pronase-treated PCA-plasma fraction	10	5.6 ± 0.4
Vehicle-treated Saline-plasma fraction	6	10.6 ± 1.3
Pronase-treated Saline-plasma fraction	6	11.7 ± 0.9
Saline	9	6.4 ± 0.5
Isoproterenol (10^{-6}M)	6	19.6 ± 1.9*

Data represent mean ± S.E.M., n represents number of determinations.
One way ANOVA $F(5,39)= 29.84$, $p < 0.001$.
Duncan's new multiple range test:
* Significant difference from saline or the corresponding pronase-treated group, $p < 0.01$; shortest range of significance between means = 4.2.

10,000) with pronase completely eliminated its renin-releasing activity [ANOVA: $F(5,39)=29.84$, $p < 0.001$; Duncan's multiple range test: shortest range of significance between means = 4.2, $p < 0.01$]. The low renin-releasing activity of the saline-plasma fraction (M.W. 5,000-10,000) was not affected by treatment with pronase. These data suggest that RRF present in the PCA-plasma is a peptide and that the increase in renin release seen with the saline-plasma is not due to a peptide factor. Saline and isoproterenol were tested in the bioassay as representative values for the low and high (stimulus induced) renin release.

The proteolytic activity of pronase was verified using a casein agar plate. Casein is one of the substrates for pronase. The pronase was mixed with the plasma fractions and a sample was added to a casein agar plate. The proteolytic activity showed up on the agar as a clear ring, indicating that pronase had digested the casein. After incubation of the pronase with the plasma fraction, the samples were boiled. Boiling inactivated the pronase and prevented any further proteolysis; this was also verified using the casein agar plate.

E. Studies of the renin-releasing factor in rat brain

1. Extraction of RRF from rat brain tissue

1a. Test of different extraction media on the recovery of renin-releasing activity from whole brain (Table 15)

One of the preliminary studies for the distribution of RRF in rat brain included the testing of different extraction media on the recovery of renin-releasing activity from whole rat brain. Whole rat

brains were extracted as described in Method section E part 1 in different extraction media, and added to the kidney slices. The brains that were extracted with cold perchloric acid (0.1 N) had the highest renin-releasing activity [One way ANOVA: $F(6,59)=4.428$, $p < 0.001$]. This increase was statistically significant from the saline control and the hot and cold hydrochloric acid extract groups (Duncan's multiple range test: shortest range of significance between means = 5.9, $p < 0.01$). Brains that were extracted with either cold 0.1 N HCl/ethanol (20:80) or with hot distilled water also had a significant concentration of renin-releasing activity when compared with the saline group (Duncan's multiple range test: shortest range of significance between means = 4.5, $p < 0.05$). Hot perchloric acid (0.1 N) extract and hot and cold hydrochloric acid (0.1 N) extracts had renin-releasing activity that did not differ from the saline control values. From these results it appears that cold perchloric acid, cold 0.1 N HCl/ethanol and boiling distilled water were the most effective in recovering renin-releasing substances from whole rat brain.

1b. Comparison of extraction with cold 0.1 N perchloric acid and boiling distilled water on the recovery of RRF from rat hypothalamus (Table 16)

Rat hypothalamic tissue was tested for the recovery of renin-releasing substances using cold 0.1 N perchloric acid or boiling distilled water as the extraction media. The extracts were added to the kidney slices and the renin-releasing activity of the two extracts were compared. The hypothalamic tissue that was homogenized in the

TABLE 15 Comparison of different extraction media on renin-releasing activity from whole rat brains.

Extraction media	n	Renin release (ng ANG I/mg kidney/hr)
Cold hydrochloric acid (0.1 N)	9	7.9 ± 0.3
Hot hydrochloric acid (0.1 N)	9	7.2 ± 0.3
Cold 0.1 N HCl/ethanol (20:80)	9	11.9 ± 2.2*
Cold perchloric acid (0.1 N)	10	13.8 ± 2.1**
Hot perchloric acid (0.1 N)	10	10.4 ± 1.6
Hot distilled water	8	11.0 ± 0.6*
saline	12	6.2 ± 0.6

Data represent mean ± S.E.M.
One way ANOVA: $F(6,59) = 4.428$, $p < 0.001$.
Duncan's new multiple range test:
Significant difference from saline control group,
* $p < 0.05$, shortest range of significance between means = 4.5.
** $p < 0.01$, shortest range of significance between means = 5.9.

TABLE 16 Comparison of 0.1 N perchloric acid and distilled water on the recovery of renin-releasing factor (RRF) from rat hypothalamus.

	Renin release (ng ANG I/mg kidney/hr)
cold 0.1 N perchloric acid	11.1 ± 1.7
hot distilled water	22.4 ± 4.2*

Data represent mean ± S.E.M. n=4.
* Significant difference from cold perchloric acid extract group, $p < 0.05$ (Student's unpaired t-test, two-tailed, t=2.516, df=6).

boiling distilled water had significantly more renin-releasing activity than the hypothalamic tissue extracted with perchloric acid extract [Student's t-test (unpaired), two-tailed: $t=2.516$, $df=6$, $p < 0.05$]. These data differ from the data obtained in the previous study that indicated that perchloric acid was more effective in recovering renin-releasing activity from whole rat brain. This suggests that the renin-releasing substance in the hypothalamus and that (those) in whole rat brain may be different. One advantage in using boiling distilled water for the extraction medium is that the hypothalamic homogenates do not need the pH readjusted before addition to the kidney slices. For the following studies on RRF the tissues were homogenized in boiling distilled water.

2. Renin-releasing activity of hypothalamic, cerebellar and pituitary extracts (Table 17)

The hypothalamus, cerebellum and pituitary tissues were homogenized in boiling distilled water (see Method section E part 3) and the extracts were added to the kidney slices to test for renin-releasing activity. The hypothalamic extract produced a significant increase in renin release from the kidney slices [One way ANOVA: $F(4,46)=11.474$, $p < 0.001$] when compared with either the saline control (Duncan's multiple range test: shortest range of significance between means = 8.5, $p < 0.05$) or the pituitary extract values (Duncan's multiple range test: shortest range of significance between means = 10.9, $p < 0.01$). The cerebellar extract significantly increased renin release from the kidney slices when compared with the pituitary extract

TABLE 17 Effect of rat hypothalamic, cerebellar and pituitary extracts on renin release from kidney slices.

Substance added	Renin Release (ng ANG I/mg kidney/hr)
Saline (13)	9.7 ± 0.6
Hypothalamic Extract (16)	19.1 ± 2.1**†
Cerebellar Extract (10)	15.3 ± 2.1*
Pituitary Extract (8)	5.7 ± 0.6
Isoproterenol 10^{-6}M (5)	19.7 ± 2.6**†

Each data point represents mean ± S.E.M. The number of determinations (n) is represented in parentheses.
One way ANOVA: $F(4,46) = 11.474$, $p < 0.001$.
Duncan's new multiple range test:
† Significant difference from the saline group, $p < 0.05$, shortest range of significance between means = 8.5.

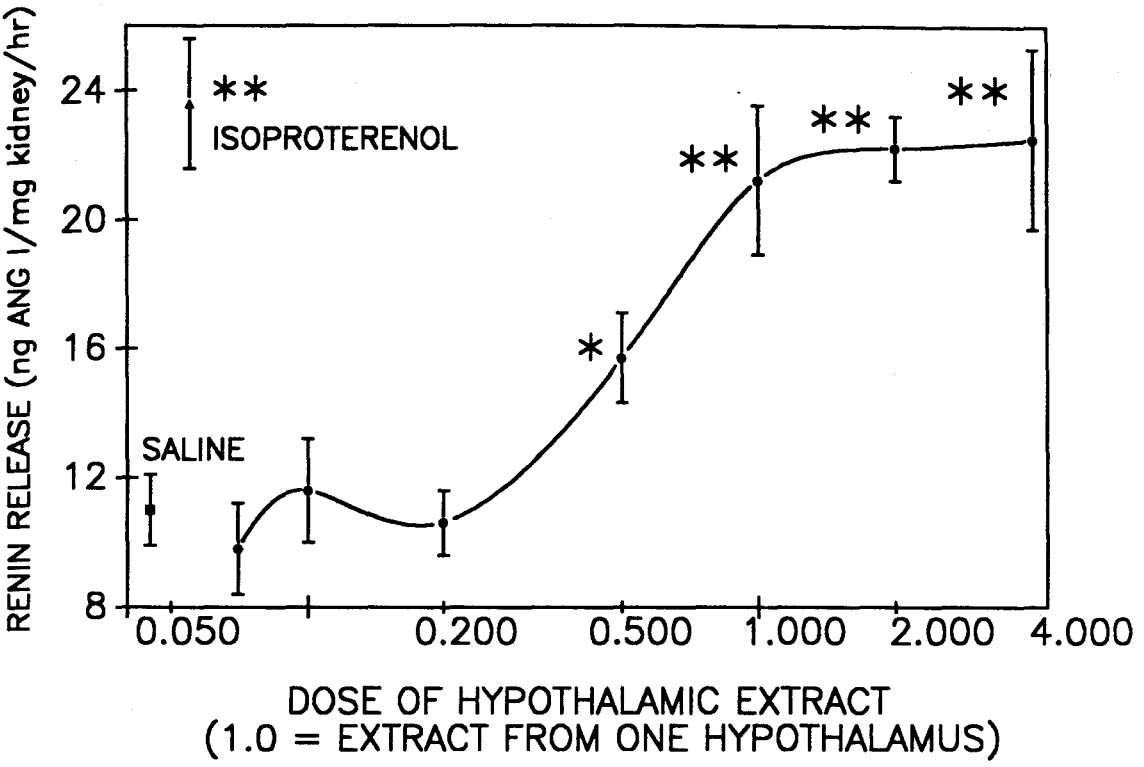
Significant difference from pituitary extract
* $p < 0.05$, shortest range of significance between means = 8.5.
** $p < 0.01$, shortest range of significance between means = 10.9.

(Duncan's multiple range test; $p < 0.05$) but the effect was not significant when compared with the saline group. Pituitary extract produced a small, but statistically insignificant, decrease in renin release when compared with the saline values. Saline and isoproterenol (10^{-6} M) were added to the kidney slices to demonstrate the high and low values of renin release for the bioassay. These results suggest that the hypothalamus and cerebellum contain renin-releasing substances. The pituitary may contain substances that inhibit renin release in vitro.

3. Dose-response effect of the renin-releasing activity of hypothalamic tissue (Figure 3)

The hypothalamus has been shown in the previous study to contain renin-releasing substances. Rat hypothalami were prepared (Method section E part 4) and added to the kidney slices in dilutions that corresponded to the content of 0.07, 0.1, 0.2, 0.5, 1.0, 2.0 and 4.0 hypothalami. Addition of hypothalamic extract to the kidney slices produced a dose-dependent increase in renin release. The content of 0.5 hypothalamus produced a significant increase in renin release that was also significantly different from the equivalent of 0.2 hypothalamus (Duncan's multiple range test: shortest range of significnce between means = 6.3, $p < 0.05$). There was a significant, and maximal, increase in renin release when the equivalent of the content of one hypothalamus was added to the kidney slices [One way ANOVA: $F(8,50)=10.520$, $p < 0.001$; Duncan's multiple range test: shortest range of significance between means = 8.2, $p < 0.01$]. The

FIGURE 3 Dose-response effect of rat hypothalamic tissue extract on renin-releasing activity.



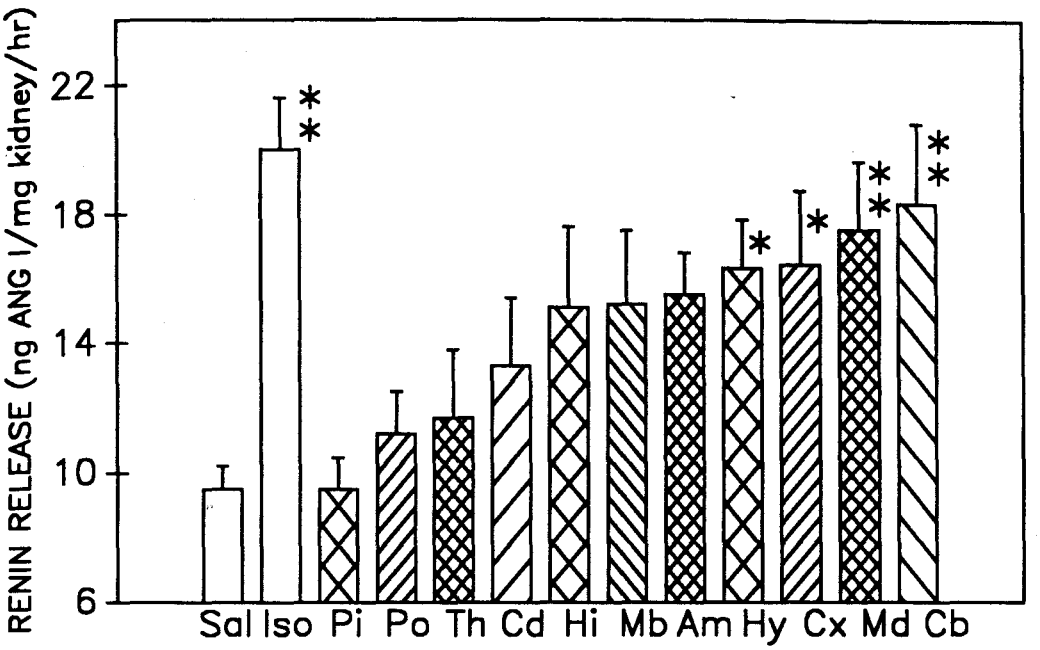
Data represent mean ± S.E.M.
n=6, except for isoproterenol (10^{-5}M) where n=12.
One way ANOVA: $F(8,50)=10.527$, $p < 0.001$.
Significant difference from saline as determined by Duncan's new multiple range test:
* $p < 0.05$, shortest range of significance between means = 6.3.
** $p < 0.01$, shortest range of significance between means = 8.2.

maximal response was also obtained when the equivalents of 2 and 4 hypothalami were added to the slices. These values were also significantly different from the equivalent of 0.5 hypothalamus. Saline and isoproterenol were added as low and high (stimulus-induced) controls for the bioassay. These results indicate that the kidney slices can respond with a maximal stimulation of renin release when the equivalent of the content of one hypothalamus is added to the kidney slices. For subsequent studies, tissue extracts were added to the kidney slices in the volume that corresponded to the equivalent of the content of one hypothalamus (0.02 ml of tissue extract in a 1 g/ml dilution with boiling distilled water).

4. Distribution of RRF in rat brain (Figure 4)

Rat brains were dissected and the individual parts were prepared as indicated in Method section E part 5. The extracts were added to the kidney slices to test for renin-releasing activity. Figure 4 shows the distribution of renin-releasing activity in different brain regions. The data are arranged in order of increasing renin-releasing activity [ANOVA: $F(14,110)=4.693$, $p < 0.005$]. Saline and isoproterenol (10^{-5} M) were tested along with the samples to control for the low and high levels of the bioassay. The following brain regions had RRF activity that was statistically different from the saline control or the pituitary extract groups: the hypothalamus, cerebral cortex (Duncan's multiple range test: shortest range of significance between means = 6.2, $p < 0.05$), medulla oblongata and cerebellum (Duncan's multiple range test: shortest range of significance between means = 7.0,

FIGURE 4 Distribution of renin-releasing activity in different regions of the rat brain.



Data represented as mean ± S.E.M. n=8.
One way ANOVA: $F(14,110)=4.693$, $p < 0.005$.
Significant difference from saline and pituitary extract as determined by Duncan's new multiple range test:
* $p < 0.05$, shortest range of significance between means = 6.2.
** $p < 0.01$, shortest range of significance between means = 7.9.

List of abbreviations:

S	saline	Mb	midbrain
Iso	isoproterenol 10^{-5}M	Am	amygdala
Pi	pituitary	Hy	hypothalamus
Po	pons	Cx	cerebral cortex
Th	thalamus	Md	medulla oblongata
Cd	caudate	Cb	cerebellum
Hi	hippocampus		

$p < 0.01$). There were no significant differences in the renin-releasing activity between these groups. The pituitary had the least amount of renin-releasing activity followed by, in increasing order, the pons, thalamus, caudate, hippocampus, midbrain, and amygdala. These values were not significantly different from the saline control value. These data demonstrate that there is an unequal distribution of renin-releasing activity in the rat brain. The areas that contain the highest amounts of renin-releasing activity are the cerebellum followed by the medulla, cerebral cortex and hypothalamus.

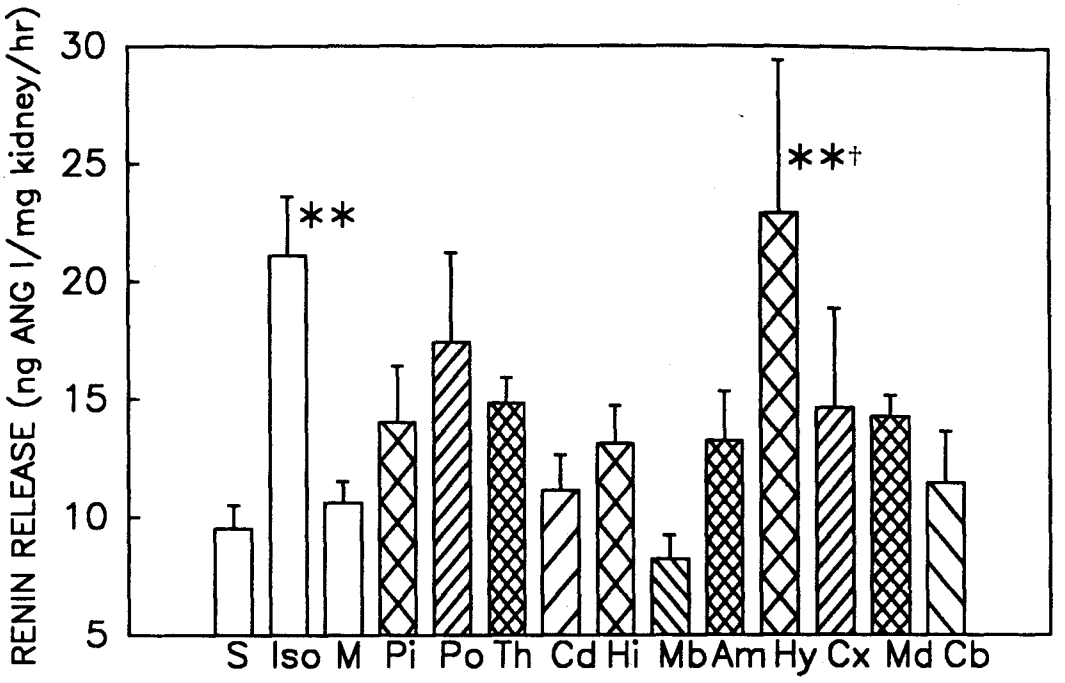
5. Distribution of RRF in brains of colchicine-treated rats (Figure 5)

5a. Test of colchicine-treated brain extracts

The results from Figure 4, cannot distinguish RRF in cell bodies from RRF in nerve terminals. In order to identify RRF in the cell bodies, a group of rats were treated with colchicine (48 hours), a drug that inhibits axonal transport of cell products toward the nerve terminals. Therefore, treatment of rats with colchicine would prevent the movement of peptides, such as RRF, from the cell body to the nerve terminals. The brains from colchicine-treated rats were dissected, homogenized and added to the kidney slices to test for renin-releasing activity as was performed with the control brains.

Figure 5 shows the distribution of renin-releasing activity in colchicine-treated rat brains. When compared with Figure 4, there was a redistribution of renin-releasing activity. The most visible difference after colchicine-treatment was that the hypothalamus was the only brain region that exhibited significant renin-releasing activity

FIGURE 5 Distribution of renin-releasing activity in brains of colchicine-treated rats.



Data represented as mean ± S.E.M. n=8.
One way ANOVA: $F(13,78)= 2.997$, $p < 0.005$.
Significant difference from saline, caudate, cerebellum, midbrain and muscle controls as determined by Duncan's new multiple range test:
** $p < 0.01$, shortest range of significance between means = 10.8.

Significant difference from pituitary, hippocampus, amygdala cortex and medulla as determined by Duncan's new multiple range test:
† $p < 0.05$, shortest range of significance between means = 8.3.

List of abbreviations:

S	saline	Hi	hippocampus
Iso	isoproterenol 10^{-5}M	Mb	midbrain
M	skeletal muscle	Am	amygdala
Pi	pituitary	Hy	hypothalamus
Po	pons	Cx	cerebral cortex
Th	thalamus	Md	medulla oblongata
Cd	caudate	Cb	cerebellum

[One way ANOVA: $F(13,78)=2.997$, $p < 0.005$]. This activity was significantly different from saline, caudate-putamen, midbrain, muscle and cerebellar extracts (Duncan's multiple range test: shortest range of significance between means = 10.8, $p < 0.01$) and from the pituitary, hippocampus, amygdala, cortex and medulla extracts (Duncan's multiple range test: shortest range of significance between means = 8.3, $p < 0.05$). The renin-releasing activity of the hypothalamus was also slightly, but not significantly, increased above the value for isoproterenol-stimulated renin release. In addition to the hypothalamus, the renin-releasing activity of the pituitary, pons and thalamus in colchicine-treated rats was slightly but not significantly increased when compared with the corresponding areas from the brains of non-treated rats (Figure 4). The renin-releasing activity previously observed in the cerebral cortex, medulla and cerebellum was reduced, and the value for renin-releasing activity in the cerebellum was at the level of saline control values. The renin-releasing activity of the caudate-putamen, hippocampus, midbrain and amygdala was also decreased slightly after colchicine treatment in comparison with the non-treated controls. Saline and isoproterenol (10^{-5} M) were run as controls for the bioassay. Skeletal muscle extract was also tested in the bioassay as a tissue control. These results suggest that RRF cell bodies are located in the hypothalamus and that the renin-releasing activity that was observed in the cerebral cortex, medulla and cerebellum was probably due to the presence of RRF in nerve terminals.

5b. Test for colchicine interference in the renin release bioassay (Table 18)

Before the actual bioassay was performed with the colchicine-treated brain parts, a preliminary kidney slice assay was performed to test for the possible interference of colchicine in the release of renin from the kidney slices. Colchicine (0.083 mg/ml extract) was added to either saline, isoproterenol (10^{-5} M) or cerebellar extract to test if colchicine would interfere in either basal or stimulus-induced renin release from the kidney slices. Isoproterenol (Duncan's multiple range test: shortest range of significance between means = 6.2, $p < 0.05$) and cerebellar (Duncan's multiple range test: shortest range of significance between means = 8.4, $p < 0.01$) extract produced significant increases in renin release from the kidney slices when compared with the saline control group [One way ANOVA: $F(5,23)=7.330$, $p < 0.001$]. There were no significant differences in renin-releasing activity between the colchicine and saline groups for each test substance. This dose of colchicine (0.083 mg/ml) was calculated as the theoretical dilution of drug in the brain. This theoretical value maybe larger than the actual distribution since this calculation did not take into account the leakage of the drug from the CSF (cerebrospinal fluid) to the periphery. Therefore, the data demonstrate that the dose of colchicine used in the brain scan experiment would not have interfered with renin release from the kidney slices in the RRF bioassay.

TABLE 18 Failure of colchicine to alter renin release from the kidney slices.

	Renin release (ng ANG I/mg kidney/hr)	
	saline	colchicine
saline	13.5 ± 1.6	13.4 ± 1.4
isoproterenol 10^{-5}M	21.7 ± 2.4*	19.7 ± 1.1*
cerebellum extract	25.5 ± 2.0**	24.4 ± 2.6**

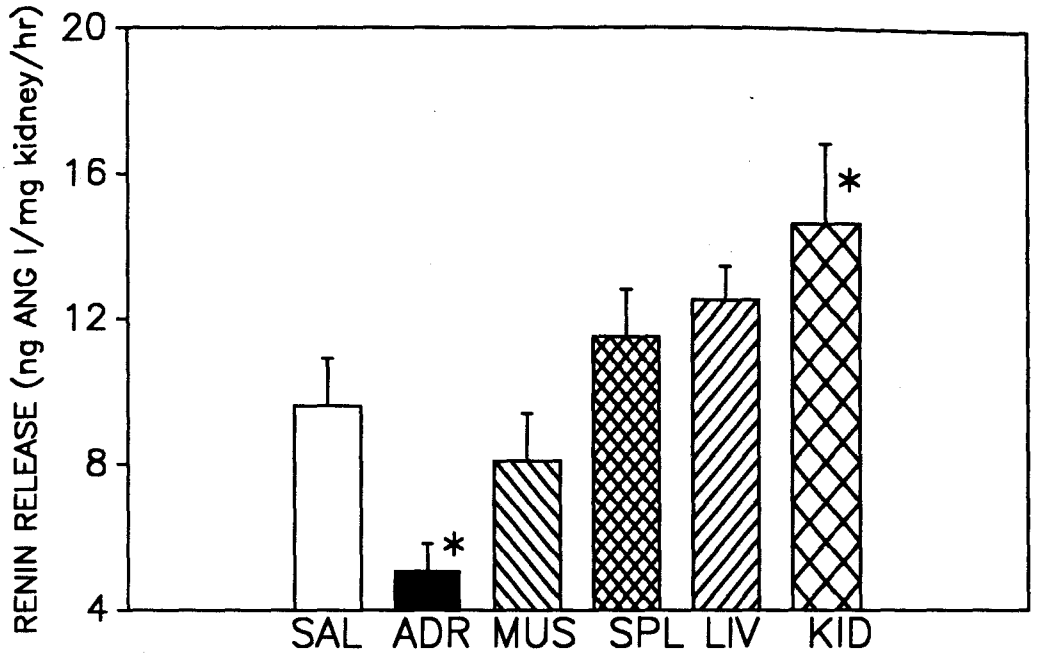
Data represent mean ± S.E.M. n=5.
One way ANOVA: $F(5,23)=7.330$, $p < 0.001$
Significant difference from saline-saline or colchicine-saline control groups Duncan's new multiple range test:
* $p < 0.05$, shortest range of significance between means = 6.2.
** $p < 0.01$, shortest range of significance between means = 8.4.

F. Distribution of renin-releasing activity in peripheral tissues (Figure 6)

Since a number of peptides are distributed in the central nervous system and in peripheral tissues, some peripheral tissues were tested for renin-releasing activity. The adrenal gland, skeletal muscle, spleen, liver and kidney were homogenized in boiling water and the extracts were added to the kidney slices. Figure 6 shows the renin-releasing activity of different peripheral tissues. The skeletal muscle, spleen and liver extracts did not exhibit any significant renin-releasing activity. The adrenal gland extract produced a significant decrease in renin release from the kidney slices [ANOVA: $F(5,28)=6.318$, $p < 0.005$; Duncan's multiple range test: shortest range of significance between means = 4.5, $p < 0.05$]. The kidney extract produced a significant increase in the amount of ANG I detected.

It was possible that not all the renin in the kidney extract was inactivated by the boiling procedure. Therefore addition of kidney extract to the kidney slices may have increased the amount of renin in the sample. To test this possibility, kidney extract, at a concentration that equalled the dilution of the kidney extract in the Krebs-Ringer buffer during the kidney slice bioassay (0.02 ml sample/2.0 ml), was incubated with renin substrate (0.1 ml nephrectomized plasma). The kidney extract was able to generate 73.1 ng ANG I/ml. When this value was divided by the average weight of the kidney slices for that bioassay (15.7 mg), this resulted in an addition of approximately 4.6 ng ANG I/mg kidney/hr. Subtracting this number from the value for renin release with kidney extract (14.6 ng ANG I/mg

FIGURE 6 Distribution of renin-releasing activity in peripheral tissues.



Data represented as mean ± S.E.M. n=7.
One way ANOVA: $F(5,28)=6.318$, $p < 0.005$.
Significant difference from saline and muscle control groups as determined by Duncan's new multiple range test:
* $p < 0.05$, shortest range of significance between means = 4.5.

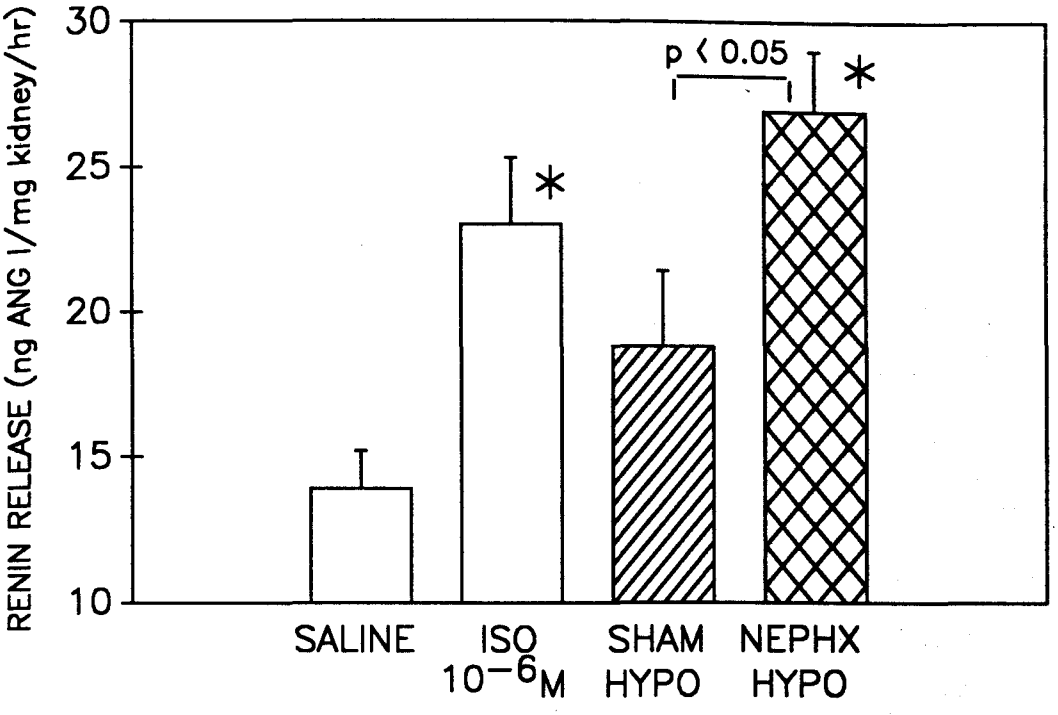
List of abbreviations:
SAL saline
ADR adrenal
MUS muscle
SPL spleen
LIV liver
KID kidney

kidney/hr) would have yielded a final value of 10 ng ANG I/mg kidney/hr, and this value would not have been statistically different from the saline control group. Therefore it is likely that the increase in ANG I with the kidney extract was due to the presence of renin in the extract.

G. Effect of nephrectomy on the hypothalamic content of RRF (Figure 7)

The colchicine experiment indicated that the hypothalamus contains RRF cell bodies. If there were a feedback loop involved in the regulation of RRF release, then removal of the kidneys should result in a change of hypothalamic RRF. The hypothalami from nephrectomized (NEPHX) and sham-operated rats were homogenized in boiling water and the extracts were added to the kidney slices for the incubation period. The hypothalamic extract from nephrectomized rats produced a significant increase in renin release from the kidney slices [ANOVA: $F(3,22)=8.09$, $p < 0.005$] when compared with either the saline control (Duncan's multiple range test: shortest range of significance between means = 8.7, $p < 0.01$) or the hypothalamic extract from sham-operated rats (Duncan's multiple range test: shortest range of significance between means = 6.5, $p < 0.05$) groups. The hypothalamic extract from sham-operated rats produced a small increase in renin release, however, this increase was not statistically significant when compared with the saline control. Saline and isoproterenol (10^{-6} M) were also added as controls for the bioassay. The data show that removal of the kidneys from rats results in an increase in the hypothalamic content of RRF. These findings may suggest that the kidney may be involved in a

FIGURE 7 Effect of bilateral nephrectomy (24 hours) on the hypothalamic content of renin-releasing factor (RRF).



Data represent mean ± S.E.M. n=6 except for nephrectomized group n=8.
One way ANOVA: $F(3,22)=8.09$, $p < 0.005$.
Duncan's new multiple range test:
$p < 0.05$, significant difference from sham-operated control group, shortest range of significance between means = 6.5.
* Significant difference from saline control group, $p < 0.01$, shortest range of significance between means = 8.7.

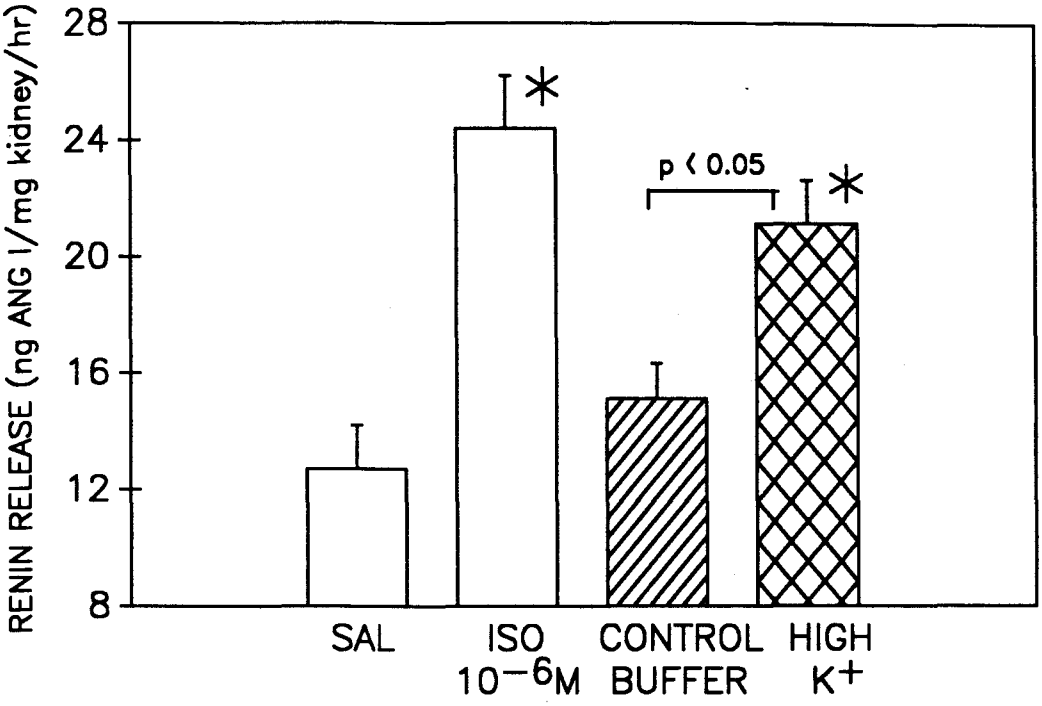
feedback loop that regulates the production and release of RRF from the hypothalamus.

H. Test for the neuronal release of RRF: Study with superfused hypothalami (Figure 8)

The study with the colchicine-treated rats suggest that RRF cell bodies are localized in the hypothalamus. If this is a true neuroendocrine system, then the neurons containing the peptide (RRF) should be able to be stimulated to release the factor. This possibility was tested by using hypothalamic explants. Hypothalami or rat hypothalamic-pituitary explants were superfused for a 30 minute control period followed by a 30 minute test period with a high-potassium Krebs-Ringer solution to stimulate the release of the peptide. The samples were collected, concentrated by ultrafiltration and the fraction that was retained on the filter (molecular weights greater than 500) were added to the kidney slices to assay for renin-releasing activity.

The samples from the hypothalamic explants that were superfused for the 30 minute control period with regular (control) buffer had a value for renin release that was slightly increased, but not significantly different from the saline control value. The superfusates from the hypothalamic explants that were superfused with the high potassium Krebs-Ringer solution had a significantly higher renin-releasing activity than when they were superfused with the control buffer [One way ANOVA: $F(3,26)=9.894$, $p < 0.001$; Duncan's multiple range test: shortest range of significance between means =

FIGURE 8 Secretion of renin-releasing factor (RRF) from superfused hypothalamus: Effect of high potassium (60 mM).



Data represented as mean ± S.E.M. n=8.
One way ANOVA: $F(3,22)= 9.894$, $p < 0.001$.
Duncan's new multiple range test:
$p < 0.05$, significant difference from control buffer group, shortest range of significance between means = 4.7.
* Significant difference from saline, $p < 0.01$, shortest range of significance between means = 6.4.

4.7, $p < 0.05$]. The value for renin-releasing activity of the potassium stimulated hypothalamic explant was not statistically different from the isoproterenol group. Both of these groups had renin release values that were significantly increased over the saline and control buffer superfused groups (Duncan's multiple range test: shortest range of significance between means = 6.4, $p < 0.01$). As a routine saline and isoproterenol were added to kidney slices as controls. This result indicates that the hypothalamus can be stimulated to secrete renin-releasing substances.

CHAPTER V

DISCUSSION

A. Summary

The results of this dissertation support the hypothesis for the existence of a renin-releasing factor (RRF) that is released from the brain into the circulation. Using an improved and reliable method for measuring renin release from kidney slices, these studies indicate that RRF is a heat stable peptide having a molecular weight between 5,000-10,000. These data support previous *in vivo* studies indicating that a factor in the plasma (M.W. 500-10,000) of PCA-treated rats is capable of increasing plasma renin activity (PRA) in recipient rats (Van de Kar *et al.*, 1982a). Stimulation of serotonergic receptors and stress triggers the release of RRF into the blood. These studies suggest that the hypothalamus contains RRF which produces a dose-dependent increase in renin release from kidney slices. Further investigation revealed that RRF is unevenly distributed within the brain with the cerebellum, medulla oblongata, cerebral cortex and the hypothalamus exhibiting the highest concentration of RRF. Studies using brains from colchicine-treated rats suggest that RRF cell bodies are localized in the hypothalamus. It is also noted that the renin-releasing activity previously observed in the cerebellum, medulla and cerebral cortex was

reduced which suggests that RRF in these brain regions is present in nerve terminals. Superfusion of rat hypothalamus explants with high potassium medium demonstrated that RRF neurons in the hypothalamus can be stimulated to release RRF. The hypothalamic content of RRF was significantly increased after bilateral nephrectomy suggesting the possible presence of a feedback loop from the kidneys to the hypothalamus which is responsible for the maintenance of RRF at a homeostatic level.

Bioassay of several peripheral tissues indicated that the spleen, liver, skeletal muscle and kidney do not contain RRF. Adrenal extract produced a decrease in renin release from kidney slices which may be due to the presence of inhibitory peptides or transmitters in the adrenal gland. The study further determined that parathyroid hormone (PTH) and neurophysin II (NPII) are not RRF.

B. Renin release bioassay

1. In vivo bioassay

The first investigations on RRF were performed in conscious rats in a series of cross-transfusion experiments (Van de Kar et al., 1982a). The plasma from PCA-treated rats was concentrated and injected into conscious recipient rats. Plasma renin activity (PRA) was measured in the recipient rats to determine whether there was a factor in the blood from PCA-treated rats that increased renin secretion. The results from these experiments indicated the presence of a blood-borne factor with a molecular weight between 500-10,000. Injecting plasma fractions into conscious rats could have altered either sodium balance,

plasma osmolarity or even blood pressure which, in turn, would have altered plasma renin activity. Therefore, it was possible that physiological changes in the recipient rats could have interfered with the effect of RRF. To eliminate these influences, an in vitro renin release bioassay was developed so that the effect of RRF could be observed directly on the kidney.

2. In vitro kidney slice bioassay

In vitro kidney slice methods have been used to study the direct effects of various stimuli on renin release (Henrich and Campbell, 1984; O'Dea et al., 1984; Fray and Laurens, 1981; Katz and Malvin, 1982a). The kidney slice method used in these studies was based on the method used by Katz and Malvin (1982a). After experimenting with the methods of slicing and oxygenation, the resulting method of measuring renin release in vitro was an improvement over the other methods. Cutting the tissue by hand (Churchill and Churchill, 1982; Henrich and Campbell, 1984) or with a Stadie-Riggs microtome (Katz and Malvin, 1982a) could cause variation in slice thickness and deformation of the kidney due to changes in the amount of pressure that is put on the kidney during slicing. Using a vibratome to slice the kidney tissue ensured that each slice was of uniform thickness, primarily because the vibratome cuts the kidney without applying pressure to the kidney tissue or deforming it. The uniformity in slice size could be correlated with the kidney slice weight (Table 2).

Another modification of the method involved coronal sectioning of the kidney. By cutting the kidney coronally instead of sagittally each

slice contained equal amounts of cortical tissue (both outer and inner cortex) and medullary tissue. The highest content of renin secreting cells is located in the outer cortex (Katz and Malvin, 1982b; Jones et al., 1979). The renal medulla however, has virtually no renin activity (Katz and Malvin, 1982b) and thus, was removed. The kidney slices then contained primarily inner and outer cortical tissue. Medullary dissection ensured that the slices were homogenous with respect to renin content. Using this coronal method of slicing more than 30 slices per kidney could be obtained which was far more than the 2-4 slices which were previously obtained using sagittal cuts (Katz and Malvin, 1982a; Churchill and Churchill, 1982; Cho and Malvin, 1979).

Proper oxygenation of the vials is important to the viability of the slices. Cho and Malvin (1979) demonstrated that direct bubbling of the Krebs-Ringer buffer surrounding the kidney slices decreased the amount or activity of renin present in the medium and also prevented the accumulation of renin activity over time. The decrease in renin was due to inactivation of the enzyme at the air-liquid interface. The values for renin release using the bubbled kidney slice method were lower and less consistent than those presented using the modified (unbubbled) method (Table 1). By changing to the unbubbled method the values for renin release became more consistent and reproducible.

Histological and immunocytochemical studies were performed to verify the viability of the kidney slices both before and after the incubation periods (Van de Kar et al., 1987). Histological analysis revealed that the glomerulus and adjacent juxtaglomerular apparatus appeared healthy both before and after incubation. Immunocytochemical

staining for immunoreactive renin was localized to segments of the afferent arterioles. Again, there were no differences between the staining in the slices either prior to, or after, 2.5 hours of incubation. These findings suggest that the content of renin was not depleted during the incubation period. Other reports indicate that the renal content of renin is not depleted by a similar incubation period (De Vito et al., 1970). These analyses of the kidney slices indicate that the juxtaglomerular cells in the kidneys remain healthy during the RRF bioassay.

An indicator for a viable kidney slice method is the determination of lactate dehydrogenase (LDH) in the medium surrounding the kidney slices. LDH is a cytoplasmic enzyme that is commonly used as a indicator for the leakage of cell contents into the medium. Reports by Churchill (1979) and Lyons (1980) showed that LDH activity in the medium did not parallel the release of renin from the slices. LDH did accumulate during the incubation, however, the rate of accumulation was not affected by factors that increased renin release. The values for LDH reported for the unbubbled kidney slice method discussed above were lower than reported in the literature (Fray et al., 1983) and were not different between the treatment groups that received the PCA-plasma or saline-plasma fraction (M.W. 5,000-10,000). Even though the kidney slices that received the PCA-plasma fraction released more than two times as much renin as the vials that received the saline-plasma fraction, the LDH values were the same.

An additional precaution taken with the kidney slice bioassay was to use young rat kidneys for the slices. The kidneys used for the

bioassay were taken from rats that weigh less than 250 g. It has been noted in our laboratory that as the rats age their kidney slices often hypersecrete renin into the Krebs-Ringer medium which masks any treatment effect during the incubation. Using rats between 150-250 g increases the probability that the kidneys will be responsive to a stimulus. Corman et al. (1985) saw that as the rats grew older their kidneys increased in weight. They noticed that the increase in kidney size is due to enlargement of the glomerulus and proximal tubules. Furthermore, the filtering capabilities of the kidney is decreased in older rats. Fray (1978) noted that the kidneys from sodium-depleted rats had higher basal levels of renin release than kidneys from controls rats. It is possible that there are alterations in the handling of sodium that causes the kidneys to release more renin. The aging of the kidney may not be related to a change in one function but may be the result of changes in several processes. It is possible that the alterations in renin release, in vitro, from older kidneys are due not only to changes in the renin-angiotensin system but to age related nephropathies.

To test the reliability of the kidney slice bioassay isoproterenol was added to the kidney slices. Addition of isoproterenol (10^{-5}-10^{-6} M) is known to cause renin release from the kidney (Henrich and Campbell, 1984; O'Dea et al., 1984; Katz and Malvin, 1982a; Churchill and Churchill, 1982) by stimulating beta receptors (Capponi and Vallotton, 1976; Weinberger et al., 1975). An increase in renin release was observed using the unbubbled kidney slice method (Table 3) after a 1 hour incubation with isoproterenol. Using the new method, a

10^{-7}M dose of isoproterenol was able to produce a significant increase in renin release whereas, with the bubbled kidney slice method, a 10^{-5} M dose did not produce a statistically significant increase. The literature generally cites increases of 1.3-2.2 times the control values for isoproterenol-induced renin release after 1-2 hours of incubation. Addition of 10^{-7} M isoproterenol produced more than a three-fold increase in renin release over a period of 3 hours in the unbubbled renin release bioassay (Urban and Van de Kar, 1986). When compared with the values in the literature, this is a larger increase in renin release produced with a smaller dose of isoproterenol; this illustrates that the bioassay is sensitive and responds to a stimulus.

One limitation to this bioassay is the narrow dynamic range for assessing renin release. It is not often that values for renin release are larger than 25 ng ANG I/mg kidney/hr. It is likely that there is more renin being released than can be detected by this bioassay. One possibility is that renin may be degraded in each of the kidney slice vials during the incubation period, since we have not measured the rate of accumulation and degradation of renin in these vials.

C. In vivo studies on plasma RRF

1. Effect of 5-HT agonists on PRA, PRC and plasma RRF concentration

Since PCA stimulates the release of RRF, the serotonin agonists MK-212 and TFMPP were administered, in addition to PCA, to conscious rats to test if these 5-HT agonists could stimulate the release of RRF. PCA, MK-212 and TFMPP increased PRA, PRC and plasma RRF levels. For both PRA and PRC, the levels produced by MK-212 were higher than the

PCA- and TFMPP-treated groups (Table 6). Both MK-212 and TFMPP are known to bind to 5-HT receptors. However, their relative potency at the receptor subtypes differ. MK-212 is a serotonin agonist (Clineschmidt, 1979) that also has been shown to increase phosphoinositol turnover in cortical slices (Conn and Sanders-Bush, 1985). The serotonin-induced increase in phosphoinositol turnover is coupled to 5-HT_2 receptors whereas stimulation of 5-HT_1 receptors results in an increase of adenylate cyclase activity (Sanders-Bush and Conn, 1985). The selective 5-HT_2 antagonist LY53857 prevented the increase in PRA after either treatment with MK-212 or fenfluramine supporting the hypothesis that 5-HT_2 receptors regulate renin secretion (Lorens and Van de Kar, 1987). Studies by Clineschmidt et al. (1978) showed that MK-212 can inhibit the serotonin uptake system at the dose that was used in this study (10 mg/kg, i.p.). They demonstrated that pretreatment of rats with MK-212 prevents the depletion of serotonin observed 72 hours after treatment with PCA. PCA gains access to the neuron via the reuptake pump. Pretreatment with an uptake inhibitor prevents PCA from entering the nerve terminal and thus inhibits its action. It is possible that by blocking the serotonin uptake pump, MK-212 increases the amount of serotonin available at the synapse (Wong et al., 1985). Assuming that the renin response, seen with the 10 mg/kg dose of MK-212 is not due to maximal occupation of the receptors, it is likely that by a combination of both stimulating 5-HT_2 receptors and increasing the synaptic availability of serotonin, MK-212 could produce a potentiation of the renin response.

Administration of the 5-HT_{1B} receptor agonist, TFMPP, produces

significant increases in PRA and PRC, although the increase in PRC is not as elevated as that produced by either MK-212 or PCA. A number of studies have demonstrated that TFMPP binds preferentially to 5-HT_{1B} receptors (McKenney and Glennon, 1986; Asarch et al., 1985). This result is in disagreement with the theory that only 5-HT_2 receptors are involved in the regulation of renin secretion. However, administration of RU24969, a non-selective 5-$HT_{1A\&B}$ agonist, produces dose-dependent increases in PRA and PRC that are inhibited by pretreatment with LY53857 (Van de Kar et al., 1987). This indicates that the putative 5-HT_1 agonist, RU24969 also has affinity and activity at the 5-HT_2 receptor or that LY53857 also blocks the 5-HT_{1B} receptor. In a study by Pettibone and Williams (1984), TFMPP and similar piperazine-containing compounds, were found to act as serotonin releasers and displace endogenous stores of serotonin. Fuller et al. (1981), have reported that TFMPP can act as an uptake inhibitor since it prevented the depletion of brain serotonin by PCA. Furthermore, Conn and Sanders-Bush (1985) have indicated that TFMPP can produce an increase in phosphoinositol hydrolysis, an effect that is associated with 5-HT_2 receptors. It is possible that TFMPP produces an increase in PRA, PRC and RRF levels by an action that is not attributable to its 5-HT_1 activity. TFMPP could release enough serotonin to increase renin secretion, or it is even possible that TFMPP may have activity at the 5-HT_2 receptor site in addition to its 5-HT_{1B} activity.

Administration of the serotonin agonists MK-212 and TFMPP and the serotonin releaser, PCA, all increased the plasma concentration of RRF. The difference in the mechanism of action of the drugs can be noticed

in the PRA and PRC values, however, the values for plasma RRF concentration are equally elevated, indicating that each drug released about the same amount of RRF. It is difficult to explain why the RRF values do not reflect the difference PRA and PRC values. It is evident that serotonin receptors were stimulated to release RRF into the plasma. One reason for the consistent values for RRF concentration is that the kidneys may have been stimulated maximally with the addition of the plasma samples so that any difference in RRF concentration between the drug treatments (measured as renin release from the kidney slices) was not detectable by the bioassay. Isoproterenol was not added in this bioassay so it is difficult to define the dynamic range of this bioassay. It is not likely that the activity of RRF was digested by proteases since the plasma samples were boiled for 20 minutes and the larger proteolytic enzymes would not have survived the boiling process. Another possibility is that some RRF was inactivated by brush border enzymes present in the proximal tubule of the renal cortex. A study by Ward and Johnson (1978) showed that kidney extracts inactivated substance P faster than the extracts of either liver or lung. The highest activity of substance P degradation occurred in the brush border of the proximal tubules. The bioassay is performed on renal cortical slices that contain a high concentration of proximal tubules (Van de Kar et al., 1987). Therefore, it is likely that some RRF could have been catabolized during the bioassay since no precautions were taken to inactivated these enzymes.

2. Role of stress on plasma RRF concentration

Rats that are subjected to a variety of stress paradigms have increased levels of corticosterone, prolactin and renin (Paris et al., 1987; Richardson-Morton et al., 1986; Van de Kar et al., 1984; 1985; Eljarmak et al., 1982). The effect of a stressor such as conditioned emotional response (Richardson-Morton et al., 1986) and immobilization (Gotoh et al., 1987) on renin secretion can be prevented by electrolytic lesions in the hypothalamic paraventricular nucleus (PVN). This result does not appear to be due to decreased levels of renin substrate that would occur as a result of interrupting the adrenocortical axis since the corresponding PRC values, measured with a saturating concentration of exogenous renin substrate, are also decreased (Richardson-Morton et al., 1986). Further studies have indicated that cell bodies in the PVN are responsible for mediating the stress-induced increase in PRA and PRC (Richardson-Morton et al., 1987). Furthermore, electrolytic lesions in the PVN also prevented the increase in PRA after administration of PCA (Gotoh et al., 1987). Since the PVN contains neurons that can release peptides into the circulation, it is likely that the release of RRF may be stimulated by stressful conditions. Stress produces an increase in PRA and plasma RRF concentration when compared with the renin-releasing activity of plasma from control (non-stressed) rats (Table 7). That the increase in PRA was about four times greater than the change observed with the RRF concentration. There are many factors that can influence the PRA values in stressed rats. PRA is a measurement that reflects changes in central and peripheral sympathetic nerve activity, changes in sodium

levels and blood pressure, in addition to increases due to RRF. When measuring the plasma concentration of RRF, only the direct effect of the factor is being detected at the kidney level which is evaluated by incubating the plasma fraction with renal cortical slices. Furthermore, the renal cortical slice is only a fraction of the total renin-releasing ability of the kidney. If more kidney tissue were added to the bioassay, the increase in renin-releasing activity could have been greater.

D. Characterization of plasma RRF in vitro

Administration of PCA to rats produced an increase in plasma renin activity that was due to the presence of a blood-borne renin-releasing factor (RRF; Van de Kar, et al., 1982a). RRF was identified to be present in the plasma fraction with molecular weights of 500-10,000. The PCA-plasma fraction (M.W. 500-10,000) was also tested in the renin release bioassay and produced dose-dependent increases in renin release. Further studies using the PCA-plasma fraction with smaller molecular weight ranges indicated that RRF is within the 5,000-10,000 molecular weight range. RRF is also heat stable. These studies were performed using the bubbled kidney slice method (Urban et al., 1985). Although there were fairly large increases in renin release among the treatment groups (0.1 $\pm$ 0.06 and 0.66 $\pm$ 0.38 ng ANG I/mg kidney/hr), these differences were not significant. This was due in part to the fact that there was a low number of samples in each group (n=3), and there was also a large variation among the values for renin release within each group. This was reflected in the relatively large standard

error of the mean (S.E.M.). The large S.E.M. values indicated the need for a more reliable method for measuring renin release. Therefore, the unbubbled method, that proved to be reliable and reproducible was developed and used for the rest of the experiments.

The studies using the plasma fractions were repeated using the new unbubbled method. The results indicate that the 5,000-10,000 molecular weight PCA-plasma fraction had the highest amount of renin-releasing activity. This replicates the results found using the previous bubbled bioassay method. Thus, we obtained similar results using both the bubbled and unbubbled in vitro renin release bioassays and an in vivo renin release bioassay (Van de Kar et al., 1982a). This suggests that even though the bubbled method was not very reliable for measuring renin release, it showed a response to an applied stimulus. More importantly, replication of the results using different methods, reinforces the validity of the observation.

Nephrectomy is known to increase plasma angiotensinogen (renin substrate) levels (Carretero and Gross, 1967). Therefore plasma from nephrectomized rats would be expected to contain increased amounts of angiotensinogen. It is not likely that angiotensinogen would influence renin release from the kidney slices since the molecular weight of angiotensinogen is approximately 60,000 (Tewksbury, 1983) and the plasma fraction that increased renin release contained solutes with molecular weights from 5,000-10,000. Furthermore, angiotensinogen is denatured by heat and would not have survived the boiling process prior to fractionation of the plasma. For the same reasons, renin would not be present in the plasma fractions: renin is heat labile and also has a

larger molecular weight (40,000). Moreover, the plasma renin activity values in rats that are anephric are at the sensitivity limit of the assay indicating that there is little, if any, renin present in the plasma. In addition, if renin or angiotensinogen were responsible for the renin-releasing activity, these substances would be expected to be present in the saline-plasma fraction and would have produced a similar increase in renin release.

Serotonin (5-HT) and PCA were not capable of directly producing an increase in renin release from the kidney slices indicating that these drugs are not renin-releasing factors. Furthermore, these molecules are small (PCA: M.W.=206; 5-HT: M.W.=179) and would have been filtered through the lower molecular weight membrane (M.W. cutoff 5,000). Circulating catecholamines which are known to alter renin release are also not likely to be the renin-releasing factor since they also have low molecular weights and are heat labile.

The renin-releasing activity of the PCA-plasma fraction (M.W. 5,000-10,000) is destroyed by incubation with pronase; in addition with the heat stability of RRF these studies suggest that RRF is a peptide. Pronase non-selectively digests peptide bonds (Narahashi, 1970). The renin-releasing activity of this PCA-plasma fraction (M.W. 5,000-10,000) is lower than in the previous experiment (Table 12 versus Table 14). This difference in potency may be partially due to the presence of calcium chloride (5 mM) that was present in the pronase vehicle (borate buffer). The final concentration of the calcium chloride that was added to the kidney slice buffer was approximately 5.5×10^{-5} M. The presence of calcium at this dose has been shown to inhibit renin

secretion (Park et al., 1986; Antonipillai and Horton, 1985). Therefore, this increase in the calcium content of the kidney slice buffer may have altered the response of the kidneys to the PCA-plasma fraction. The renin-releasing activity of the corresponding saline-plasma fraction (M.W. 5,000-10,000) was not affected by treatment with pronase, suggesting that the substance in this fraction that increases renin release is not a peptide. The substance present in the saline-plasma fraction might therefore be different from the factor present in the PCA-plasma fraction. Furthermore, the other plasma fractions (M.W. 1,000-5,000 and 10,000-20,000) also exhibited some renin-releasing activity. It is possible that the increase in renin release seen with these other plasma fractions may be due to incomplete fractionation of RRF or due to a change in the osmolality of the buffer bathing the kidney slices. The ultrafiltration method employed is not an absolute method for determining molecular weight but is primarily used to concentrate solutes within different molecular weight ranges. Some of RRF may have been retained in the large molecular weight fraction and, if the molecular weight of RRF is closer to 5,000, it could have been filtered through into the lower (1,000-5,000) molecular weight fraction. This explanation may account for some of the activity present in the other PCA-plasma fractions (M.W. 1,000-5,000 and 10,000-20,000). However, in the saline-plasma fractions there is also some renin-releasing activity. Administration of PCA stimulates release of RRF, but it is also likely that there may be a small basal release of the peptide that would be detected in the plasma of the saline-treated rats. However, this possibility is not supported by the data obtained

from the pronase experiment. If the saline-plasma fraction (M.W. 5,000-10,000) contained the same substance as the PCA-plasma then pronase should have destroyed the renin-releasing activity present in the saline-plasma fraction.

Since these fractions contain solutes with different molecular weights it is difficult to conceive that one substance produces these increases. One possibility may be that these fractions change the osmolality of the Krebs-Ringer buffer when they are added to the kidney slices which would affect renin release. Studies have indicated that decreases in osmolality stimulate the release of renin *in vitro* suggesting that the release of renin may be regulated by the cell volume of the juxtaglomerular cells (Skott, 1986; Frederikson *et al.*, 1975). If the plasma fractions do alter the osmolality of the Krebs-Ringer buffer, it would most likely result in a relative increase in osmolality since these fractions are concentrated plasma and contain high concentrations of protein. According to the hypothesis of Frederikson *et al.* (1975) and Skott (1986) these concentrated plasma fractions should decrease renin release from the kidney slices. Hall and Guyton (1976) reported that infusions of hypertonic dextran or albumin increased renin secretion rate in anesthetized dogs. Similarly, Fray and Laurens (1981) reported that addition of albumin to juxtaglomerular cells increases renin release from the cells probably by sequestering, and lowering, the extracellular calcium concentration. It could be possible that there may be substances in the PCA-plasma and saline-plasma fractions that could lower the amount of extracellular calcium thereby increasing renin release over the saline control

values.

Recent evidence has indicated that in addition to active renin, an inactive form of renin is present in the plasma (Bouhnik et al., 1985; Inagami et al., 1983). Inactive renin, or prorenin, is primarily produced in the kidney (Taugner et al., 1987; Bouhnik et al., 1985) and circulates in the plasma where it is activated. There are a variety of conditions that can activate prorenin, such as cold activation (Wilczynski and Osmond, 1983), proteolysis with trypsin (Takada et al., 1986; Wilczynski and Osmond, 1986), and acidification (Wilczynski and Osmond, 1983). The actual stimulus for activation of prorenin in the plasma in vivo has not been identified. One possibility for the increased amount of renin activity seen with the addition of the PCA-plasma (M.W. 5,000-10,000) could have been that the plasma fraction contained an activator that would convert prorenin in the medium surrounding the kidney slices to active renin. PCA-plasma and saline-plasma fractions (M.W. 5,000-10,000), when added to the pooled Krebs-Ringer buffer that was previously incubated with kidney slices, did not produce an increase in renin activity above the saline control group (Table 4). This indicates that the increased amount of renin release from the kidney slices was not due to activation of prorenin by the plasma fractions.

The plasma fractions were also tested for possible renin-like activity that would have resulted in higher values for ANG I generated in the renin assay. The PCA-plasma and saline-plasma (M.W. 5,000-10,000) fractions were incubated with renin substrate to test if they could generate ANG I. The plasma fractions were not capable of

producing any measurable amounts of ANG I, which demonstrates that there was no renin-like enzyme activity present in these plasma fractions. These two tests verify that the increase in the amount of ANG I generated from the samples was due to the release of renin from the kidney slices and not to activation of prorenin or the presence of renin-like enzymes in the plasma fractions. This conclusion is logical since the molecular weights of renin and cathepsin D, a renin-like enzyme, have molecular weights around 40,000.

E. Studies of RRF in rat brain

1. Extraction media

Rat hypothalami that are homogenized with boiling distilled water yield a higher amount of renin-releasing activity than hypothalami that are homogenized with cold perchloric acid (0.1 N). Since RRF is a heat stable peptide, the brain tissue could be homogenized in boiling water to denature other larger proteins, leaving the renin-releasing activity of RRF unaffected. It would be expected that the whole brain extracts and hypothalamic extracts would exhibit similar values for renin-releasing activity with the different extraction media. The comparison of extraction media with whole brain homogenates indicate that the cold perchloric acid (0.1 N) is the better solution for extracting renin-releasing activity. Boiling distilled water and cold 0.1 N hydrochloric acid/ethanol (20:80) were equally effective in recovering renin-releasing substances from whole brain tissue. The results from the whole brain versus hypothalamic tissue homogenates are not in total agreement. These data imply that extraction of whole brain with cold

perchloric acid may extract or activate other renin-releasing substances not located in the hypothalamus.

2. Central nervous system (CNS) distribution of RRF

Rat hypothalamic extract produces dose-dependent increases in renin-release from the kidney slices (Figure 3). The equivalent of the content of one hypothalamus, when added to the kidney slices, produces a maximal stimulation of renin release. This indicates that there are receptors on the kidney that can respond to different concentrations of RRF. This also is an important finding because it made it possible to standardized the amount of tissue extract that was added to the kidney slices so that the relative concentration in renin-releasing activity of different brain regions could be compared. The renin-releasing activity of other brain regions were compared to the renin-releasing activity present in one hypothalamus.

Bioassay of rat brain regions showed a differential distribution of renin-releasing activity (Figure 4). In addition to the hypothalamus and cerebellum which have been shown previously to contain renin-releasing substances (Van de Kar et al., 1987b), the cerebral cortex and medulla oblongata also have significant amounts of renin-releasing substances. Other brain regions (pons, thalamus, caudate, hippocampus, midbrain and amygdala) do not contain significant concentrations of RRF although there is some renin-releasing activity present in these regions.

Addition of pituitary extract produced a small decrease in renin release from the kidney slices. Since the whole pituitary (both neural

and anterior lobes) was homogenized, the decrease in renin release may be due to the presence of vasopressin in the neural lobe. Vasopressin is known to inhibit renin secretion (Schwartz and Reid, 1985; Vander, 1968). The high vasopressin content in the pituitary may have masked any renin-releasing activity. The hypothalamic PVN and SON (supraoptic nucleus) contain vasopressin cell bodies, however, an increase in renin release is still observed when the hypothalamic extract is added to the kidney slices. Within the PVN and SON neurons, vasopressin is synthesized and processed along with neurophysin and a smaller glycoprotein. Vasopressin is separated from the neurophysin as they are transported down the axon to the nerve terminals where they are released (Camier et al., 1985; Masse et al., 1982; Russell et al., 1982). It can be hypothesized that when vasopressin is bound to the carrier protein (neurophysin) it may not interact at the kidney receptor and thus will not suppress renin release. Therefore, based on this conjecture, the renin-releasing activity of the hypothalamus would not be masked by the inhibitory action of vasopressin.

Colchicine is known to inhibit microtubule transport (Andreu and Timasheff, 1986). Treatment with colchicine has been used to prevent the axonal transport of peptides from the cell body in order to concentrate the peptide within the cell body (Emanuele et al., 1985; Kawata et al., 1985). Therefore brain areas that have increased renin-releasing activity should indicate the presence of RRF in cell bodies. The brain areas that have a decrease in activity would indicate the presence of RRF in nerve terminals. After intracerebroventricular injections of colchicine, the renin-releasing activity in the rat brain

was redistributed. The hypothalamus was the only brain region that had renin-releasing activity that was significantly increased over all other brain regions except the pons and thalamus. These results suggest that the cell bodies containing RRF are located in the hypothalamus.

Using an HPLC (high performance liquid chromatography, GPC-100 column) system and a G50 Sephadex gel chromatography column, rat hypothalamic extract was separated into different molecular weight fractions and the renin-releasing activity of the fractions was determined. There was a significant concentration of renin-releasing activity in three peaks within the molecular weight range of approximately 4,800-6,000 (Van de Kar, Brownfield and Urban, unpublished observations). These fractions have not been tested for susceptibility to pronase digestion.

In addition to the presence of RRF in the rat CNS, bovine hypothalamus also contains renin-releasing substances (Van de Kar et al., 1987b). Bovine hypothalamic extract was filtered to obtain different molecular weight fractions which were tested in the renin release bioassay for the presence of RRF. These fractions were treated with pronase to test whether there were similar renin-releasing substances in the hypothalamus. We determined that the bovine hypothalamus contains renin-releasing peptides within the molecular weight ranges of approximately 1,000-5,000 and 5,000-10,000 (Van de Kar et al., 1987b). Discovering the presence of renin-releasing peptides in bovine hypothalamus with a similar molecular weight range as the rat plasma fractions, suggests that the rat may not be the only species to

have RRF. The bovine hypothalamic fraction (M.W. 5,000-10,000) may contain the same or similar renin-releasing peptides as the rat plasma fraction. One dissimilarity between the rat plasma and bovine hypothalamus fractions is that the bovine hypothalamus contains renin-releasing activity in a fraction with a molecular weight range of 1,000-5,000. Renin-releasing activity was only present in the rat plasma fraction within the 5,000-10,000 molecular weight range. It is possible that in the bovine hypothalamus, the smaller peptide may be a metabolite of the larger peptide that does not reach the bloodstream, or it may even be a different substance altogether.

F. Distribution of RRF in peripheral tissues

Many neuropeptides are not only localized to the brain but also exist in peripheral tissues. For example, vasoactive intestinal polypeptide (VIP), neurotensin, substance P, neuropeptide Y (NPY) and atrial natriuretic factor (ANF) are peptides that can be identified in neurons in the brain and also are distributed in different peripheral tissues. The test for renin-releasing activity in various peripheral tissues indicated that only the kidney extract produced an increase in the amount of ANG I detected in the RRF bioassay. This effect was likely due to excess ANG I that was generated by renin in the kidney extract which escaped denaturation before it was added to the kidney slices.

Addition of adrenal extract to the kidney slices inhibited renin release. The adrenal medulla contains a high content of catecholamines. These are not likely to be involved in the decrease in

renin release since catecholamines are not heat stable and are likely to be destroyed by addition of water and heating at 100°C for 20 minutes. However, if the catecholamines did survive the treatment, it could be likely that they would decrease renin release by stimulating alpha receptors.

Glucocorticoids and mineralocorticoids are localized in the adrenal cortex. These hormones play a role in the maintenance of glucose and sodium balance and plasma volume. Administration of steroids decreases the elevated levels of plasma renin activity that occurs after adrenalectomy (Nasjletti and Masson, 1969). Infusion of prednisolone, a glucocorticoid with some mineralocorticoid activity, also decreases plasma renin activity in normal subjects. The suppression of renin after administration of glucocorticoids in conscious animals probably occurs secondarily to the increase in plasma volume. Receptors for corticosterone (Lee et al., 1983) and aldosterone (Scholer et al., 1979) have been identified by binding studies to be localized along the nephron and on distal segments of the cortical tubules, respectively. De Vito et al. (1970) reported that addition of aldosterone to kidney slices produces about a 30% decrease in renin release from the slices whereas addition of deoxycorticosterone has no effect on renin release.

In addition to catecholamines and steroids, immunocytochemical methods have identified other transmitters and peptides in the adrenal medulla. Serotonin (Holzwarth and Brownfield, 1985), VIP (Hokfelt et al., 1981; Holzwarth, 1984), neurotensin (Lundberg et al., 1982), substance P (Hokfelt et al., 1977), neuropeptide Y (NPY; Varndell et

al., 1984; Majane et al., 1985), somatostatin and enkephalin (Lundberg et al., 1982) were identified in the adrenal medulla. In the case of VIP, some fibers were also found in the adrenal cortex. In our studies, 5-HT has been demonstrated not to alter the release of renin from the kidney slices, therefore 5-HT would not produce the decrease in renin release produced by the adrenal extract. VIP stimulates the secretion of renin both in vivo (Porter et al., 1985) and in vitro (Porter et al., 1983). Therefore any VIP that is present in the adrenal homogenate would not be responsible for decreasing renin release from the kidney slices. On the otherhand, NPY (Hackenthal and Taugner, 1986), substance P (Gullner and Bartter, 1979; Gullner et al., 1979) and somatostatin (Izumi et al., 1979) have been shown to decrease renin release in vitro. Substance P, even though it is a potent vasodilator which would reflexly increase PRA, produced a decrease in PRA at doses that do not alter blood pressure (Izumi et al., 1979). NPY slightly decreases plasma renin activity in conscious animals (Pfister et al., 1986) at doses that do not influence mean arterial pressure or heart rate. Neurotensin also produces hypotension like substance P, but may not affect renin release (Gullner and Bartter, 1979). Based on these findings it is probable that the decrease in renin release from the kidney slices produced by adrenal extract may be due to the presence of NPY, substance P or somatostatin which are known to suppress renin release.

G. Evidence for a RRF neuroendocrine system

1. Superfusion of rat hypothalamus

Since the main grouping of RRF cell bodies have been localized to the hypothalamus, hypothalamic explants were stimulated to test for the release RRF. Superfusion of the hypothalamic explants with a high potassium solution (60 mM), which depolarizes the neurons, results in increased release of RRF. This observation indicates that RRF neurons can respond to a stimulus and that the nerve terminal regions are likely to be present in the hypothalamus. For a neuropeptide to be released from the hypothalamus there are essentially three pathways: 1.) The neuron may send a projection down the pituitary stalk to the posterior pituitary lobe, 2.) the neuron may terminate in the region of the median eminence where the peptide is released into the portal blood and 3.) the peptide may be released into the third ventricle where it will enter the cerebrospinal fluid (CSF). Based on the earlier in vivo studies of the PCA-induced increase in renin secretion, the role of the pituitary in the release of the peptide still needs to be resolved. Hypophysectomy prevented the PCA-induced increase in PRA after 22 days but did not affect PRA 4 days after surgery (Karteszi et al., 1982; Van de Kar et al., 1982). Both hypothalamic explants and hypothalamic-hypophyseal explants that had the pituitary and infundibulum attached, were used for the superfusion study. The results suggest that there was no need for the hypothalamus to have an intact pituitary stalk in order to release RRF. This suggests that the terminals involved in the release of RRF were intact and may terminate in the median eminence which would not have been destroyed in the

dissection procedure. These results do not rule out the involvement of the pituitary gland in the release of RRF. However, they do lend credibility to the hypothesis that RRF is part of a neuroendocrine system.

2. Effect of bilateral nephrectomy on hypothalamic content of RRF

Another characteristic of a neuroendocrine system is that the release of the factor may be regulated by the end product or hormone. The release of corticotropin releasing factor (CRF) is regulated by circulating levels of corticosterone. High levels of corticosteroids will inhibit the release of ACTH and CRF from the hypothalamus to control the plasma levels of CRF (Keller-Wood and Dallman, 1984). When the adrenal gland is removed, thereby removing corticosterone feedback, the hypothalamic content of CRF is increased (Plotsky and Sawchenko, 1987; Piekut and Joseph, 1986; Sawchenko and Swanson, 1985). The studies by Piekut and Joseph (1986) and Sawchenko and Swanson (1985) were performed in rats that were adrenalectomized for at least seven days. However, the recent study by Plotsky and Sawchenko (1987) indicate that 24 hours after adrenalectomy there is a significant increase in CRF, as determined by radioimmunoassay, and in CRF staining in the PVN. This study suggests that nephrectomy performed 24 hours before sacrifice may also be a long enough time to observe an increase in the hypothalamic concentration of RRF.

The data suggest that removal of the kidneys eliminates an inhibitory input for RRF secretion and production. By removing the kidneys, there is a reduced capacity to inhibit the synthesis and

possibly, the release of RRF from the hypothalamus. Therefore, RRF cells will continue to synthesize and secrete RRF as long as there is no feedback stimulus from the kidney to reduce their activity. This feedback factor may be a product of the renin-angiotensin system, possibly ANG II or aldosterone. Based on these results we could speculate that ANG II may be the mediator of the feedback loop from the kidney to the brain. The subfornical organ (SFO) is a circumventricular organ that is located in the third ventricle and is responsive to changes in the circulating levels of ANG II (Gross et al., 1985). The SFO has been documented to send projections to the PVN (Tanaka et al., 1986) that are implicated in the control of blood pressure (Gutman et al., 1985). In an earlier study, Tanaka et al. (1985) demonstrated that stimulation of the neurons in the SFO produced an inhibition in the activity of 50% of the PVN neurons tested. This suggests that the SFO sends projections to the PVN that can regulate the firing rate of neurons in the PVN. More studies need to be performed to characterize this RRF feedback loop. However, it is probable that elevated levels of ANG II produce by increased renin release, may stimulate neurons in the SFO which project to the PVN to maintain the release of RRF at a homeostatic level.

It is important to note that in the bilateral nephrectomy and the colchicine studies, the levels of renin-releasing activity in the hypothalamus is elevated over the isoproterenol control values. Even though these increases are not significantly different, this may give an indication as to whether or not RRF stimulates a renal beta receptor. It is probable that isoproterenol is maximally stimulating

the renal beta receptors since different doses of isoproterenol (10^{-5}-10^{-7} M) produce similar increases in renin secretion. This indicates that the beta receptors may be saturated since the more concentrated dose (10^{-5} M) does not produce a further increase in renin release from the slices when compared with the 10^{-7} M dose of isoproterenol. The elevations observed with isoproterenol usually produce increases in renin release around 20-23 ng ANG I/mg kidney/hr. However, with the PCA-plasma fraction (M.W. 5,000-10,000; Table 8) and the hypothalamic extracts, increases in renin release from the kidney slices range from 23-32 ng ANG I/mg kidney/hr. These results are not sufficient evidence for the existence of separate RRF receptors, but may give hint on the possibility of selective RRF receptors in the kidney.

When the beta antagonist, d,l-propranolol, is added to the kidney slices prior to addition of the PCA-plasma fraction or plasma from stressed rats, there is a slight inhibition of both basal and RRF-stimulated renin release which may be due to the membrane stabilizing effects of d-propranolol. However, there still is a significant increase in renin release when the PCA-plasma fraction is added to the kidney slices. Other beta blockers have been tested. Nadolol and atenolol, on the otherhand, prevented the renin-releasing activity of the PCA-plasma fraction (Urban and Van de Kar, unpublished results). These data are conflicting and do not conclusively state whether or not RRF stimulates renal beta receptors. Clearly, the receptor that mediates the effect of RRF on renin release from the kidney needs to be studied further.

H. Possible renin-releasing peptides

There are a number of peptides that stimulate renin release. A few of them are within the 5,000-10,000 molecular weight range. At the time that these studies were performed, the molecular weight of RRF was identified to be in the range of 5,000-10,000. It was not until after these studies were performed that the molecular weight of RRF was identified to be within the 4,800-6,000 molecular weight range. Insulin (M.W. 6000) and parathyroid hormone (PTH, M.W. 9000) are two hormones that are within the molecular weight range of 5,000-10,000 and are known to increase renin release.

Insulin is primarily synthesized in the pancreas, however, recent evidence has accumulated indicating the presence of insulin-like immunoreactivity and binding sites in the brain and, of particular interest for this study, in the hypothalamus (Baskin et al., 1983; Van Houten et al., 1980). Induction of diabetes by alloxan injection produces a decrease in PRA that is restored to normal when the diabetic rats are supplemented with insulin (Pratt et al., 1985). The levels of sodium remain normal. However, potassium levels are increased in the diabetic rats and it is likely that the decreased PRA values were produced by the increased plasma concentration of potassium (Vander, 1970). Infusion of insulin produces increases in plasma renin activity (Otsuka et al., 1970) that is prevented by either pretreatment with propranolol (Assaykeen et al., 1970) or by the prostaglandin synthesis inhibitor, indomethacin (Campbell and Zimmer, 1980). The distribution of insulin in the rat CNS appears to be concentrated around the periventricular area (Baskin et al., 1983; Dorn et al., 1981).

Considerable staining for immunoreactive insulin has been identified in the ependymal cells lining the wall of the third ventricle and in the hypothalamus (Dorn et al., 1981). The cerebellum and brainstem contain small, almost nondetectable concentrations of insulin as determined by radioimmunoassay. Before insulin is ruled out as a putative RRF, insulin should be tested in an in vitro renin release bioassay to test its renin-releasing activity without the effects of potassium and increases in circulating catecholamines that can occur in vivo.

Parathyroid hormone (PTH) produces increases in PRA (Smith et al., 1979; Powell et al., 1978; Broulik et al., 1986). PTH infusion did not alter plasma calcium levels or blood pressure but pretreatment with a beta blocker (metipranol) attenuated the renin response to PTH in humans (Broulik et al., 1986). The PCA-induced increase in PRA was not affected by removal of the parathyroid gland indicating that PTH is not the RRF. If PTH were the RRF then removal of the parathyroid gland would have blocked the effect of PCA on renin secretion.

There are other peptides that are known to regulate renin secretion. Even though some of the peptides are not within the molecular weight range of the PCA-plasma fraction that has renin-releasing activity, recent evidence with the sephadex G-50 and HPLC gel filtration (GPC-100 column) indicated that there may be hypothalamic substances with molecular weights below 5,000 that are capable of inducing renin release from the kidney slices (Van de Kar, Brownfield and Urban, unpublished observations). Glucagon (M.W. 3350), has been shown to increase renin secretion from the kidney (Ueda et al., 1978; Vandongen et al., 1973). When infused into the renal artery at a

relatively low dose, glucagon produced increased heart rate, renal blood flow and glomerular filtration rate. At a higher dose, glucagon produced similar changes in the renal hemodynamic parameters and in addition produced a significant increase in renin release (Ueda et al., 1978). Pretreatment with propranolol did not prevent the increase in renin release produced by glucagon (Ueda et al., 1978; Vandongen et al., 1973). Glucagon and isoproterenol stimulate adenylate cyclase in the renal medulla (Mulvehill et al., 1976). However, the effect of glucagon on adenylate cyclase activity is not inhibited by propranolol, indicating a glucagon specific adenylate cyclase. Therefore it is likely that glucagon may stimulate renin secretion by activating adenylate cyclase in the renal cortex.

Vasoactive intestinal peptide (VIP; M.W. 3326) increases renin release both in vivo (Porter et al., 1983; 1985) and in vitro (Porter et al., 1983). There is a high density of VIP-immunoreactive neurons in the hypothalamus, specifically, the suprachiasmatic nucleus (SCN) of the rat (Loren et al., 1979; Sims et al., 1980; Samson et al., 1979). The SCN is known to receive serotonergic inputs from the midbrain raphe nuclei (Azmitia and Segal, 1978; Bobillier et al., 1976; Van de Kar and Lorens, 1979). Reduction of serotonergic inputs into the SCN, by injection with 5,6-dihydroxytryptamine (5,6-DHT), produced a decrease in the amount of VIP but not of vasopressin in the SCN (Kawakami et al., 1985). These results from this study (Kawakami et al., 1985) are suspect because the neurotoxin 5,6-DHT produces non-specific damage to neurons (non-serotonergic) surrounding the injection site (Baumgarten et al., 1973). Shimatsu et al. (1982) have shown that intraventricular

(icv) injection of serotonin stimulates the release of VIP into the portal blood. The plasma concentration of VIP was also increased after icv injection of serotonin. It is possible that VIP could be the RRF, based on the hypothalamic localization of the cell bodies and fibers, and the fact that serotonin can stimulate VIP release from the hypothalamus.

However, when comparing the distribution of RRF with that of VIP in the CNS, there is a discrepancy between the distribution of VIP and that of RRF. One of the primary differences is that the cerebellum dose not contain any VIP-immunoreactivity (Loren et al., 1979; Samson et al., 1979). The cerebellum, in control rats, had significant renin-releasing activity that was reduced in colchicine-treated rats. Furthermore, the cerebral cortex contains a high concentration of VIP cell bodies (Loren et al., 1979; Sims et al., 1980) but the RRF concentration in the cerebral cortex was significantly reduced after colchicine-treatment when compared with the hypothalamic extract. This decrease in renin-releasing activity suggests the presence of RRF in nerve terminals, not in cell bodies. From the current data, it is noticeable that the patterns of distribution for RRf and VIP are not similar, since the molecular weights of these peptides differ also, it seems unlikely that VIP and RRF are the same peptide.

Hauger-Klevene et al. (1970) have shown that adrenocorticotropic hormone (ACTH) produces an increase in PRA, that is inhibited by blockade of the sympathetic nervous system with pentolinium, a ganglionic blocker. The release of ACTH from the pituitary has been shown to be influenced by serotonergic neurotransmission (Fuller, 1981;

Szafarczyk et al., 1980). However, it is not likely that ACTH is the RRF, since hypophysectomy does not prevent the PCA-induced increase in PRA (Karteszi et al., 1982). Lesions of the dorsal raphe nucleus prevent the PCA-induced increase in PRA, but do not alter the increase in corticosterone levels (Van de Kar et al., 1982b). Posterolateral deafferentation of the hypothalamus also blocked the effect of PCA on PRA but not on corticosterone (Van de Kar et al., 1985a; Karteszi et al., 1982). This indicates that the PCA-induced release of renin and ACTH may be mediated by different pathways.

Atrial natriuretic factor (ANF) plays a role in regulating blood pressure and sodium balance. In 1981, De Bold et al. reported that peptides in the cardiac atria contain a natriuretic factor. ANF produces an increase in sodium excretion accompanied by a decrease in plasma volume and a decrease in blood pressure. ANF is, therefore, the antithesis of the renin-angiotensin-aldosterone system. Infusion of ANF to conscious animals results in an increase in sodium excretion (Murray et al., 1985) and a decrease in blood pressure and renin release (Burnett et al., 1984; Sosa et al., 1986; Seymour et al., 1985). Studies by Deray et al. (1987) show that infusion of ANF into nonfiltering canine kidneys does not alter the increase in renin release produced by addition of either norepinephrine or prostacyclin, whereas adenosine did attenuate the renin response to these agents. Based on these studies the authors conclude that ANF does not have a direct action on the juxtaglomerular cells.

Autoradiographic studies using kidney tissue localized ANF receptors to the renal outer cortex with concentrations of binding

sites over the glomerulus (Healy and Fanestil, 1986; Murphy et al., 1985). There is also a light distribution of binding sites in the renal medulla. In vitro studies have not clearly determined to what extent ANF regulates renin secretion directly from the kidney. Using a juxtaglomerular cell culture and renal cortical slices, Kurtz et al. (1986) and Henrich et al. (1986) showed that addition of ANF decreases renin secretion. This effect is independent of calcium, since pretreatment with verapamil did not alter the ANF-induced decrease in renin secretion. Other studies using similar doses of ANF indicate no change in renin release (Rodriguez-Puyol et al., 1986). However ANF further potentiated the ANG II-induced inhibition of renin release (Antonipillai et al., 1986). Stimulation of renin release by isoproterenol was unaffected by ANF. Contrary to these findings are the results obtained by Hackenthal et al. (1985) and Hiruma et al. (1986) who reported that ANF stimulates renin release. It is not likely that ANF is the RRF since its physiological actions are directly opposed to renin. So far, the stimuli that increase the plasma concentration of RRF also increase PRA and PRC. If ANF were the RRF, it would most likely result in an initial decrease in PRA since the natriuresis would reflexly inhibit renin secretion by increasing the delivery of sodium chloride to the macula densa.

The existence of a hypertension-producing, aldosterone-secreting factor (ASF) has been reviewed by Carey and Sen (1986). ASF is a glycoprotein with a molecular weight of approximately 26,000 with a smaller active fragment of 4,000 (Sen et al., 1981). Sen et al. (1986) report that ASF does not alter PRA, ANG II or angiotensinogen

concentration in the rat. Therefore, it does not seem likely that RRF is ASF. Furthermore, immunofluorescence studies have localized ASF to the anterior pituitary with no significant binding in the hypothalamus, cerebral cortex, cerebellum, brainstem or peripheral tissues (kidney, spleen, nerves, adrenal gland, thyroid, gastrointestinal tract; Sen et al., 1977). This distribution pattern in the CNS does not parallel that of RRF and therefore, it can be concluded that ASF and RRF are different factors.

For some peptides, such as prolactin and somatostatin, the presence of a disulfide bonds are necessary for these hormones to exert their physiological effect (Wehrenburg et al., 1983; Palkovits et al., 1982). In an attempt to determine if RRF contains disulfide bonds, cysteamine was administered before PCA. Reduction of possible disulfide bonds in the RRF could change the configuration of the molecule and thus destroy its renin-releasing activity. The results indicate that cysteamine did not alter the PCA-induced increase in PRA or PRC. However, PCA did not produce a significant increase in renin levels after cysteamine treatment when compared with either the corresponding cysteamine control group or the saline-PCA group. In these same animals, cysteamine reduced prolactin immunoreactivity. Therefore, there may be another action of cysteamine that alters resting renin levels. We have shown, that at the dose used in this experiment, cysteamine does not alter the renin-angiotensinogen interaction as described previously (Poisner and Hong, 1977). It could be possible that the increase in renin secretion may be due to a stress effect. At the time that the animals received the second injection,

lesions were observed at the injection site in some of the animals that received cysteamine. By reviewing the data again, it is seen that there is more variability among the values for PRA and PRC in the cysteamine groups. This is reflected in the S.E.M. However, the only information this data provides is that cysteamine was not able to inhibit thee renin-releasing activity of RRF that might be necessary for its renin-releasing activity.

Cysteamine is capable of reducing the immunoreactivity of prolactin (Sagar et al., 1985; Wehrenberg et al., 1983) and somatostatin (Palkovits et al., 1982; Webb et al., 1986; Cameron and Fernstrom, 1986) but not that of vasopressin or oxytocin (Palkovits et al., 1982; Cameron and Fernstrom, 1986) which also contain disulfide bonds. Therefore it appears that the ability of cysteamine to reduce disulfide bonds in a molecule may depend, in part, on the position of the disulfide bond in the molecule and in part, on the importance of the disulfide bond in maintaining a functional conformation. A study by Cameron and Fernstrom (1986) demonstrated that cysteamine treatment can affect the in vivo incorporation of [^{35}S]cysteine into somatostatin, oxytocin and vasopressin. This indicates that cysteamine might also interfere with the synthesis of proteins which may result in a reduction of activity. Treatment with cysteamine did not alter the renin-releasing activity of RRF. Therefore it can be concluded that either RRF does not contain disulfide bonds or that the bonds are not necessary for RRF to maintain its activity. Further studies should be done to establish the presence of disulfide bonds in RRF.

I. Role of RRF as a neurotransmitter

It is possible that in addition to its renin-releasing activity, RRF may also function as a neurotransmitter in the central nervous system. This is supported in part by the fact that there are RRF nerve terminals in the cerebral cortex, cerebellum and medulla. Since these brain regions are not known to be involved in releasing peptides into the blood, it is reasonable to think that RRF may be acting as a neurotransmitter. Werman (1966) has established eight criteria that need to be fulfilled in order to classify a substance as a neurotransmitter:

1. The substance must be present in neuronal tissue with an uneven distribution in the CNS.
2. Stimulation of identified neurons should cause release of this putative neurotransmitter.
3. Direct application of the substance should produce responses that are similar to those produced by stimulating the neurons.
4. & 5. Receptors should be identified that interact with the substance and stimulation of these receptors should produce a postsynaptic effect.
6. Precursors and synthetic enzymes must be demonstrated in the neuronal element.
7. Inactivating mechanisms should exist that terminate the action of the neurotransmitter.
8. A substance (agonist) should be able to mimic the natural transmitter.

Using these criteria and applying them to the results that we have observed for RRF, it becomes evident that RRF fulfills some of the criteria. First, we have demonstrated that RRF is differentially distributed within the brain. Release of RRF has been demonstrated from hypothalamic explants *in vitro* and stimulation of serotonergic neurons *in vivo* has increased the plasma concentration of RRF. Both the PCA-plasma fraction (M.W. 5,000-10,000) and the hypothalamic extract produced dose-dependent increases in renin release from the

kidney slices suggesting a direct action on renal receptors. The dose-response effect agrees with the occupancy assumption theory that presumes that as more receptors on the kidney are filled there is a larger increase in the effect of the neurotransmitter. These results satisfy requirements 3, and 4 & 5 (to a lesser degree), identifying receptors that respond to application of RRF. ALthough the kidney is not a post-synaptic element, there are receptors on the kidney that respond to application of RRF. The other criteria have not been addressed in this dissertation and have not, as of yet, been studied in this, or any other, laboratory. As the work continues with RRF, future studies may address these other issues and confirm the role of RRF as a neurotransmitter. However, with the data that we have collected so far it is conceivable that RRF may be part of a neuroendocrine system and may in fact be a neurotransmitter.

1. Possible role of RRF in the CNS

The renin-releasing activity of the pons, pituitary and thalamus was slightly elevated after colchicine treatment. The renin-releasing activity that was present in the cerebral cortex, medulla oblongata and the cerebellum was decreased suggesting that RRF in these areas is present in nerve terminals. These data may give some indication for possible RRF pathways outside the hypothalamus. The studies by Gotoh et al. (1987) and Richardson-Morton et al. (1986) have indicated that lesions in the PVN prevent the PCA-induced and stress-induced increases in renin secretion. Since RRF cells bodies are localized in the hypothalamus it is possible that RRF may be contained in hypothalamic

PVN neurons and that some projections of RRF neurons may parallel those of already identified PVN pathways.

Recent studies have focussed on the PVN as an integrator of neuroendocrine and autonomic responses (Sawchenko and Swanson, 1981). It is conceivable that if RRF is contained in the PVN, it too, may act as a regulator. The presence of RRF in the medulla, and in the pons, suggest that RRF may be involved in the regulation of blood pressure since these areas of the brain also monitor blood pressure (Nakai et al., 1982). We have demonstrated that RRF releases renin both in vivo and in vitro. However, its importance in regulating blood pressure remains to be determined since we have not observed any increases in blood pressure in rats that have received doses of concentrated PCA-plasma fractions (Van de Kar and Urban, unpublished results). It is equally likely that RRF may exert an action that is independent of cardiovascular regulation. The brainstem also is the site of integration of tracts and nuclei of the cranial nerves, primarily the hypoglossal, glossopharyngeal, vagus and accessory nerves. Therefore, the RRF could also integrate messages from the cranial nerves that may be involved with olfaction, taste, muscle movements or even respiration.

The role of the cerebellum in mediating autonomic functions has been studied in other laboratories. Stimulation of the fastigial nucleus of the cerebellum results in increased levels of plasma renin activity (Koyama et al., 1980) and increases in heart rate and blood pressure (Del Bo et al., 1983). Renal sympathetic nerve discharge, heart rate and blood pressure, in the decerebrate rabbit, are all

increased when the cerebellum (uvula) is electrically stimulated (Bradley et al., 1987). The uvula receives auditory, somato-sensory and proprioceptive inputs, the authors speculate that the cerebellum may be involved in a startle response resulting in cardiovascular changes. Our results suggest that RRF terminals are present in the cerebellum and release of plasma RRF is increased during stress. It can be speculated that in addition to sending a projection to the pituitary or median eminence, that there is a collateral RRF projection to the cerebellum. This pathway may be activated during either a startle or stress response which might have some influence on the cerebellar neurons that regulate cardiovascular function in addition to releasing RRF into the circulation.

2. Role of RRF in stress

The role that RRF plays in stress-induced renin secretion needs to be further defined. It appears that the effect of stress on renin secretion is not regulated by a serotonergic mechanism, since injections of the serotonin-selective neurotoxin, 5,7-DHT into the dorsal raphe nucleus did not affect the stress-induced increase in PRA nor did pretreatment with the 5-HT_2 antagonist LY53857 (Lorens et al., 1986). Stress is a stimulus that increases sympathetic nerve activity resulting in an increase in PRA (Jindra et al., 1984; 1980) and hypertension (Dobrakovova et al., 1984). Privitera et al. (1979) found that administration of propranolol intracisternally to rats suppresses renin secretion and produces hypotension. Identical doses of propranolol intravenously do not produce the same effects. Renal

denervation selectively attenuates the decrease in renin secretion but not the hypotensive effect of propranolol. Contrary to these results is the finding that injections of the catecholamine neurotoxin 6-hydroxydopamine (6-OHDA), combined with adrenal medullectomy did not prevent the stress-induced increase in PRA (Richardson-Morton et al., unpublished observations). This study indicates that the peripheral sympathetic nervous system is not the sole mediator of the stress-induced increase in PRA. However, intraperitoneal administration of propranolol greatly attenuates the renin response to stress (Van de Kar et al., 1985). Propranolol crosses the blood-brain barrier. Therefore, this study does not differentiate between central and peripheral beta blockade. Further studies by Richardson-Morton et al. (1987) have shown that injections of 6-OHDA into the PVN attenuates the stress-induced increase in PRA, suggesting that a central catecholaminergic mechanism of action is involved. The PVN appears to play a pivotal role in the regulation of the stress-induced increase in PRA (Richardson-Morton et al., 1987; 1986; Gotoh et al., 1987) and may also be involved in the regulation of the release of RRF since lesions of this nucleus prevent the PCA-induced increase in PRA (Gotoh et al., 1987). Therefore I would speculate that the serotonergic and catecholaminergic systems converge on the PVN to differentially regulate the release of RRF from the hypothalamus. It is also likely that catecholamine receptors on cell bodies in the PVN are activated during stress and trigger the release of RRF.

J. CONCLUSION

These studies provide evidence for a blood-borne renin-releasing factor. RRF cell bodies are localized in the hypothalamus and RRF is released from the hypothalamus when these neurons are depolarized. The present studies have shown that the release of RRF is stimulated by activation of serotonergic receptors and stressful stimuli. Further studies to identify other physiological factors, such as changes in sodium balance or blood pressure, that may increase the release of RRF need to be performed.

We postulate a neural circuit for the regulation of the release of RRF. This circuit is represented in Figure 8. From the studies performed by Van de Kar et al. (1982) and Karteszi et al. (1982) it has been demonstrated that the dorsal raphe nucleus sends a serotonergic projection to the mediobasal hypothalamus which stimulates renin secretion. Data presented by Richardson-Morton et al. (1987) and Gotoh et al. (1987) suggest that neurons in the PVN mediate both the stress-induced and PCA-induced increases in renin secretion. Since the PVN receives very little serotonergic innervation, we have considered the possibility that the projection from the dorsal raphe may synapse on an interneuron in the arcuate nucleus. The arcuate nucleus receives considerable serotonergic innervation from the dorsal raphe nucleus and is known to project to neurons in the PVN. We propose that stimulation of neurons in the PVN results in the release of RRF into the circulation. RRF then circulates in the blood to the kidney and causes the release of renin. An increase in PRA increases the circulating levels of ANG II. ANG II would then feedback on the CNS system that

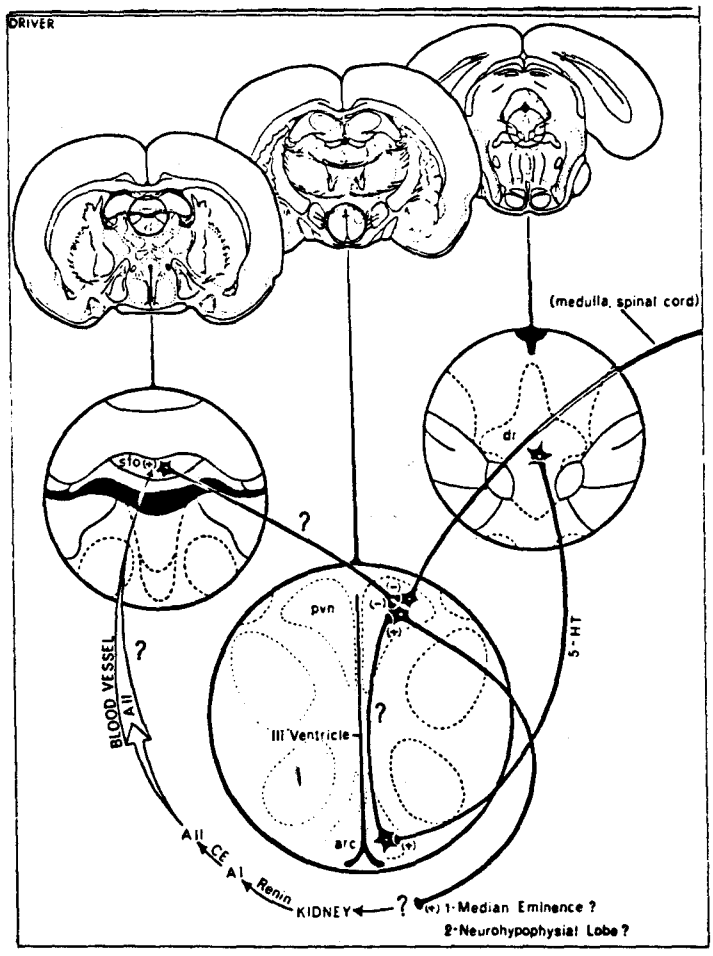


Figure 9. Postulated neuronal circuit for the regulation of RRF release.

regulates renin secretion by stimulating neurons in the SFO. Tanaka et al. (1986) have indicated that there are projections from the SFO to the PVN that can regulate the firing of PVN neurons. As the work continues in this area, it is possible that this neuronal circuit may change with future results.

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