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# Characterization of renin release from partially purified rat renal cortical plasma membranes: Effects of trypsin, hypophysectomy, sodium deprivation, calcium and other ions

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City University of New York, 1987

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# CHARACTERIZATION OF RENIN RELEASE FROM PARTIALLY PURIFIED RAT RENAL CORTICAL PLASMA MEMBRANES: EFFECTS OF TRYPSIN, HYPOPHYSECTOMY, SODIUM DEPRIVATION, CALCIUM AND OTHER IONS

by

Sr. Shirley Marie Russo

A dissertation submitted to the Graduate Faculty in Biology
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## ABSTRACT

CHARACTERIZATION OF RENIN RELEASE FROM PARTIALLY PURIFIED RAT RENAL CORTICAL PLASMA MEMBRANES: EFFECTS OF TRYPSIN, HYPOPHYSECTOMY, SODIUM DEPRIVATION, CALCIUM AND OTHER IONS

by

Sr. Shirley Marie Russo

Advisor: Professor John C.S. Fray

A rat renal cortical cell suspension enriched in juxtaglomerular (JG) cells was used to facilitate the isolation of highly purified rat renal cortical plasma membrane vesicles (PMVs) using isotonic medium and Percoll self-forming gradient centrifugation. The cells exhibited normal stimulatory responses to forskolin, cAMP and ionomycin. The vesicles were characterized by enzyme markers, an ATPase/ouabain vesicle sidedness and integrity assay , and electron microscopy. Contamination by mitochondria, lysosomes, granules, and ER membranes was minimal as indicated by the low specific activities of the respective enzyme markers. Forcefully passing the membrane fraction through a 26G hypodermic needle several times resulted in PMVs of >85% inside-out orientation. Trypsin treatment (2mg/ml) increased the renin specific activity of the isolated PMVs twofold over the untreated controls. Fifty percent of membrane-bound renin is in an inactive, but trypsin-activatable form. Other factors and maneuvers which are known to affect renin storage and secretion were investigated. For example, sodium deprived and hypophysectomized (Hx) rats had a higher renal renin content than controls. However, renin released from Hx rat kidneys was substantially lower than controls and sodium deprived, suggesting that the plasma membrane may be defective in releasing renin in the Hx rats and may store a large amount of renin. Supporting this hypothesis, renin specific activity of the PMVs was significantly greater in the sodium deprived and Hx rats which also had a high renal renin content compared to the normal controls. Lowering media Na caused a twofold increase in renin release from isolated PMVs whereas chloride was without effect. Neither EGTA nor calmodulin affected renin release from PMVs, but in combination they caused a substantial release with a subsequent decrease in membrane-bound

renin. A dose dependent loss of renin specific activity in the extravesicular compartment (EVC), in part a function of media Ca, was observed below 140mM K, with a gain above 140mM K. These results support the hypothesis that the membrane plays a role in the storage and secretion, and perhaps in the uptake of renin. The advantages of this new PMV isolation method include the use of isotonic media throughout, a high proportion of vesicles with inside-out polarity, and a relatively short isolation time. Based on both biochemical and morphological criteria, plasma membranes prepared by this method are of greater purity compared to those prepared by sucrose gradients and lengthy differential centrifugations; and the yields are comparable or several fold higher. This method may be useful in providing insights into the process of renin secretion or renin uptake.

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Part of this dissertation has been published (Russo and Fray, 1987), and portions have been presented in abstract form at the 36th Annual Fall Meeting of the American Physiologial Society, Niagara Falls, NY (Russo & Fray, 1985).

Finally, I am in debt to Srs. Marie Dugas, Michele Jacques, Bernadette LaVoie, and Joyce Snyder for organizational assistance, and to David J. Lush, Claude Saint-Come, and David H. Sigmon for their friendship, encouragement and stimulating scientific dialogue in the course of my studies.

## DEDICATION

This dissertation is dedicated to my family,whose love and confidence are a constant source of strength and to the Sisters of Saint Anne, whose encouragement and sacrifice have permitted me to complete this work.

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# INTRODUCTION

## A. Thesis rationale.

Rouillier and Orci (1971) have suggested that renin secretion occurs by three pathways: exocytosis, lysosomal related (granule-granule fusion?), and cytoplasmic solubilization. Evidence has been provided to confirm the former two (Taugner and Hackenthal, 1981; Skøtt, 1986; Park et al., 1987), but little is known as to the cytoplasmic solubilization pathway. Thurau and Levine (1971) have proposed that renin is released from secretory granules in the juxtaglomerular (JG) cells into the cytoplasmic space and some recent evidence has confirmed this proposition by showing that lowering extracellular Ca stimulates renin secretion and increases renin in the cytoplasmic soluble space (Fray et al., 1983b; Fray and Lush, 1984). This renin may originate from the secretory granules since lowering extragranular Ca stimulates renin release from renin granules (Sagnella and Peart, 1979; Sigmon and Fray, 1985). Baumbach and Leyssac (1977) have suggested that renin is transported out of the JG cells across the plasma membrane and that Ca regulates the permeability of the renin transport system. Nishimura et al. (1980b) have supported this suggestion by localizing a substantial amount of renin in the plasma membrane. The implication of these lines of evidence is that one pathway of renin secretion is from secretory granule to cytoplasmic space and then across the plasma membrane by a Ca-dependent transporting system. Precedence for a Ca- dependent transporting system in the plasma membrane for the secretion of other substances has been provided (Israel and Manaranche, 1985; Tauc, 1982), and at least in one study the transporting system has been characterized (Israel et al., 1986). However, comparable demonstration of the involvement of the plasma membrane in renin secretion is lacking. This is due primarily to the paucity of information on the role of the plasma membrane in the renin secretory process and of the need for a suitable preparation in which to gain further information of this important pathway. This study attempts to remedy this deficiency by developing a simple and rapid technique for isolating plasma membranes from renal cortical cells enriched with JG cells and to show that this preparation may provide useful information about the renin secretory process.

The special advantages of using isolated plasma membrane vesicles (PMVs) to investigate the renin secretory process include the ability to study the role of this final membrane barrier in the absence of the homeostatic influence of the cytosolic compartment allowing precise control of both intracellular and extracellular environmental substrates, pH, and temperature effects. The study of asymmetric membrane properties is made possible by a well defined orientation of the membrane preparation permitting biochemical modifications by group specific reagents, by electrolytes, by proteases, or by enzymatic substances normally involved in the posttranslational regulation of biological processes. The extravesicular surface can be exposed to nonpenetrating reagents, permeant reagents or the use of detergent concentrations and osmotic shock. The latter technique has been used successfully by several investigators (Biber et al., 1983; Gmaj et al., 1984) to load PMVs with a modifying agent to determine its effect from the intravesicular compartment.

There are significant disadvantages to the use of isolated PMVs which also need to be addressed. Isolated PMVs are almost never identical in size and membrane composition. Size differences might be related to the preparation conditions used, whereas differences in carrier density, receptor density, and other membrane binding proteins are intrinsic properties usually related to differences in the cell location within a given tissue. Compared to the intact cell, vesicles have a very high surface to volume ratio and this needs to be taken into account when quantitating responses from isolated PMVs. It is difficult to predict exactly the intravesicular composition, even at the beginning of an experimental incubation. Despite all of these limitations, studies with vesicles can contribute significantly to the understanding of cellular mechanisms and membrane transport across the final membrane barrier of the cell. Thus, the experiments of this thesis have been designed to test the responsiveness of partially purified PMVs to factors known to affect renin secretion in vivo.

Trypsin is known to have several plasma membrane-localized effects (Walter et al., 1979; Lingappa et al., 1979; Toogood et al., 1983; Dux et al., 1985 ) and has been used extensively as a proteolytic probe of membrane translocation activity (Walter et al., 1979; Lingappa et al., 1979). Trypsin has also been reported by several investigators to activate inactive renin (Gallagher et al., 1980; Barrett et al., 1982; Madeddu et al., 1985; Ohashi et al., 1985; Derkx et al., 1987); thus the trypsin experiments were

designed to determine if trypsin could activate plasma membrane-bound renin.

Hypophysectomized (Hx) and sodium deprived rats are known to have an elevated renal renin content, yet Hx rats exhibit an abnormally low plasma renin activity (PRA) (Honeyman et al., 1983), while sodium deprived rats have an increased plasma renin activity (Fray, 1978). This seems to suggest that the sensitivity of the kidney to release renin may have been suppressed in the absence of the pituitary. It was therefore of interest to investigate the effect of hypophysectomy and sodium deprivation on plasma membrane-bound renin to determine if this pool of renin was involved in the altered PRA of both hypophysectomized and sodium deprived rats. These animal models provide a test of the usefulness of the PMVs preparation in the study of renin secretion.

Several ions are known to affect renin secretion in vivo such as Na, Cl, K, Ca, and Mg (reviewed by Fray, 1977). Since membrane transport is driven by ion gradients involving these same ions (Semenza & Kinne [eds.], 1985), and several membrane-localized enzyme systems are known to be regulated by K (Kernan, 1980) and are Ca- calmodulin-dependent, several experiments were designed to test the effect of ions on membrane-bound renin. Furthermore, several investigators have proposed that functional alterations of the plasma membrane, particularly in the handling of ions and ion transport, are involved in the pathogenesis of several types of both human and rat hypertension (David-Dufilho et al., 1986; Postnov and Orlov, 1985; Haddy, 1983).

Finally, one of the most recent areas of investigation in renin research is the demonstration of immunoreactive renin or reninlike enzymes (angiotensinases, isorenin) in extrarenal tissues such as blood vessels, uterus, testes, adenohypophysis, salivary gland and brain (Ganten et al., 1976; Hirose et al., 1980; Re et al., 1981; 1982; Doi et al., 1984; Naruse et al., 1985; Dzau & Re, 1987). The histogenesis of the extrarenal cells containing renin is uncertain, but the renin-granules in most of these cells are probably lysosomes (Barajas & Salido, 1986) and since the lysosomal system functions in both uptake and secretion, it is conceivable that renin is avidly taken up and stored throughout the body for use in the control of local vascular phenomena. Sealey and associates (1983; 1985) have demonstrated that prorenin can be found circulating under a variety of conditions. They postulate a possible role for prorenin in reproductive physiology (Sealey et al., 1985) in view of its high concentration in amniotic

fluid and in the placenta, a highly vascularized tissue. As suggested by Sealey (1983), prorenin could be a transport form of renin. Perhaps prorenin binds to cell membranes, is interiorized in the phagosomal system and is activated intracellularly by fusion with a primary lysosome. If this is true, then renin should be found in the plasma membrane fraction of the extrarenal tissues reported to contain renin. The last series of experiments in this thesis offer preliminary evidence in support of this hypothesis by the demonstration of extrarenal plasma membrane-bound renin.

Experiments with vesicles can be taken as a biochemical or biophysical approach to membrane physiology. If, however, studies with vesicles represent an experimental tool to explain organ function at the membrane level, then the findings must be integrated with those obtained under more physiological conditions. It is realized that vesicles represent highly artificial systems and therefore, it cannot be assumed a priori that the observations obtained in this artificial system reflect a property of the intact membrane under more physiological conditions. Experiments are required which show a correlation between membrane function in vesicles and membrane function in the intact cell. It was therefore considered necessary to isolate a population of JG cells and to demonstrate its physiological responsiveness to known renin secretogogues. This series of experiments was designed to provide an intact cell system for the comparative analysis of PMVs data and to validate the use of a Percoll self-generating gradient in the isolation of renin-secreting cells with an intact cell membrane and by inference, therefore, the use of this gradient medium for JG cell membrane isolation.

In conclusion, the following literature review is provided to survey the development of the field of renin research and the multifactorial dimensions of the renin secretory process. Without a thorough understanding of all the major mechanisms proposed for the regulation of renin secretion from the most significant in vivo experiments as well as the in vitro investigations using isolated perfused kidneys, renal cortical slices, isolated glomeruli and afferent arterioles, it would not be possible to appreciate fully the complexity of this problem. All of the foregoing in vivo and in vitro experimental tools represent complex systems lacking the primary advantage of isolated PMVs, i.e. an in vitro system allowing "direct" access to the final stage of the secretory process, the movement of the renin zymogen across the plasma membrane.

**B. Overview of the renin-angiotensin system and hypertension.**

The history of the investigation of the renin-angiotensin system has been controversial from its earliest days. Even the stimulus for Tigerstedt and Bergman's (1898) interest in the kidney as an endocrine organ has often been mistakenly attributed to Richard Bright (Gibbons et al., 1984). As early as the beginning of the 19th century, Bright, a remarkably astute observer, noted that hypertension and the kidneys were linked since patients who eventually died with dropsical effusion also had an elevated pulse and in most cases secreted albuminous urine which he connected with the disorganized state of their kidneys (Bright, 1827). On autopsy the kidneys were found to be small, hard, distinctly granulated and on sectioning had an exceedingly thin cortex. The heart, on the other hand, was remarkably enlarged though the valves were often perfectly healthy (Bright, 1827). Since patients with such chronic glomerulonephritis also had hypertrophied hearts, he postulated that the quality of the blood must be altered in some way such that the force it exerts on the walls of blood vessels as it circulates is greater than normal in these patients and therefore, the heart had to work harder to pump the blood (Bright, 1836). With the development of the sphygmomanometer (Riva-Rocci, 1896), it soon became apparent that the quality that Bright was seeking was blood pressure. These early reports, however, were not the immediate impetus for Tigerstedt and Bergman's search for a pressor agent in the kidney. In the introductory paragraph of their classic paper of 1898 they clearly state that it was Brown-Sequard's (1892) early endocrine studies that animated them: "The ingenious idea of Brown-Sequard, that various organs release substances into the blood which do not belong to the usual catabolic products, but are formed by specific activity of the tissues and have a basic importance for the overall functioning of the body, has been brilliantly established through numerous investigations (Tigerstedt & Bergman, 1898)."

Tigerstedt and Bergman (1898) subsequently designed a series of imaginatively planned and carefully executed experiments to determine whether substances released from the kidney did indeed influence the circulatory system. In so doing they added experimental evidence to Bright's proposed link of kidney disease with hypertension by demonstrating that an elevation of the arterial pressure could be induced in rabbits by injecting a crude saline extract of kidney. They named this vasopressor substance

"renin" and found that it's pressor effect was localized in the renal cortex. They further characterized renin to be water soluble, alcohol insoluble, and non-dialyzable.

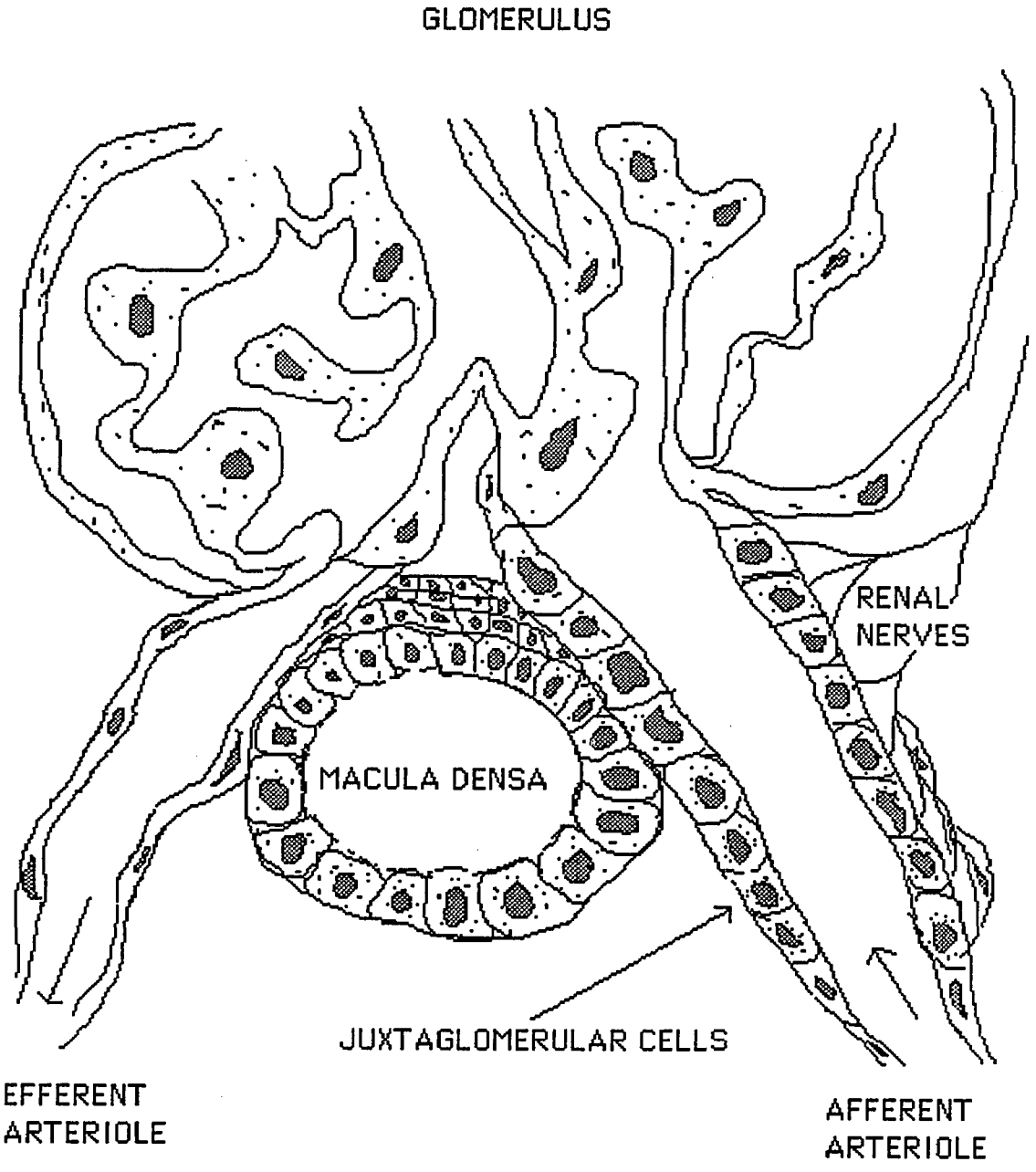
These remarkably accurate experiments in light of current knowledge were disreputed by contemporary scientists and thus the controversy concerning renin was born. The earliest arguments were published by Max Lewandowsky (1899) of the Physiological Institute of Berlin. He wrote a scathing and sarcastic rebuttal of their initial report emphasizing that the adrenal medullary extract of Oliver and Schafer (1895) was far more powerful than the proposed renal pressor substance. Moreover, he noted that hypotension and death ensued when the adrenal glands were removed, whereas no one had yet demonstrated that nephrectomy led to a significant drop in blood pressure. In support of Lewandowsky's arguments, other investigators in the early 1900's, such as Pearce (1909), reported finding primarily depressor substances in kidney extracts. Unfortunately, investigators who reviewed the literature of the day and quoted Pearce's negative results apparently failed to notice that most of Pearce's studies were done with a dried filtrate of an alcoholic extract dissolved in saline solution. Tigerstedt and Bergman had clearly shown that renin was alcohol insoluble. The crowning blow may have come from T. C. Janeway (1913), the doyen of American internists at that time specializing in the kidney. Janeway published a plenary denunciation of Tigerstedt and Bergman's work in his review of nephritic hypertension in which he tersely concluded, "I think it is reasonable to dismiss them from consideration." In the 1924 obituary of Tigerstedt printed in the Skandinavisches Archiv fur Physiologie, the journal Tigerstedt had edited for many years, no reference to his renin hypothesis was even mentioned by his long-time colleague and successor, Santesson (1924). The concept of a renal pressor substance was thus denounced a priori and forgotten for over three decades.

Much of what we know about human and experimental hypertension today has evolved from the pioneering work of Goldblatt and his associates in the early 1930's. These studies provided the model that ultimately led to the appreciation of the role of renin in hypertension and the major endocrine function of the kidney as the source of renin. Using the now famous adjustable silver renal artery clamp Goldblatt and his coworkers (1934) showed that moderate constriction of both renal arteries or of one artery following contralateral nephrectomy, resulted in a persistent form of hypertension in the dog. This same team later demonstrated that this experimentally induced hypertension could be prevented by complete

sympathectomy, by pithing of the spinal cord, or by transplantation of the clamped denervated kidney to the neck. Removal of the hypophysis, thyroid, pancreas or gonads had no effect on the development of hypertension following clamping, but experimental hypertension could not be produced in the absence of the adrenal cortex unless adequate substitution therapy was given. Goldblatt further demonstrated that the induced hypertension was abolished if the renal veins were ligated. The animal became normotensive, but eventually died of uremia (Goldblatt , 1947). Thus Goldblatt demonstrated not only the renal origin of hypertension, but also its humoral nature.

During this same period the highly specialized structure of the juxtaglomerular apparatus (JGA, Figure 1) including the assumed secretory nature of the JG cells were extensively studied and described by Goormaghtigh (1932, 1937, 1939, 1940, and 1944). These juxtaglomerular cells which had been described by Ruyter (1925) as modified smooth muscle cells due to the presence of myofilaments and attachment bodies near the plasmalemma, were postulated to be the site of renin synthesis and release. Today it is known that they are located in the media of the afferent arteriolar wall, and less frequently in the efferent arteriolar wall as well (Barajas & Latta, 1963). Indirect evidence for their secretory nature included the presence of stainable, well-defined granules and the fact that changes in granule content could be effected by certain experimental conditions such as renal ischemia (Goormaghtigh, 1939) adrenalectomy (Dunihue, 1949), variations in salt intake (Hartroft & Hartroft 1953) and changes in blood pressure (Hartroft, 1957 Tobian et al., 1958). Hartroft & Hartroft (1953; 1961) developed a reproducible method to quantitate the degree of JG granularity and subsequently correlated this with the renin content of the kidney in normal and experimental conditions. This physiomorphologic evidence for their secretory nature was further corroborated by the electron microscopic studies of Hartroft and Newmark (1961). These observations revealed an extensive endoplasmic reticulum with abundant granules; a well defined Golgi apparatus; many characteristic mitochondria; a large ovoid, sometimes indented nucleus with prominent nucleoli; and a plasma membrane, frequently involuted with its folds penetrating the interior of the cell and in some cases appearing continuous with the endoplasmic reticulum. More direct evidence of the association between renin-containing cells and the JGA was provided by Cook and Pickering (1959). On separation of the glomeruli from the rest of the kidney, they found that renin activity was associated with cells near the

**Figure 1.** The juxtaglomerular apparatus with attached glomerulus. Computer drawing adapted from Davis (1971).


GLOMERULUS
RENAL NERVES
MACULA DENSA
JUXTAGLOMERULAR CELLS
EFFERENT ARTERIOLE
AFFERENT ARTERIOLE

glomerulus. In 1961, Edelman and Hartroft were able to localize renin convincingly in the JG cells by immunofluorescence. The final proof that these cells are the source of renin in the kidney was provided by Cook (1968), who demonstrated that granules removed from the JG cells contained renin. Thus, these cells exhibit characteristics of both endocrine and smooth muscle function.

Inasmuch as there was such a large amount of renin in kidney tissue, it seemed logical to suspect that renin was the causative agent in hypertension. Indeed an important function of the kidney as an endocrine organ has turned out to be its ability to secrete renin. It is now known that the mechanisms controlling renin release are closely related to the morphological structure of the juxtaglomerular apparatus.

A particularly significant but very puzzling observation was made by Friedman et al. (1938), who discovered that renin did not cause vasoconstriction in the isolated dog tail perfused with Tyrode's solution (a modified Lock's solution). How, then, could it exert such a powerful pressor action?

The answer to this important question was forthcoming within two years from two widely separated laboratories initiating a controversy which was to last for nearly two decades. Page and Helmer (1940) working in the United States and Braun-Menendez and associates (1940) working in Argentina simultaneously demonstrated the proteolytic nature of renin. They reported that renin acted on a substance in the plasma to produce a heat stable, dialyzable peptide that had both vasoconstrictor properties and a brief, but powerful pressor effect. This peptide was the product of the enzymatic cleavage by renin of a plasma localized substrate. This was the first significant step in the elucidation of the renin-angiotensin system. Because the two investigative groups named their substrate and product differently, the world according to renin was divided into two camps, one using the term, hypertensinogen for the substrate and hypertensin for the enzymatic product as published by the Braun-Menendez team and the other using renin- activator (later renin substrate) and angiotonin respectively as reported by the American team. It was nearly twenty years before Drs. Braun-Menendez and Page (1958) conceded in a published statement that they were discussing the same molecules and therefore agreed to a hybrid term, angiotensin, which recognized the work of both groups amd thus ended the controversy.

The first to present supporting evidence that renin may be the causative agent in experimental

hypertension was Pickering and Prinzmetal (1938a, 1938b). They reported that saline extracts of alcohol dried kidney in unanesthetized rabbits produced a strong pressor effect. However, in spite of many attempts, neither renin nor angiotensin, or any other pressor agent for that matter, had been shown in unequivocal fashion to be present in the circulating blood of hypertensive humans or animals. All that was known of angiotensin was that it was heat and acid stable, soluble in alcohol and dialyzable (Page and Helmer, 1940; Braun-Menendez et al., 1940). The only way it could be recognized was by its effect on the blood pressure of an experimental animal. Continued progress in hypertension research depended on the isolation of angiotensin from the blood of hypertensive humans or animals.

Skeggs and coworkers (1949) developed the first plate-type artificial kidney and it was therefore natural for them to attempt to dialyze angiotensin out of the blood of hypertensive dogs with their equipment. They equilibrated 300 ml of isotonic dialyzing solution with the circulating arterial blood of dogs over a 90-minute period and then purified, concentrated and assayed the dialysate in a highly sensitive rat preparation. Using this procedure they clearly demonstrated the presence of angiotensin in the blood of hypertensive dogs (Skeggs et al., 1951). The fact that it was also present in small amounts in the blood of half of the normotensive controls was disconcerting and prevented Skeggs and coworkers from concluding, as they had hoped, that angiotensin was the causative circulatory pressor substance. Today, considerable evidence exists to conclude that renin and angiotensin are involved in some but not all cases of hypertension.

The minimal amount of angiotensin in the plasma of hypertensive animals hindered its purification. In 1954, Skeggs et al. accomplished this task by the in vitro incubation of large amounts of crude renin isolated from hog kidneys with crude renin substrate derived from horse plasma. Amino acid analysis subsequently showed it to be a decapeptide containing nine different amino acids with histidine the only residue repeated in the sequence (Skeggs et al., 1955). This sequence has proven to be remarkably conserved in all animal species thus far studied (Peart, 1956; Elliott and Peart, 1957; Bumpus et al., 1957; Arakawa & Nakamura, 1967).

Quite early in the investigation of angiotensin, Skeggs and his collaborators (1954b) found that it existed in two forms that could be separated by countercurrent distribution, a decapeptide that they

called angiotensin I and an octapeptide, angiotensin II. Angiotensin II was formed by the action of proteinases on the precursor peptide, angiotensin I. Incubating angiotensin I with crude horse plasma renin substrate in the presence of sodium chloride resulted in the production of angiotensin II, soon discovered to be the active end-product of the renin-angiotensin system (Skeggs et al., 1956). Through this experiment, it was learned that a chloride-activated enzyme, a metalloprotein subsequently named angiotensin-converting enzyme (ACE), was present in both the plasma and the crude renin substrate and functions to convert angiotensin I to angiotensin II. Thus, if renin is incubated with plasma in the absence of chloride, the product formed is angiotensin I, whereas if chloride ions are present, the product is angiotensin II. This conversion is accomplished by the enzymatic cleavage of a dipeptide (His-Leu) from the carboxy-terminus of angiotensin I (Lentz et al., 1956). Ng and Vane (1967) later demonstrated that this angiotensin II generation in the plasma was actually a slow process and that the lung was the most important site of ACE activity. Ryan and coworkers (1975; 1979) have since shown that the enzyme is located on the luminal surface of the pulmonary endothelial cells and that both the plasma and urine contain dialysable peptide inhibitors of the converting enzyme. Skeggs and coworkers' use of dialysed preparations in their original work, may explain why they were misled into assuming that conversion in vivo occurred in the plasma.

Angiotensin converting enzyme activity has subsequently been shown to exist in several other tissues including the placenta (Johnson et al., 1984); the choroid plexus of the brain (Rix et al., 1981); the microvilli of the gut (Ward et al., 1980); the kidney (Hall et al., 1976); and in the plasma membrane of vascular endothelial cells (Kreye & Gross, 1971; Aiken & Vane, 1972; Rabito et al., 1972; Ryan et al., 1976). In view of its widespread distribution, it seems reasonable to assume that some conversion must occur wherever the enzyme is found; i.e. in peripheral vascular beds, in the tubule system of the kidneys, and within the central nervous system. ACE is now known to be identical to kininase II which inactivates bradykinin (Lindop and Lever, 1986). Thus, it generates a vasoconstrictor (the most potent pressor substance known, i.e. angiotensin II) and inactivates a vasodilator (a potent depressor substance, i.e. bradykinin).

In vitro studies by Helmer (1957) using rabbit aortic strips established that angiotensin II was a

potent vasopressor agent, whereas angiotensin I was much less effective requiring at least three orders of magnitude greater concentration to equal the vasopressor activity of angiotensin II. The observed vasopressor activity of angiotensin I in vivo was subsequently demonstrated with specific inhibitors to be the result of converting enzyme activity. It thus seemed to have no direct pressor action of its own and its apparent activity was believed to be due entirely to its conversion to angiotensin II (Greene et al., 1972).

Immediately following this observation, the development of specific inhibitors to block the conversion of angiotensin I to angiotensin II, have provided evidence suggesting that the decapeptide, angiotensin I, may have significant biologic activity in the kidney and within primitive structures of the nervous system (Buckley, 1972; Bryant and Falk, 1973; Vandongen and Greenwood, 1975).

Angiotensin II is a very potent peptide with a vast spectrum of biologic activities. The major actions of ANG II, all of which serve to elevate blood pressure include: a vasoconstrictor effect by direct action on vascular smooth muscle (Ribeiro & Krakoff, 1976; Saragoca et al., 1983; Wilson, 1986); a cardiac effect consisting of an increase in contractile force (Lefer, 1967); a liberation of epinephrine from the adrenal medulla and of norepinephrine from stores in sympathetic nerve endings in various tissues, an effect contributing to, but not solely responsible for, the vasoconstrictor and cardiotonic effects (Page & Bumpus, 1974). The positive inotropic effect of angiotensin is also dependent on the ionic environment. When the rate and maximum magnitude of tension development are low (low Ca or high Na), the peptide exerts a greater inotropic action than under conditions where the degree of activation of the contractile elements is already high (Koch-Weser, 1965; Lefer, 1967); and finally, ANG II receptors have been localized in the rat kidney using the high-affinity agonist analog $^{125}$I-labeled [Sar$^{1}$]ANG II as a probe for in vitro autoradiography (Mendelsohn et al., 1986). A high density of receptors was found in the glomeruli, exhibiting a cortical gradient, highest in the superficial and midcortical glomeruli and lowest in juxtamedullary glomeruli, all diffusely distributed, and thus consistent with a mesangial localization. Low levels of tubular ANG II binding were seen in the outer cortex and a very high density was found in longitudinal bands in the inner zone of the outer medulla in association with vasa recta bundles. Finally, a moderate density of receptors occurs diffusely throughout the inner zone of the outer

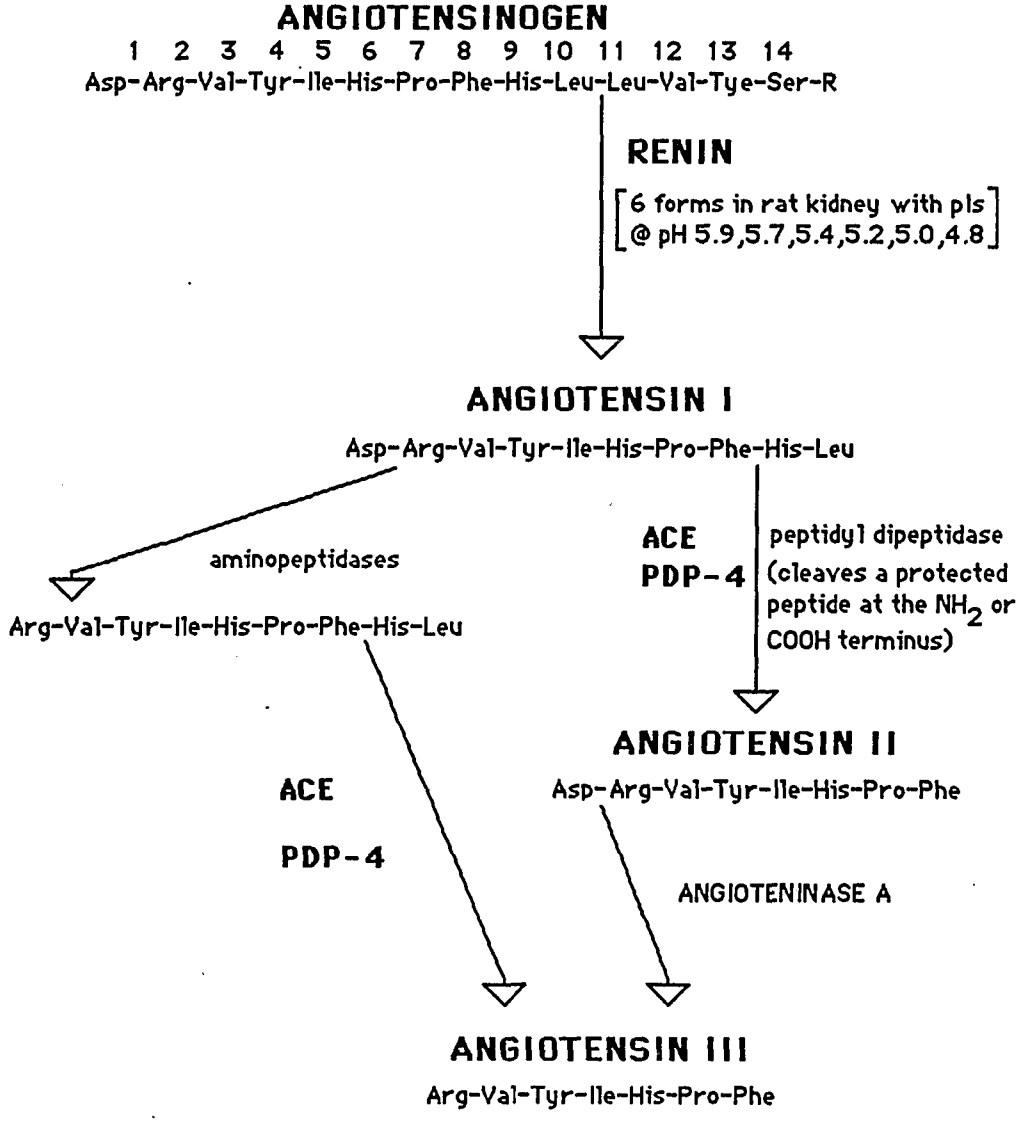
medulla in the interbundle areas. Many of these ANG II receptors exhibited a down-regulation on low sodium intake. These results suggest that ANG II exerts a number of different intrarenal regulatory actions. Wilson (1986) recently demonstrated that ANG II causes a severe focal constriction of afferent arterioles, and that this constriction cannot be attributed primarily to a pressure-induced autoregulatory response in the renal vasculature. Steinhausen et al. (1987) showed that acute intravenous injections of ANG II caused severe constriction of afferent arterioles in rat kidneys with postischemic hydronephrosis, an experimental model in which tubular continuity has been interrupted and tubuloglomerular feedback mechanisms are effectively eliminated.

The heptapeptide angiotensin III (ANG III) apparently has a much shorter biological half-life than does ANG II (Peach, 1977), but it has been shown to be equally as potent as ANG II in suppressing renin release by a direct effect on the kidney (Freeman et al., 1975). Yet to be defined are the possible functions of the nonapeptide formed by removal of aspartic acid from ANG I. In experiments to date, this peptide has been active only when converting enzyme was present. The nonapeptide is readily cleaved by converting enzyme to the heptapeptide ANG II (Peach, 1977). The biochemical relationships of the renin-angiotensin cascade in the light of the current understanding of the system are shown in Figure 2.

## C. <u>The control of renin secretion</u>.

Goldblatt's hypothesis that renal ischemia was responsible for renin release dominated the thinking on renin secretion mechanisms for more than a decade. However, several investigators later reported that reduction in renal perfusion pressure, as first intimated by Blalock and Levy (1937), appeared to be the more important variable. In the mid 1940's, Goormaghtigh (1944; 1945) observed increased granulation of the JG cells after constriction of the renal artery. This observation coupled with Goldblatt et al.'s (1934) earlier induction of renovascular hypertension provided the impetus to search for the hemodynamic factors responsible for stimulating renin secretion. In 1958, Kolff noted in working with bilaterally nephrectomized dogs with normal transplanted kidneys that neither pulsatile flow to the transplanted kidney nor

**Figure 2.** Biochemical relationships of the renin-angiotensin system. R = ∂-globulin; ACE = angiotensin converting enzyme; PDP-4 = peptidyl dipeptidase-4.


ANGIOTENSINOGEN
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tye-Ser-R
RENIN
[6 forms in rat kidney with pIs
@ pH 5.9,5.7,5.4,5.2,5.0,4.8]
ANGIOTENSIN I
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
aminopeptidases
ACE
PDP-4
peptidyl dipeptidase
(cleaves a protected
peptide at the NH2 or
COOH terminus)
Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
ANGIOTENSIN II
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
ACE
PDP-4
ANGIOTENINASE A
ANGIOTENSIN III
Arg-Val-Tyr-Ile-His-Pro-Phe

nonpulsatile flow gave a pressor response. When the perfusion pressure was lowered, the renin response the renin response occurred. The following year, Tobian et al (1959) observed a marked (42%) degranulation in the JG cells with a rise in perfusion pressure (167 - 192 mmHg) in normal isolated kidneys; whereas a control group being perfused at 82 - 85 mmHg showed no change in JG granulation. Tobian (1960) subsequently suggested the existence of a renal "baroreceptor" that responds to stretching of the afferent arteriole.

Today there are five mechanisms reported to control renin release. These include: (1) the intrarenal baroreceptor; (2) the sodium signal; (3) renal sympathetic nerve activity; (4) humoral factors including endogenous catecholamines, antidiuretic hormone, prostaglandins, angiotensin II, atrial natriuretic peptide; and (5) plasma electrolytes.

## 1. THE INTRARENAL BARORECEPTOR

According to Tobian's hypothesis, lowering renal perfusion pressure or raising tissue pressure stimulates renin secretion (Tobian, 1960). Citing the work of Pitcock and Hartroft (1958), he further noted that granulation of the JG cells observed on autopsy could be correlated with the level of plasma Na during the last week or so of life such that low Na concentrations were associated with increased granularity and higher Na concentrations with decreased granularity. He then postulated that diets containing very little Na lower extracellular fluid volume and blood volume, and as this occurred in afferent arterioles, there would be a diminished stretch of the JG cells and hence a stimulus to hypergranulation and hypersecretion. It's disappointing, as well as most surprising to find that in Tobian's extensive review of the interrelationship of electrolytes, JG cells and hypertension, he fails to even consider the possibility of Ca involvement. In 32 pages, while extensively discussing the influence of all the other major electrolytes on tonus or contraction of the arterial smooth muscle, on arterial pressure, granularity and renin content of the JG cells, and on the pathogenesis of hypertension, a mere two lines is devoted to Ca. Tobian obviously did not consider the Ca ion worthy of discussion.

Nevertheless, in support of Tobian's stretch receptor hypothesis, Skinner, McCubbin and Page

(1963) demonstrated an increased renin release in response to suprarenal aortic constriction. The possibility that renin release occurred secondary to changes in glomerular filtration rate could not be discounted in this study. Thus, Blaine and coworkers (1970, 1971a, 1971b) and Witty and associates (1971) subsequently published a series of experiments in which the renal afferent arteriole was systematically isolated from the influence of the macula densa, the renal nerves and the circulating adrenal catecholamines and provided convincing evidence for an autonomous afferent arteriolar baroreceptor in control of renin release. Since transmural pressure is the difference between perfusion pressure and tissue pressure, lowering the transmural pressure appeared to be the sought after hemodynamic stimulus for renin release from Tobian's stretch receptor (Eide et al., 1973). However, in 1976, Fray presented a stretch receptor model based on his studies of the perfused rat kidney, in which a mathematical analysis of the mechanics of the afferent arteriole revealed a much more complex hemodynamic stimulus to stretch than simple transmural pressure. In this model, renin release is related to the elastic modulus of the afferent arteriole, the internal and external hydrostatic pressures, and more importantly, to the ratio of the internal and external radii of the afferent arteriole. Fray proposed that vasodilation, induced by papaverine which blocks autoregulation, or high pressure may stretch the afferent arteriole and depolarize the granular cell membrane, inhibiting renin release; whereas low pressure or vasoconstriction may decrease stretch and thus hyperpolarize the cell membrane and increase renin secretion.

Keeton and Campbell (1981) attempted to challenge Fray's hypothesis by arguing that renal artery hypotension is accompanied by autoregulatory arteriolar dilatation and that intrarenal infusion of vasodilators other than papaverine (e.g. dopamine, prostaglandins, etc.) stimulate renin secretion. However, since vasoconstriction is the autoregulatory sympathetic response to acute hemorrhage, and hypovolemia is also accompanied by an increase in renin release (Skinner et al., 1963), a simple inverse relationship between afferent arteriolar stretch or autoregulatory control and renin release probably does not apply. Furthermore, since literally hundreds of different cellular control functions have been postulated for the different prostaglandins (Guyton, 1981), their vasodilator role may well be compromised by their other effects. On the other hand, in support of Fray's hypothesis and to counter Keeton & Campbell's challenge, a recent analysis of the hypothesis shows that the stretch receptor responds most consistently

to changes in cytosolic Ca rather than wall tension per se (Fray et al., 1986). Wall tension and renin secretion are associated as a consequence of the involvement of Ca in determining both the physical (wall tension) and physiological (renin secretion) responses. Under most circumstances, a decreased wall tension is accompanied by increased renin secretion, but when extracellular Ca is removed (thus by implication lowering intracellular Ca), an increased wall tension is accompanied by increased renin secretion.

One of the limitations of this model is that it greatly simplifies the forces governing membrane deformation. In the expression of the model determining intracellular Ca, channel proteins are undoubtedly influenced by other membrane events such as the involvement of membrane potential. It is conceivable that an increased Ca permeability could occur in response to stretch-induced depolarization, as has been demonstrated for muscle stretch receptors (Hunt et al., 1978).

Two major features of the stretch receptor hypothesis are: that the JG cell itself is the stretch receptor which regulates its secretory activity in response to physical deformation and humoral agents (Fray, 1976), and that the secretory process is initiated by a lowering of the cytoplasmic concentration of free Ca (Fray et al., 1983a) and completed by a process involving the release of renin from the secretory granule into the cytosolic space (Thurau & Levine, 1971) and its eventual transport across the plasma membrane by a Ca-regulated mechanism (Baumbach & Leyssac, 1977).

Thus, in contrast to secretory processes in other cells, there seems to be an inverse correlation between renin secretion and intracellular free Ca concentration. It has been shown that hemodynamic and humoral factors which generally increase net influx of Ca inhibit renin secretion (Churchill and Churchill, 1982a, 1982b; Naftilan & Oparil, 1982; Fray et al., 1983a) and that secretagogues which promote Ca efflux stimulate renin secretion (Fray, 1980b; Churchill & Churchill, 1982a, 1982b; Freeman & Davis, 1983)), whereas agents which block Ca efflux inhibit secretion (Fray et al., 1983).

The ultimate test of the persistently controversial stretch-receptor hypothesis must be conducted on the proposed stretch-receptor itself, i.e. the juxtaglomerular cells in isolation from other renal, nervous and endocrine influences. In 1984, Fray and Lush adapted a preparation enriched in JG cells and designed experiments in which isolated JG cells attached to gas-pressure-stretched latex reduced their secretory activity in response to stretch. In the presence of 8(N,N- diethylamino) octyl-3,4,5-

trimethoxy-benzoate (TMB-8), an agent which lowers cytosolic Ca by blocking calcium release from intracellular sequestration sites (Malagodi and Chiou, 1974; Charo et al., 1976), renin secretion was stimulated. In addition, melittin, an agent which activates renin from renal cortical cell plasma membrane fractions (Nishimura et al., 1980a) and kallikrein, an endogenous peptide found in the same fractions (Nishimura et al., 1980b), both stimulated renin secretion by a Ca-dependent mechanism (Fray and Lush, 1984).

The most recent support for the stretch-receptor hypothesis came from the laboratory of Buhrle and coworkers (1986a). Using hydronephrotic kidney slices devoid of macula densa tubuloglomerular feedback signals and exhibiting no change in the percentage, morphology, or normal responses of renin-positive afferent arteriolar vessels, these investigators demonstrated that the membrane potential of JG cells ($-73 \pm 10.3$mV) does not significantly differ from that of vascular smooth muscle cells isolated from the distal end of the renal afferent arteriole ($-70.4 \pm 9.4$mV). Furthermore, agents inhibiting renin secretion also depolarized the JG cell membrane, as for example, superfusing with a high potassium medium. Presumably Ca influx in response to depolarization-induced or stretch-induced opening of Ca channels triggered the inhibitory response (Fray et al., 1986). Of special interest is the observation that increased stretch of stretch receptors (Hunt et al., 1978; Edwards et al., 1981), epithelial cells (Wong and Chase, 1986) and red blood cells (Larsen et al., 1981) increases the membrane permeability to Ca. Verapamil and its methoxyderivative, D600, both block these Ca channels and inhibit the negative renin response of high perfusion pressure and high K depolarization (Fray and Park, 1979). Depolarization of JG cells and the consequent inhibition of renin secretion has been demonstrated using high concentrations of K in isolated perfused kidneys (Fray, 1980a; Edwards, 1983), renal cortical slices (Fishman, 1976; Park & Malvin, 1978; Park et al., 1981; Churchill and Churchill, 1982b; Naftilan & Oparil, 1982; Baxter et al., 1985), isolated glomeruli (Baumbach & Leyssac, 1977), and isolated JG cells (Fray & Laurens, 1981; Kurtz et al., 1984).

Inhibition of renin secretion by high perfusion pressure and high K depolarization are known to share many similar features. For example, they are both Ca dependent, inhibited by verapamil (Fray, 1980b), reversed by forskolin, the direct activator of adenylate cyclase, and blocked by calmodulin

inhibitors (Fray & Park, 1986); furthermore they both inhibit isoproterenol-stimulated renin release (Fray, 1978).

The mechanism by which reduction of renal perfusion pressure stimulates renin secretion is still a subject of controversy though the cellular processing and possible modes of secretion have been addressed in several studies. Lowering renal perfusion pressure causes an increased renal renin content suggestive of increased renin synthesis and a possible constituitive secretion pathway (Pratt et al., 1983). Renin secretion with decreased pressure is short-lived even with continued stimulation (Fray et al.,1977; Travis & Ricanati, 1982) and peaks at 3-5 minutes (Travis & Ricanati, 1982) suggesting release from a readily available, presynthesized storage pool. The half-time of the constituitive pathway, the shortest secretory route in other cells is on the order of 10 minutes (Kelly, 1985). Lowering perfusion pressure also selectively releases the more acidic of the isoelectric forms of renin (Sessler & Malvin, 1985). Barajas (1966) has suggested that upon lowering renal perfusion pressure, small granules in the vicinity of the Golgi fuse to form one large conglomerate which then becomes a mature granule. One could speculate that this granule-granule fusion could be facilitated on a physical level by the decreased stretch of the JG cells and the resultant decreased intracellular space for granule dispersion. Barajas (1966) has also demonstrated swelling in renin granules suggesting a resultant release of their contents into the cytoplasmic space and Faraggiana et al. (1982) using immunohistochemistry has demonstrated renin in this compartment of the JG cells of humans with renal artery stenosis. This suggests that one route of renin secretion upon reduction of renal perfusion pressure may include a soluble cytoplasmic route. Thus, it may be concluded that low pressure stimulates the secretion of a variety of renins that may originate from different cellular pools.

## 2. THE SODIUM SIGNAL

The first reports of the beneficial effects of salt restriction in hypertension appeared in 1904 in a publication by Ambard & Beaujard. It is interesting to note that investigators at that time felt that chloride but not Na restriction was beneficial in reducing blood pressure, an idea resurrected and gaining prom-

inence in recent research efforts (Whitescarver et al., 1984; Whitescarver et al., 1986). Plasma renin activity is known to vary inversely with dietary intake of Na in all species examined (Kaufman et al., 1980; Munoz-Ramirez, 1980; Sessler, 1985; Atlas et al., 1986), but the mechanism by which Na alters renin release remains a subject of controversy (Gibbons et al., 1984). The effect may be a direct action of Na on the JG cells, or an indirect action mediated by humoral or neural signals induced by alterations in fluid volume or extracellular Na concentration. Of the myriad hypotheses proposed for regulation of renin secretion by Na intake, the macula densa theory has received the greatest scrutiny. Anatomically, the macula densa is that portion of the nephron linking the thick ascending limb of the loop of Henle to the early distal convoluted tubule (Figure 1). Goormaghtigh (1932) was the first to suggest an endocrine role for the macula densa of the kidney and to recommend that a functional relationship might exist between the macula densa and the juxtaglomerular cells (Goormaghtigh, 1939). He also proposed that glomerular filtration rate is regulated by the composition of renal tubular fluid at the macula densa (Goormaghtigh, 1945). From the late 1950s and throughout the 1960s, in view of the intimate anatomical relationship of the macula densa to the juxtaglomerular cells of the renal afferent arteriole (Barajas and Latta, 1967; Hartroft and Hartroft, 1961), a number of investigators suggested that renin secretion is also regulated by the ionic composition of tubular fluid at the macula densa. However, because of the difficulty in functionally isolating the macula densa from other mechanisms, the precise role of this tubular segment in controlling renin release has been difficult to establish. Since the macula densa is inaccessible to micropuncture, the exact composition of tubular fluid at this point, and therefore, the nature of the perceived signal, can only be inferred.

These uncertainties resulted in two opposing hypotheses regarding the precise role of the macula densa in mediating renin release. According to Vander & Miller (1964) renin release is inversely related to the rate of Na delivery to the macula densa, or the Na load. On the assumption that cellular transport of Na is directly related to the Na load, it was suggested that renin release is inversely related to Na transport into or across the macula densa cells (Vander & Miller, 1964; Nash et al., 1968; Vander & Carlson, 1969). In contrast to this concept, a second hypothesis postulated that renin release is directly proportional to tubular fluid Na concentration at the macula densa (Meyer et al.,1968; Cooke et al., 1970).

Opposing this latter hypothesis, Fray (1976), using isolated rat kidneys, varied the Na concentration of the fluid perfusing the kidneys from 85 to 204 mM and reported no significant change in the rate of renin secretion, despite large changes in Na excretion. In support of this data and consistent with the Vander hypothesis, Beeuwkes and Rosen (1979) demonstrated, histochemically and by electron probe micro-analysis, that little or no (Na/K)- ATPase activity is found within the macula densa cells regardless of dietary Na intake. If the ability to establish high transepithelial ion gradients is required for transduction, then these results are inconsistent with a transducer role for the macula densa cells. Furthermore, Sottiurai and Malvin (1982), also using electron probe microanalysis, observed no correlation between Na concentration in macula densa cells and plasma renin activity since the Na concentration in these cells did not vary during maneuvers that changed renin release over a wide range.

To complicate further the elucidation of the precise sodium signal in renin secretion, in vitro work using cortical slices, isolated glomeruli with and without attached afferent arterioles; isolated afferent arterioles with and without attached macula densa elements; and isolated cell suspensions have produced equally conflicting results. Renin secretion has been shown to be proportional (Braverman et al., 1971; Lyons and Churchill, 1975; Capponi and Vallotton, 1976), inversely proportional (Galla et al., 1981; Itoh et al., 1985; Atlas and Laragh, 1986) or unrelated (Baumbach and Skott, 1986; Rostand et al., 1985; Khayat et al., 1981) to the concentration of Na in the incubation medium.

From another perspective, the influence of a low Na diet has been intensely studied at several levels of organization including the systemic level, the organ and tissue levels, and most recently, the cellular and biomembrane levels. While a number of specific effects have been reported and confirmed, the plenary physiological consequences of Na depletion on the renin angiotensin system and in blood pressure regulation remains controversial.

In a recent series of experiments by Lindop & Lever (1986), Na depletion greatly increased the percentage of renin-positive efferent arterioles in the superficial cortex of the rat kidney. Apparently renin-containing cells increase in response to Na depletion and subsequently renin synthesis and release increases. This could quite effectively protect the organism against an imbalance in Na homeostasis.

Under a Na-deficient diet, JG cells have abundant rough endoplasmic reticulum, appearing as

elongated or round saccules studded with ribosomes and containing very pale, flocculent material (Morimoto et al., 1979). In biochemical analysis of isolated renal cortical cell organelles, D-glucose-6-phosphatase activity was significantly higher in cells from rats on low Na diet compared to rats on normal salt intake, further suggesting an extensive development of the endoplasmic reticulum fraction. The mechanism of induction of this endoplasmic reticulum marker activity with a low salt diet is unknown (Morimoto et al., 1979); however, it does provide evidence for increased renin synthesis in response to salt restriction. Succinate dehydrogenase and acid phosphatase activities showed no change with Na depletion. Thus mitochondrial and lysosomal content of the renin secreting cells were unaltered by Na restriction. The Golgi apparatus is much more plentiful in JG cells from Na depleted rats than in normal JG cells, being comprised of numerous saccules and vesicles, which often occupied a paranuclear position (Desormeaux et al., 1982). The renin system thus augmented by hyperplasia of juxtaglomerular cells and its organelles is apparently summoned to activity by low or absent salt intake. However, despite this extensive development of the intracellular machinery for an increased protein synthesis, no change was observed in the density of the renin granules isolated from rats on a low Na diet compared to those isolated from rats on a normal diet. In fact, the protein concentration of the granule fraction was slightly decreased in JG cells from low Na rats (Morimoto et al., 1979), even though the number of granules increased.

According to Fray (1978) the primary effect of Na depletion is to increase renin release rather than renin synthesis. Fray reported that while Na-deprived rats stored 3-fold more renin than Na-loaded rats (a secondary indication of increased renin synthesis), the Na-deprived rats released 10-fold more renin. Thus, the increase in renin release was much greater than the increase in renin content. Fray proposed that Na deprivation increased the responsiveness of the release mechanism more than it stimulated renin synthesis.

However, in isolated cell suspensions enriched in JG cells, a significant loss of cell viability was reported with NaCl concentrations less than 110mEq/L (Khayat et al., 1981). Examination of the cells revealed that most were dead and many had ruptured, especially at very low NaCl concentrations. Since, in these experiments, no correction was made for differences in osmolarity, and altering the NaCl concentration had no appreciable effect on either renin release or renin synthesis, the results indicated that neither renin secretion nor cellular renin activity was affected by changes in osmolarity in the absence of the

macula densa (Khayat et al., 1981). Such results lend support to the postulated involvement of the macula densa cell in the mediation of the Na influence on renin release.

In experiments with isolated rat afferent arterioles, the JG cells sensitive to changes in media NaCl concentration were found to be located within or very close to the glomerulus (i.e. close to the macula densa). These cells represent no more than some 20% of the entire JG cell population (Baumbach & Skøtt, 1986).

At the kidney nephron level, the increased renin release associated with a low salt diet has been found to be suppressed by all chloride salts tested, while Na salts in the absence of chloride, with the exception of NaBr, had no effect on the elevated renin release stimulated by the low salt diet (Kotchen et al, 1976; Kirchner et al., 1978). Thus bromide was the only anion that could substitute for chloride in affecting renin release. To a greater extent than that of other halides, bromide transport resembles that of chloride (Walser & Rahill, 1966; Imai & Kokko, 1976), and inhibition of renin by both NaCl and NaBr further suggests a link between reabsorptive chloride transport at the macula densa and renin release (Kirchner et al., 1978).

It is now recognized that the chloride ion can be specifically transported by a variety of tissues and exert its own biological effects ( Wilcox, 1979; Koletsky et al., 1981; Zadunaisky, 1982). The pathogenesis of NaCl-dependent hypertension might therefore depend on the dietary intake of Cl as much as, or rather than, Na (Zehr et al., 1980; Kurtz & Morris, 1983). In support of this hypothesis, Kurtz and Morris (1983) found that rats given desoxycorticosterone and NaCl had a greater increase in blood pressure than rats given desoxycorticosterone and $NaHCO_3$ or Na ascorbate.

In these and other early studies reviewed elsewhere (Davis & Freeman, 1976), however, one can not completely exclude the effect of changes in chloride intake, excretion or in plasma chloride on the baroreceptor mechanism, nor the influence of renal nerves, circulating humoral agents or a change in hepatic clearance of renin. To elucidate further whether changes in chloride influence renin release only via a tubular receptor, Rostand and coworkers (1985) employed an isolated perfused rat kidney preparation which permitted examination of renal function and renin release in the absence of neural, humoral and volume influences. They subsequently reported a significant negative correlation between renin and

absolute tubular reabsorption of chloride, with no such relationship with absolute Na reabsorption. On the basis of these findings and Fray's (1976) previous report showing no effect of variations in perfusate Na concentration on renin release, they concluded that chloride reabsorption in the thick ascending limb of the Loop of Henle is the critical signal for renin release via the macula densa (Rostand et al., 1985).

Contrary to these reports, a recent study (Whitescarver et al., 1986) compared the effects of dietary NaCl loading and selective chloride loading (without concomitant Na) on renin release and blood pressure in Dahl salt-sensitive hypertensive rats and in NaCl-deprived Sprague-Dawley rats with one-kidney, one-clip hypertension. In the latter model, elevated arterial pressure is renin-dependent rather than Na-dependent (Kotchen & Guthrie, 1980). These investigators found that selective dietary chloride loading (without Na) did not decrease plasma renin activity and did not alter blood pressure in either salt-sensitive or renin-dependent hypertension (Whitescarver et al., 1986). Resolution of these apparently contradictory results will not be possible until the transport properties of the renal tubular epithelium and the precise effect of tubuloglomerular feedback on renin release are better understood.

At the system level of organization, the effect of renal innervation on renal adaptation to dietary Na restriction and renin release has also been studied (DiBona, 1985). Renin secretion was found to be unaffected by $alpha_1$- or $alpha_2$-adrenoceptor blockade. Therefore intact renal innervation was proposed to be necessary for normal renal adaptation to dietary Na restriction in conscious rats (DiBona, 1985). Evidence in support of this hypothesis included the observation that before bilateral renal denervation, changes to a low Na diet were associated with diminishingly negative cumulative Na balance for 3 days, but Na balance became increasingly positive thereafter. However, after bilateral renal denervation, changes to a low Na diet were linked with a continuously and progressively negative cumulative Na balance (DiBona, 1985). The adrenergic effects will be considered in greater detail in the subsequent section.

About the same time this report appeared, another group of investigators (Skøtt & Baumbach, 1985) demonstrated that renin release <u>in vitro</u> could be inhibited by a mechanism independent of a functioning nephron. In fact, by a metabolic factor independent of changes in hemodynamics, Na excretion or sympathetic nervous activity. Skøtt & Baumbach reported that the signal for a change in renin release is not the NaCl load itself, but rather the ribonucleoside, adenosine, a metabolic factor which reflects the

amount of work done by the macula densa cells to reabsorb NaCl. Renal arterial infusion of hypertonic saline induces increased formation of adenosine through the increased hydrolysis of ATP required in the transport of excess NaCl at the macula densa. Thus, Na depletion may not increase renin release through a specific macula densa-mediated mechanism per se, but rather through the absence of a metabolic inhibitor released by macula densa cells in response to an increased NaCl load. Itoh and coworkers (1985) demonstrated that Na depletion has no effect on renin release from microdissected afferent arterioles devoid of any macula densa influence. This release could be blocked by the addition of adenosine to the microdissected afferent arterioles. If the afferent arterioles were isolated with the macula densa attached, no increase in renin release was observed with Na depletion.

It is hypothesized that JG cells have both $A_1$- & $A_2$-adenosine receptors (Murray & Churchill, 1984; 1985). $A_1$-adenosine receptors have a high affinity for adenosine and couple to adenylate cyclase in an inhibitory manner (Van Calker et al., 1978; Londos et al., 1980) thus decreasing intracellular cAMP and decreasing renin release. A common feature of receptors that are negatively coupled to adenylate cyclase is their regulation by Na (Jacobs et al., 1979). Specifically, Na has been shown to shift Na-regulated receptors to antagonist high affinity states accompanied by a markedly decreased affinity for agonists (Watanabe et al., 1985). Adenosine may also inhibit renin secretion by inducing Ca influx since Skøtt and Baumbach (1985) have demonstrated that it fails to effectively inhibit renin secretion in the presence of EGTA or in the absence of extracellular Ca. This adenosine-mediated inhibition of renin secretion may not involve voltage sensitive Ca channels since verapamil or D600 were unable to block the effect (Macias-Nunez et al., 1985).

$A_2$-adenosine receptors have a lower affinity for adenosine and couple to adenylate cyclase in a stimulatory manner (Bruns, 1980; Londos et al., 1980; Woodcock et al., 1984) increasing cAMP inside the cell and increasing renin release. Thus Na depletion may stimulate renin release through the absence of inhibitory adenosine (a consequence of a decreased Na load to the macula densa) and the absence of the regulatory influence of Na resulting in a shift to the agonist high affinity state, a decreased affinity for antagonist and the subsequent activation of $A_2$-adenosine receptors. Alternatively, Berne (1980) has

suggested that since in coronary smooth muscle adenosine decreases the inflow of Ca, a similar mechanism may exist in the JG cells. This possibility is supported by the observation of Bradley and Morgan (1985) adenosine inhibits Ca influx in coronary as well as other vascular smooth muscle cells.

That a low Na diet may affect Na handling of individual cells has recently been reported by Jest and co-workers (1985) who studied the effect of prolonged salt restriction on cellular handling of Na in lymphocytes in normotensive human subjects without a history of essential hypertension. The Na efflux rate constant was reduced and attributed almost exclusively to a change in the ouabain-sensitive efflux rate constant. Simultaneously, there was a corresponding increase in intracellular Na content. These observations reflect a change in the kinetics of the Na pump and were interpreted as a primary inhibition of Na pump activity and a secondary cellular accumulation of Na. This hypothesis was supported by a significant reduction in cellular K:Na ratios. The Na/K pump had been implicated in Na depleted renin release in an earlier report by Fray et al. (1983b) in which adding ouabain, removing K, or lowering extracellular Na all inhibited high pressure-induced renin secretion in the absence of Ca. Presumably, the full expression of the Na/K-ATPase pump was limited by the lower Na concentration gradient across the JG cell membrane. An especially interesting finding of the work in Jest's laboratory was that the value of the absolute Na efflux, despite the reduction in K:Na ratio, remained unchanged indicating that a new steady state of Na transport had been established after 4-5 weeks (Jest et al., 1985). Apparently, Na depletion leads to alterations in cellular Na homeostasis.

Inhibition of Na transport across the plasma membrane and an increased intracellular Na concentration have also been reported in erythrocytes from patients with essential hypertension (Wilkins et al., 1985). The etiological significance of these observations is unknown, but Wilkins et al. (1985) clearly demonstrate an impairment of Na transport across erythrocyte plasma membranes which persists when the cells are removed from their plasma and is therefore an integral feature of these cells. In this report, Wilkins and his coworkers ( 1985) investigate the possibility that similar abnormalities of Na transport might occur in other pathological states even in the absence of changes in blood pressure.

The possibility of intracellular Na regulating renin secretion led Park and Malvin (1978) to add ouabain to renal cortical slices, to block the Na/K pump and thereby increase intracellular Na. They

observed an expected inhibition of renin secretion and thus confirmed the earlier findings of Lyons and Churchill (1975) who reported an inverse relationship between cytosolic Na and renin secretion as well as its attenuation by ouabain. However, Park and Malvin were only able to duplicate Lyons and Churchill's work in the presence of Ca. The involvement of Ca had not been considered in the earlier study. Park and associates (1981) went on to show that without Ca in the medium (or in the presence of the Ca channel blocker verapamil) ouabain failed to inhibit renin secretion. These results suggest two tentative conclusions. The first is that intracellular Na concentration alone may be an inadequate signal for the the regulation of renin secretion. In support of this idea, Sottiurai and Malvin (1982) found no correlation between the macula densa cells' concentration of Na and PRA. The second conclusion is that an increased intracellular concentration of Na may increase net Ca influx as has been shown in vascular smooth muscle (Bohr et al., 1969; Blaustein, 1977) and this increased Ca may be the signal for the macula densa cells, as suggested by Bell and associates (Bell & Navar,1982; Bell & Reddington, 1983).

As is evident from the foregoing review, the relationship of hypertension and renin release to Na and the role of Ca in the macula densa response to Na remains tantalizingly obscure. The data are still insufficient and a standardization of research techniques is sorely lacking for an accurate assessment of the precise Na signal and/or the effect of chloride salts on renin release. A role has also been postulated for K in the macula densa pathway (Kirchner & Mueller, 1982). There has even been some debate on whether the renin system itself is necessary to maintain a normal blood pressure. However, some investigators, using an angiotensin converting enzyme inhibitor to block the generation of ANG II (i.e., renin-angiotensin system blockade), have provided evidence that even in a Na repleted state, renin appears to contribute to blood pressure maintenance though the observed decrease in blood pressure was small and asymptomatic (McGregor et al., 1983). On the other hand, when salt deprivation of a sufficient magnitude exists, the renin response becomes a key factor in the maintenance of blood pressure and blockade of this single mechanism in the multifactorial interplay suffices to significantly affect blood pressure maintenance (Brunner et al., 1986).

## 3. RENAL SYMPATHETIC NERVE ACTIVITY

Histochemical fluorescence (Wagermark et al., 1968) and numerous ultrastructural studies (De Muylder, 1952; Barajas and Muller, 1973; Barajas, 1981) have established that the renin-secreting cells of the JGA have a dense sympathetic innervation and it is well recognized that nervous mechanisms are important in the stimulation of renin release (Davis and Freeman, 1976). Most nerves of the JGA of the rat are monaminergic nerves as evidenced by the almost total disappearance of catecholamine fluorescence and acetylcholinesterase activity with the injection of 6-hydroxydopamine, a substance which selectively destroys adrenergic nerves (Barajas, 1981). The histochemical demonstration of acetycholinesterase in the renal nerves has been considered to indicate the existence of a cholinergic innervation (Barajas and Muller, 1980). This observation is consistent with the evolution of the sympathetic nervous system in vertebrates as a study of the vertebrate phylogenetic tree reveals that cholinergic innervation diminished as adrenergic innervation increased in the course of vertebrate evolution (Nishimura, 1980). According to Nishimura, the participation of the adrenergic nerves in the control of renin release may have evolved in a later stage in vertebrate phylogeny and perhaps serves as a supplementary rather than a primary system of control.

It is clear that the autonomic nervous system is not necessary for the increased renin secretion observed in response to acute salt depletion. Even the collective effects of surgical denervation, local anesthesia of renal nerves, and both ∂- and ß-receptor blockade failed to prevent a renin response to chlormerodrin, a mercurial diuretic drug (Davis and Freeman, 1976). It seems evident that other renin release mechanisms are rapidly called into action in the absence of an intact renal sympathetic nervous system.

The chronic effects of renal denervation on renin secretion are variable and conflicting. Mogil et al. (1969) reported the complete inhibition of renin release in Na depleted animals with renal denervation; while others have demonstrated a delayed, but otherwise normal response in comparison with innervated kidneys (Brennan et al., 1974; Gotshall et al., 1974). The results of these early experiments are difficult to interpret since they were usually complicated by marked changes in renal hemodynamics and renal

function as well. High rates of stimulation of renal nerves cause profound reductions in RBF and renin release (Coote et al., 1972) and at lesser rates there are reductions in both GFR and Na excretion (Johns et al., 1976; Ball and Johns, 1982). However, at low stimulation rates, which do not change either RBF or GFR, both renin release and tubular Na reabaorption increase representing a direct action of the renal sympathetic nerves on the renin-containing cells of the JGA (Keeton and Campbell, 1981).

It is now clear that renal nerve regulation of kidney function also occurs when the nerves are activated reflexly by either carotid sinus pressure reduction (DiBona and Johns, 1980), stimulation of the cardiopulmonary receptors (Thames et al., 1978), or activation of the sensory receptors of the muscles and skin (Handa and Johns, 1983). Thames and DiBona (1979) have demonstrated that very low frequency renal nerve stimulation, which failed to change any aspect of renal hemodynamics or tubular function enhanced the ability of the kidney to release renin in response to renal perfusion pressure reduction to 50mmHg and administration of the diuretic, furosemide. More recently Kopp and DiBona (1984) used higher rates of electrical stimulation of renal nerves and showed an enhanced renin secretion in response to more modest reductions in renal perfusion pressure to 100mmHg. Considered together, these studies highlight the fact that the renal nerves appear to modulate the ability of the kidney to respond to renin-releasing stimuli of a nonneural origin (Johns, 1985) and lend support to a secondary subsidiary role of the renal sympathetic system in renin secretion.

Using recently developed highly specific neuroadrenoceptor blockers, there is now a clear consensus that adrenergic stimulation of renin secretion at the molecular level primarily involves ß-adrenoceptors (Keeton & Campbell, 1981). Selective blockade of $ß_1$-adrenoceptors with atenolol inhibits renin release caused by renal nerve stimulation at levels too low to evoke a significant hemodynamic response (Johns, 1981); while a similar blockade of $ß_2$-adrenoceptors with butoxamine (Osborn et al., 1981) had no effect on nerve mediated renin release. On the other hand, although direct demonstration of surface-specific receptors on the JG cells have never been reported, it is generally accepted that such receptors do exist (Fray et al., 1987) especially since ß-agonists stimulate renin secretion from isolated JG cells (Johns et al., 1975; Khayat et al., 1981; O'Dea, 1984; Kurtz et al., 1984). Several

lines of evidence support the hypothesis that ß-agonists simulate renin secretion by lowering cytosolic Ca via increased Ca efflux and sequestration into intracellular storage sites, and decreased Ca influx (Fray et al., 1983a). An increased Ca efflux prior to increased renin secretion has been shown with norepinephrine, glucagon, and renal nerve stimulation; and propranolol, a ß-antagonist, blocks both responses (Harada & Rubin, 1978). Lanthanum, an ion that blocks Ca efflux, also blocks the effect of ß-agonists (Logan et al., 1975). An important caveat to be aware of in these experiments is the involvement of the entire mixed population of renal cells, all potentially responsive to ß-agonists, of which JG cells are an extreme minority contributing to the generalized Ca efflux. The Na/K pump has also been implicated in the mechanism of increased Ca efflux (Fray, 1980a; Churchill, 1985) since isoproterenol, and presumably other ß-agonists as well, stimulates the Na/K pump in smooth muscle and thereby lowers cytosolic Ca by Na/Ca exchange (Scheid et al., 1979). Furthermore, ouabain, vanadate, low extracellular K and Na block both the Na/K pump and ß-adrenergic-induced renin secretion (Fray, 1980a; Churchill, 1985).

As ß-adrenergic agonists are known to stimulate adenylate cyclase in vascular smooth muscle cells, it appears likely that cAMP also has a central role as a second messenger in the stimulatory pathway for renin secretion (Taugner et al., 1984a). This view is supported by the observation that forskolin, a selective activator of adenylate cyclase in most mammalian cells in a receptor-independent way, at low concentrations stimulates renin release and decreased Ca influx from the isolated rat kidney (Schwertschlag and Hackenthal, 1982) and the isolated JG cell (Kurtz et al., 1984). Although Kurtz and coworkers (1984) noted that forskolin also inhibited Ca efflux slightly, the net effect was a reduction of the membrane permeability to Ca. It has been suggested that ß-agonists hyperpolarize the JG cell membrane (Fray, 1980b) and thus decrease Ca inflow. Such hyperpolarization of the JG cell membrane and subsequent renin secretion has been demonstrated with epinephrine (Fishman, 1976), but has not been confirmed by others (Buhrle et al. 1986b).

The fact that renal nerves may also affect the baroreceptor and macula densa pathways and that norepinephrine exhibits both ∂- and ß-adrenergic properties has made the precise elucidation of this pathway extremely difficult. Despite these complications, it is now generally believed that the pathway

whereby renal nerve activation increases renin secretion depends on the frequency of stimulation (Holdaas et al., 1981; Ammons et al., 1982; Thames, 1984; Blair et al., 1985; DiBona, 1985). Ganong (1972) was the first to propose that renin secretion increases presumably by direct ß-adrenergic effects on the JG cells at low frequencies (about 0.5-1.0 Hertz). This effect is mediated by a $ß_1$-adrenergic receptor (Thames, 1984; DiBona, 1985). The macula densa pathway presumably mediated renin secretion at higher frequencies (1.0-3.0 Hertz) since Na excretion decreases over this range of stimulation (Kotchen et al., 1974). At greater than 3.0 Hertz, renin secretion is mediated by a hemodynamic response via $∂_1$-adrenergic receptors since marked vasoconstriction is evident at this level of stimulation (Fray et al., 1987). Since ∂-adrenergic effects may be directed through a baroreceptor pathway (vasoconstriction) and through a neurohumoral pathway (direct effects on the JG cell), the net response in vivo may be complex involving renal blood flow and autoregulatory responses, vasoconstriction, perfusion pressure, and ultimately changes in cytosolic Ca levels (Fray et al., 1987).

The transduction of the ∂-adrenergic pathway has been defined at the cellular level and Ca has been implicated. Several investigators have shown that extracellular Ca is necessary for the inhibitory effects of ∂-agonists (Vandongen and Peart, 1974; Opgenorth & Zehr, 1983; Matsumura et al., 1985), and Ca influx through voltage-sensitive channels appears to be the critical step since blockade with verapamil and nifedipine renders ∂-agonists ineffective in inhibiting renin secretion (Opgenorth & Zehr, 1983; Churchill & Churchill, 1984; Matsumura et al., 1985). In support of this view, norepinephrine at high concentrations ($10^{-5}$M) and phenylephrine have been shown to cause substantial depolarization of the JG cell membrane (Buhrle et al., 1985).

It is interesting to note that the depressor effect of prazosin (the $∂_1$-adrenoceptor antagonist) is most pronounced among low-renin patients and the degree of first-dose depressor response is inversely correlated with basal plasma renin activity (Laragh, 1986). Furthermore, low-renin hypertensive patients exhibited a threefold greater depressor response than high-renin patients with the calcium channel blocker, nifedipine (Resnick & Laragh, 1985) and a similar response relationship was evident with diuretics (Laragh, 1986). The precise interrelationships among Ca, Na and ∂-receptors are not yet understood, but

it may be a matter of the proximity of their action sites on the cell membrane. Indeed, there is evidence at the experimental level in other cell types that calcium channel antagonists, by depleting intracellular Ca, may have a spill-over effect on nearby postsynaptic ∂-adrenergic receptors (Cavero et al., 1983; Motulsky et al., 1983; Van Zwieten et al., 1983). Conversely, ∂-adrenergic blockade may be facilitated by Ca depletion since norepinephrine-driven contraction in an isolated in vitro system is not observed unless Ca is included in the bathing media (Van Zwieten et al., 1983).

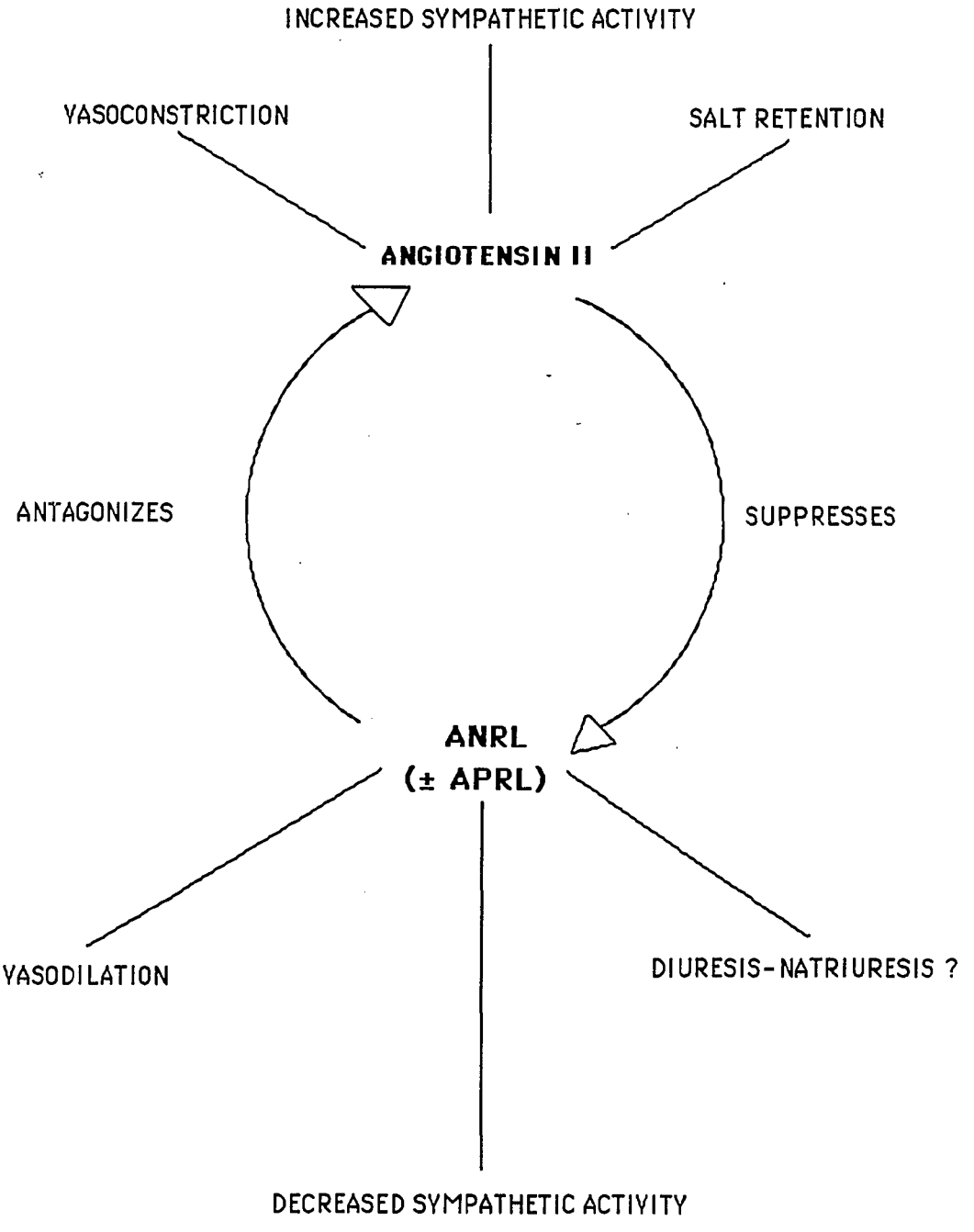
Recognizing that the sympathetic nervous system (SNS) may be critical for Na and blood pressure homeostasis during Na and fluid depletion in humans (Wilcox et al., 1983), and since recent animal studies have shown that enhanced Na reabsorption during renal SNS activation is mediated by $\partial_1$-adrenoceptors (Cotterell et al., 1984; Hesse and Johns, 1984), Wilcox and coworkers (1987) administered prazosin to block alpha$_1$- adrenoceptors and captopril to inhibit generation of ANG II during furosemide administration to normal patients on a liberal salt diet. They found that neither the alpha$_1$-adrenoceptor, nor the renin-angiotensin system are required for maintenance of Na and K ion balances during diuretic administration. However, the statistical significance of the change in renin release in the presence of furosemide alone ($p < 0.001$) was ten-fold less than in the presence of both furosemide and prazosin ($p < 0.01$) indicating that furosemide-stimulated renin secretion was considerably blunted in the presence of the $\partial_1$-adrenoceptor blocker in normotensive human subjects (Wilcox et al., 1987). Nevertheless, administration of prazosin alone failed to evoke a significant change in PRA over that measured in the absence of prazosin (Wilcox et al., 1987). Brunner and coworkers (1986) using one clip hypertensive rats reported a similar renin response to prazosin, but they noted a nearly 20-fold increase in renin secretion in DOC-hypertensive rats in the presence of phentolamine, a nonspecific ∂-adrenoceptor blocker. DOC-hypertensive rats mimic primary hyperaldosteronism in humans and are characterized by a low plasma renin activity. Hence, the precise effect of ∂-adrenoceptor blockade on the renin secretory process remains unclear; though it seems reasonable to propose that the influence of the ∂-adrenergic system on renin secretion is insignificant in normal renin states, but may be more pronounced when the RAS is impaired as for example in low-renin hypertensive states.

Most of the studies on adrenergic control of renin release have focused on epinephrine and norepinephrine, both of which have been shown repeatedly to increase renin secretion when infused intravenously or intrarenally (for an extensive review see Davis and Freeman, 1976). Recently, several investigators have reported that dopamine may also be a significant neurotransmitter in the kidney (Kopp et al., 1983; Morgunov and Baines, 1981). Mizoguchi et al. (1983) have shown that intrarenal infusion of dopamine produces a dose-related increase in renin secretion that is inhibited by specific dopaminergic blocking agents.

Very recently, Muirhead and coworkers (1986) presented evidence for a renal papillary secretion of an antihypertensive hormone that antagonizes both the sympathetic nervous system and the blood pressure-raising effect of Na volume, and causes vasodilatation; i.e. a hormone whose action is opposite that of angiotensin II. The source of the hormone appears to be the renomedullary interstitial cells (RIC). Two classes of antihypertensive lipids have been derived from these cells grown in cell culture. One is polar (the antihypertensive polar renomedullary lipid, APRL), a vasodilator causing tachycardia and increased sympathetic tone in the rat; and the other is nonpolar (the antihypertensive neutral renomedullary lipid, ANRL), a vasodilator causing bradycardia and decreased sympathetic tone. ANRL appears to be the dominant hormone and a basal renal output of ANRL (± APRL) was found by extracting the renal venous effluent of normal rats (Muirhead et al., 1982). The same extract of one kidney, one clip hypertensive rats has an inappropriate amount of ANRL (± APRL). Thus a deficiency of the hormone may contribute to the pathogenesis of this hypertensive state. Furthermore, unclipping causes degranulation of the RIC (Pitcock et al., 1981), blood pressure drops and both ANRL and APRL appear in the renal venous effluent. Renal venous effluent, as well as APRL, shifts the dose-response curve of norepinephrine to the right, indicating less sensitivity to this vasoconstrictor agent (Muirhead, 1983). These observations are consistent with the view that ANRL (±APRL) is involved in blood pressure control via ANG II and sympathetic activity. The proposed relationship between this putative antihypertensive hormone and sympathetic activity is summarized in Figure 3.

Angiotensin II has a variety of central and peripheral sympathetic nervous actions that might well potentiate its initial pressor effect (Barnes et al., 1977; Zanchetti and Bartolrelli, 1977). These

**Figure 3.** Proposed relationship between angiotensin II and the antihypertensive neutral renomedullary lipid (ANRL) ± the antihypertensive polar renomedullary lipid (APRL). Taken from Muirhead et al., 1986.


INCREASED SYMPATHETIC ACTIVITY
VASOCONSTRICTION
SALT RETENTION
ANGIOTENSIN II
ANTAGONIZES
SUPPRESSES
ANRL
(± APRL)
VASODILATION
DIURESIS-NATRIURESIS ?
DECREASED SYMPATHETIC ACTIVITY

include an excitatory action on the area postrema of the brain; stimulation of the adrenal medulla and sympathetic ganglia; facilitation of sympathetic ganglionic transmission; potentiation of postganglionic neurotransmitter biosynthesis and release, and inhibition of neurotransmitter re-uptake (Robertson et al., 1986). The neurogenic effects of ANG II have been further studied and reviewed by Clough et al. (1983).

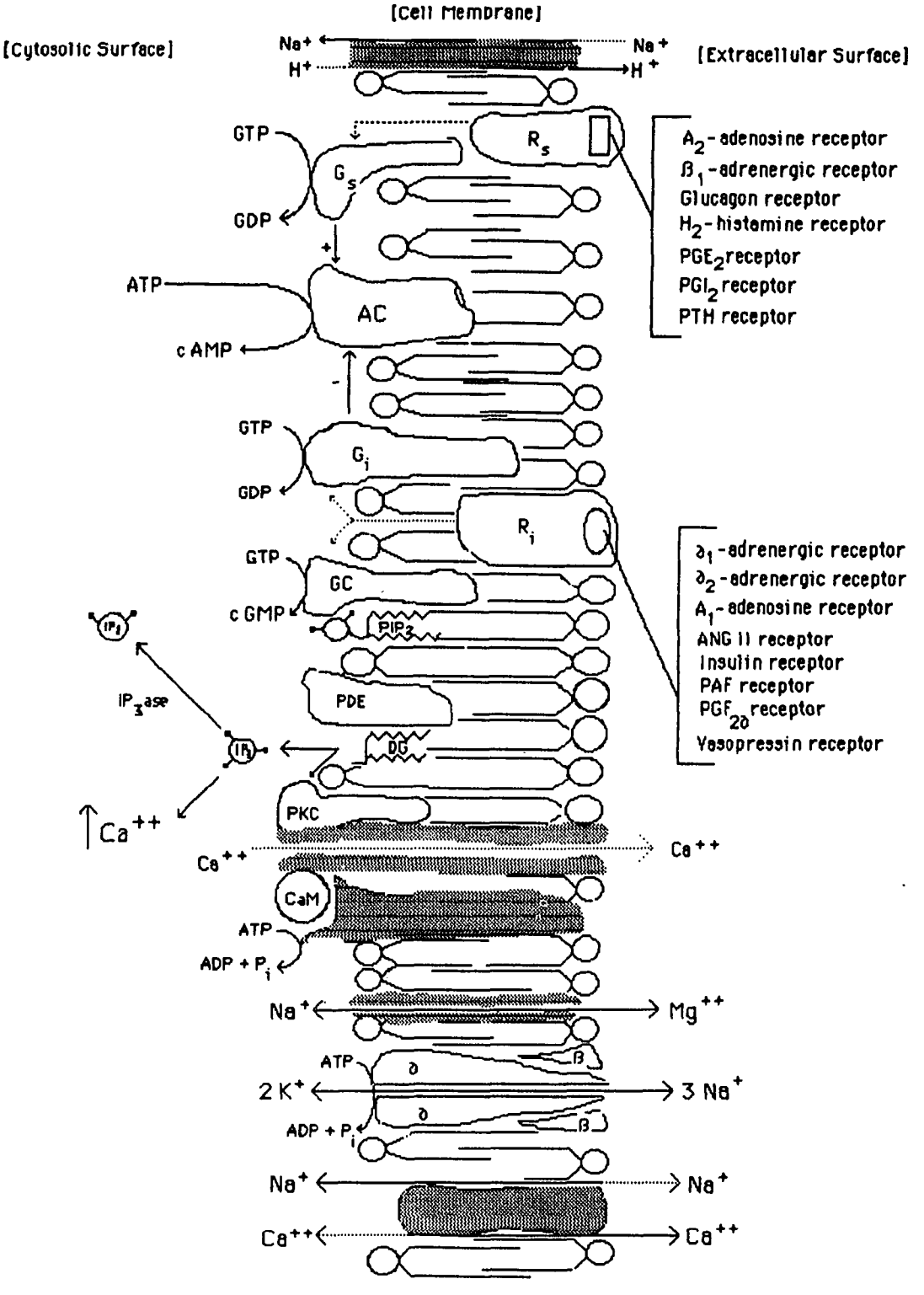
The scientific literature concerning the role of the sympathetic nervous system in the control of renin release and its pharmacological alteration is voluminous, and all facets of this subject cannot be covered in this chapter. In summary, it is clearly established that the renal sympathetic nerves exert a marked effect on renin release, and this effect is mediated by ß-adrenergic receptors located on the JG cell membrane and perhaps antagonistically by ANRL (±APRL) which depresses sympathetic acitivity.

## 4. HUMORAL FACTORS

The currently proposed plasma membrane pathways involved in regulating renin release from juxtaglomerular cells are illustrated in Figure 4. While the role of the main receptor pathways in mediating renin secretion has been extensively investigated, there is relatively little information about the exact sequence of biochemical and physiologic events that transduce the signal from the receptor into a change in renin secretion. Useful models for in vitro studies of JG cell function have not been available and studies on cellular and subcellular mechanisms involved in renin release have had to rely on the use of intact animals (Keeton and Campbell, 1981), isolated perfused kidneys (Hofbauer et al., 1976; Nakane et al., 1980; Fray, 1980b), renal cortical slices (Aoi et al., 1974; Capponi and Vallotton, 1976), isolated glomeruli (Baumbach and Leyssac, 1977) or semipurified cell suspensions derived from the renal cortex (Khayat et al., 1981; O'Dea et al., 1984). Accordingly, evidence favoring certain mechanisms must be considered circumstantial and confirmation has had to await the development of improved techniques of JG cell isolation and JG plasma membrane purification. A preparation greatly enriched in JG cells exhibiting normal physiological responses as well as a highly purified JG cell membrane model are presented in this thesis.

Despite the earlier lack of a useful in vitro model, a series of observations has been made which

**Figure 4.** JG cell membrane pathways proposed to influence renin release. This hypothetical diagram summarizes the receptor-mediated, ion channel, active transport and exchange mechanisms postulated in the current literature to affect renin release from the JG cell. Admittedly, the diagram is an oversimplication of the actual plasma membrane structure, and the literature is often controversial probably due to species variations and differences in experimental techniques. However, care has been taken to include only those structures for which published evidence is available. For clarity, the receptors have been grouped into two categories, stimulatory and inhibitory. However, each receptor-mediated agonist and antagonist undoubtedly acts through its own specific receptor (a concept difficult to pictorialize with clarity). Furthermore, interactions between membrane components and environmentally imposed secretogogues, as well as among the secretogogues themselves, may modulate the effect any one secretogogue has on renin release. Experimental evidence, direct and indirect, in support of each component of the diagram has been obtained from the following sources: Baumbach & Skøtt, 1986; Beierwaltes et al., 1980; Blaustein, 1977; Bruns, 1980; Buhrle et al., 1986; Churchill, 1985; Cohen et al., 1983; De Reimer et al., 1984; Davis & Freeman, 1976; Ettienne & Fray, 1979; Fray, 1980a; Fray et al., 1983b; Fray & Park, 1986; Garty et al., 1987; Gerber et al., 1981; Henrich et al., 1986; Herman et al., 1979; Itoh et al., 1985; Jackson et al., 1982; Johns, 1981; Keeton & Campbell, 1981; Kurtz et al., 1984, 1986a, 1986b, 1986c; Linas, 1984; Lindop & Lever, 1986; Londos et al., 1980; MacGregor, 1985; Marin et al., 1986; Matsumura et al., 1985; Mendelsohn et al., 1986; Milavec-Krizman et al., 1985; Murray and Churchill, 1984, 1985; Nilsson et al., 1985; Park and Malvin, 1978; Park et al., 1986; Pedraza-Chaverri et al., 1986; Pettinger, 1987; Pfeilschifter et al., 1985; Powell et al., 1978; Resnick & Laragh, 1985; Saier et al., 1986; Schwertschlag & Hackenthal, 1982; Seifter & Aronson, 1986; Thames, 1984; Taugner et al., 1984; Ueda et al., 1978; Van Calker et al., 1978; Vandongen et al., 1979; Vandongen and Peart, 1974; Wilcox, 1978; Woodcock et al., 1984.


[Cell Membrane]
[Cytosolic Surface]
[Extracellular Surface]
Na+
H+
Na+
H+
GTP
GDP
$G_s$
$R_s$
$A_2$-adenosine receptor
$\beta_1$-adrenergic receptor
Glucagon receptor
$H_2$-histamine receptor
$PGE_2$ receptor
$PGI_2$ receptor
PTH receptor
ATP
AC
c AMP
GTP
GDP
$G_i$
$R_i$
$\partial_1$-adrenergic receptor
$\partial_2$-adrenergic receptor
$A_1$-adenosine receptor
ANG II receptor
Insulin receptor
PAF receptor
$PGF_{2\partial}$ receptor
Vasopressin receptor
GTP
GC
c GMP
$IP_1$
PIP3
$IP_3$ase
PDE
$IP_3$
DG
PKC
$Ca^{++}$
$Ca^{++}$
$Ca^{++}$
CaM
ATP
ADP + $P_i$
$Na^+$
$Mg^{++}$
ATP
$\partial$
$\beta$
$2K^+$
$3Na^+$
ADP + $P_i$
$\partial$
$\beta$
$Na^+$
$Na^+$
$Ca^{++}$
$Ca^{++}$

has allowed the formulation of workable hypotheses on likely cellular and plasma membrane events implicated in renin secretion. A considerable amount of experimental evidence has been accumulating to suggest that humoral agents stimulating renin secretion hyperpolarize cell membranes and lower cytosolic calcium and those inhibiting secretion depolarize cell membranes and raise intracellular calcium (reviewed by Fray, 1980b; Keeton & Campbell, 1981; Freeman & Davis, 1983; Fray et al., 1983a; Fray et al., 1987).

As previously discussed, intrarenal ß-adrenergic receptors mediate an increase in renin secretion presumably by activation of adenyl cyclase and the formation of cAMP. Support for this hypothesis is found in the many observations that cyclic AMP, dibutyryl cyclic AMP analog, or inhibitors of phosphodiesterase such as theophylline stimulate renin secretion (Winer et al., 1971; Nolly et al., 1974). Furthermore, in JG cells, the ß-adrenergic receptor agonist, isoproterenol, causes a rise in cAMP levels (Lopez et al., 1978; Gaal et al. 1979) and cAMP and related agents have been shown to stimulate renin secretion (Abe et al., 1977; Ueda et al., 1978; Laychock et al., 1979; Langard et al., 1981; Churchill & Churchill, 1982a; Satoh et al., 1982). Apparently, the intracellular mediator of ß-adrenergic receptor stimulation of renin secretion is the adenylate cyclase-cAMP system, but it remains uncertain whether cAMP is the final signal which couples ß-stimulation with renin secretion (Fray et al., 1987). Intrarenal arterial infusion of pressor doses of norepinephrine inhibits renin secretion (Logan and Chatzilias, 1980), however in a Ca-free perfusion system, norepinephrine stimulates renin secretion presumably by activating ß-adrenergic receptors on the JG cell. Cyclic AMP and Ca are thought to serve as intracellular messengers in the JGC, as in many other secretory cells (Rasmussen and Barrett, 1984).

Numerous reports have supported the idea that among the multiple roles of angiotensin II (ANG II) in the kidney, it clearly inhibits renin release by a direct action on the granular JG cells (for review see Davis & Freeman, 1976). In addition as initially demonstrated by Vandongen and Peart (1974) this ANG II inhibition of basal and stimulated renin release is Ca-mediated ; i.e. ANG II increases intracellular Ca concentration and thereby elicits vasoconstriction in vascular smooth muscle cells and renin release inhibition in JG cells. A recent study by Wilson (1986) using a perfusion-fixation and vascular casting technique demonstrated that ANG II causes severe focal constriction of rat afferent arterioles, and

the major part of this constriction is not attributable to a pressure-induced autoregulatory response in the renal vasculature. Given the clearly distinctive focal, irregular and intermittent constriction pattern observed in Wilson's afferent arteriolar photomicrographs, one is tempted to speculate that the sites of constriction may be the JG cells themselves, especially since the area of the most pronounced constriction is proximal to the glomerulus; the region reported to have the highest concentration of JG cells (Taugner et al. 1984b). In direct support of this hypothesis, Kurtz et al. (1986b) have reported an increase in cytosolic Ca in isolated JG cells by ANG II and Burhle and coworkers (1986b) have shown depolarization of JG cells with ANG II.

The direct negative-feedback inhibition of ANG II on renin secretion is referred to as the "short-loop mechanism (Keeton et al., 1976) to differentiate it from the indirect inhibitory effect of ANG II-stimulated aldosterone secretion and the resultant retention of Na and ECF volume expansion eventually suppressing renin secretion, i.e. the "long-loop mechanism" (Keeton et al., 1976). The short-loop effects of ANG II provide immediate modulation of renin secretion, whereas the long-loop mechanism provides feedback control over longer periods of time. However, since aldosterone has also been shown to have vasoconstrictor effects (Purdy et al., 1982), the increased secretion of aldosterone in response to increased ANG II, may of itself, inhibit renin release thus illustrating an immediate "short-loop" within a "long-loop" mechanism of control.

Navar and coworkers (1986) recently determined that the kidney degrades about 90% of arterially delivered ANG II. Thus, most of the ANG II in renal venous blood is formed intrarenally. The concept most generally accepted is that renin release from JG granular cells leads to subsequent ANG I generation and conversion to ANG II in the interstitial spaces of the kidney (Navar et al., 1986).

ANG II receptors have been localized in rat kidney using the high-affinity agonist analog $^{125}$I-labeled [Sar$^{1}$]ANG II as a probe for in vitro autoradiography (Mendelsohn et al., 1986), and immunocytochemical studies reveal the presence of ANG II within JG cells (Taugner and Hackenthal, 1981). Furthermore, from a structural-biochemical perspective, as described earlier, ANG II receptor density shows a cortical gradient which directly parallels regional differences in renin content and from a functional perspective, receptors associated with both superficial and deep glomeruli are down-regulated

during Na depletion (Mendelsohn et al., 1986).

The conceivable involvement of postranslational mechanisms in the short-term inhibition of renin secretion by ANG II has been suggested by Nakamura and coworkers (1985). Following a one-hour infusion of ANG II in Na-depleted and captopril-treated rats, plasma renin concentration decreased by 84% whereas no significant changes in either renal renin concentration or renin mRNA content were observed. The absence of these changes suggests that the decrease in plasma renin concentration was due to posttranslational mechanisms. However, the possibility that renin gene transcription had been blocked by ANG II or that the unchanged renal mRNA content merely reflected the stability of the messenger, remains open to question. In addition, it has been demonstrated that ANG II will depress cyclic AMP, but not cyclic GMP levels in isolated tubules and glomeruli (Torres et al., 1978). It seems plausible that ANG II may be acting via the proposed inhibitory receptor ($R_i$) located on the outer surface of the cell membrane (Berridge and Irvine, 1984) which brings on a conformational change in the inhibitory G-protein ($G_i$) following GTP binding. This $G_i$ protein in turn inhibits adenylate cyclase and thus cAMP synthesis. The work of Pedrazo-Chaverri et al., (1986) lends support to this hypothesis since pertussis toxin which blocks the inhibition of adenylate cyclase via this pathway, also blocks $R_i$-mediated control of renin secretion (Figure 4).

On the other hand, it was very recently demonstrated that ANG II after binding to glomerular receptors, induces initial $PIP_2$ hydrolysis to diacylglycerol by phospholipase C and subsequent resynthesis of $PIP_2$ through phosphoinositide turnover (Ochi et al., 1987) which increases cytosolic Ca. ANG II has also been shown to stimulate $PIP_2$ hydrolysis in rat hepatocytes (Creba et al., 1983), in adrenal glomerulosa cells (Farese et al., 1984), and vascular smooth muscle cells (Smith et al., 1984). Given the growing body of evidence negatively correlating cytosolic Ca levels with renin release, it seems reasonable to propose that the inositol-lipid pathway may play a role in the inhibition of renin secretion.

In conclusion, the available evidence suggests that ANG II may act through both of the major

signal transduction pathways now known. In the cAMP pathway, ANG II may activate inhibitory receptors to effect the inhibition of adenylate cyclase and a decrease in cytosolic cAMP, i.e. a Ca-independent mechanism, ultimately decreasing renin secretion. In the inositol-lipid pathway, ANG II may activate stimulatory receptors, which transmit information through guanylate cyclase to activate a $PIP_2$ phosphodiesterase inducing hydrolysis of $PIP_2$ to diacylglycerol and inositol triphosphate ($IP_3$). $IP_3$ mobilizes stored Ca resulting in an increase in cytosolic Ca concentration and ultimately decreasing renin secretion in a Ca-dependent manner. The exact details of either of these proposed pathways in the modulation of renin secretion remains to be elucidated. However, Park et al. (1986) recently presented results supportive of a direct stimulatory action of cAMP on the renin secretory process independent of the Ca inhibitory pathway. Thus, the possibility of two independent pathways of ANG II-mediated inhibition of renin release may be at least inferred, though not firmly established.

Over the past decade there has been intense interest in determining the role of prostaglandins in stimulus-secretion coupling when the intrarenal receptors are activated. To date these studies have yielded conflicting results. There are data that support and refute a role for prostaglandins as a mediator of renin release after activation of the vascular receptor (Data et al., 1978; Berl et al., 1979; Blackshear et al., 1979; Frohlich et al., 1979; Seymour and Zehr, 1979; Freeman et al., 1982), the macula densa (Data et al., 1978; Seymour and Zehr, 1979; Gerber et al., 1981; Francisco et al., 1982; Freeman et al., 1982), and the ß-adrenoceptor (Campbell et al., 1979a, 1979b; Frohlich et al., 1979; Seymour and Zehr, 1979; Beierwaltes et al., 1980; Campbell and Zimmer, 1980; Seymour et al., 1981; Suzuki et al., 1981). Several factors could account for the discrepancies in the interpretation of these results. (1) Many studies were performed in anesthetized animals, a condition that in itself may alter both systemic and renal hemodynamics (Linas et al., 1980) as well as renin (Carvalho et al., 1975; Pettinger et al., 1975) and prostaglandin production (Swain et al., 1975; Terragno et al., 1977; Zimmerman, 1978). (2) Methods used to stimulate the intrarenal receptors were often complex and resulted in activation of more than one receptor, e.g. suprarenal aortic constriction may be associated with activation of both the vascular and macula densa receptor. (3) The prostaglandin dependency of renin production

has often been assumed to occur solely on the basis of studies utilizing cyclooxygenase inhibitors (agents which block the final oxidative cyclization step in PG synthesis) such as indomethacin. Because cyclooxygenase inhibitors have diverse effects on other enzyme systems that may also alter renin production (Dunn and Hood, 1977), it is possible that the discrepancies between these studies are the result of prostaglandin-independent effects of the inhibitors. (4) A wide range of species differences coupling PGs to renin release have been reported as well as a disparity of effects of inhibiting PG synthesis on blood pressure regulation (Jackson et al., 1982). Clearly, the common biosynthetic origins of the PGs do not connote similar functions. Rather function is linked to the structure of the prostanoid formed and the microenvironment into which it is released. (5) A diversity of methods for the measurement of renins from different animal species may also account for some of the conflicting data. Studies from several laboratories (Inagami & Murakami, 1977; Nielsen et al., 1978; Iwao et al., 1980) have shown that enzymatic activity (angiotensin I generated from renin substrate) of high molecular weight renins from hog is considerably less than that of low molecular weight renin from pig and mouse. Hence, the measurement of renin by its enzymatic activity may not always represent actual renin levels.

In an attempt to overcome these discrepancies, Linas (1984) used an isolated perfused rat kidney preparation in which the effects of systemic hemodynamics, renal nerves and circulating factors such as calcium or catecholamines could be held constant. In this system, a single receptor could be activated in the absence of activation of the other two intrarenal receptors and PG production could be determined so that any increase in renin production could be correlated with an increase in PG production. The results were then confirmed by cyclooxygenase inhibition with indomethacin. Linus presented convincing evidence that renin secretion mediated by stimulation of the ß-adrenergic receptor with isoproterenol or by a decrease in extracellular calcium, is PG independent (i.e. not associated with changes in $PGE_2$ excretion and not prevented by PG inhibition), while renin release mediated by activation of the vascular and macula densa receptors is dependent on an intact PG system (Linas, 1984). The vascular receptor (i.e. the JG cell itself) was stimulated by perfusing either filtering or nonfiltering kidneys below the autoregulatory range of pressure and both renin and PG production increased, and both increases were prevented by PG inhibition. The macula densa was activated by three methods: eliminating distal

nephron fluid delivery by perfusing with hyperoncotic albumin; perfusing at 60 mmHg in the presence of papaverine which prevents activation of the vascular receptor; and limiting chloride transport by partially replacing reabsorbable chloride, which prevents activation of the macula densa, with nonreabsorbable nitrate. In each circumstance, renin and PG production were increased and the increase was prevented by PG inhibition. Hence, one of the mechanisms PGs appear to mediate an increase in renin secretion is the macula densa pathway.

Briefly, the current consensus appears to be that $PGE_1$, $PGE_2$, $PGA_2$, $PGD_2$, $PGI_2$, $PGG_2$, $PGH_2$, and arachidonic acid stimulate the release of renin in most species , whereas $PGF_{2\partial}$ usually inhibits renin release (Jackson et al., 1982) or it may stimulate renin release when present in excess quantities in hypertensive male rats (Lennon and Poyer, 1986). It appears that the renin secretion elicited by $PGE_2$, $PGD_2$, $PGI_2$ and arachidonic acid in vivo occurs via activation of the renal baroreceptor, possibly because of an increase in renal interstitial pressure and a resultant fall in the transmural pressure gradient at the afferent arteriole (Keeton and Campbell, 1981). In view of the observation that arachidonic acid and $PGI_2$ stimulate renin secretion from renal cortical slices in vitro, these compounds seem to possess an additional direct effect on the granular JG cells (Whorton et al., 1977). Furthermore, arachidonic acid-induced renin release from renal cortical slices in vitro was antagonized by the prostacyclin synthetase inhibitor 9,11-azoprosta-5,13-dienoic acid (Whorton et al., 1980); therefore, $PGI_2$, the major PG synthesized by normotensive rats (Lennon and Poyser, 1986), is the most likely candidate for the renin-releasing metabolite of arachidonic acid. In addition $PGI_2$ has been shown to activate adenylate cyclase and increase cAMP content in the rat renal cortex (Herman et al., 1979; Lin et al., 1981). Hence at least one component of the physiological actions of $PGI_2$ may be mediated through the adenylate cyclase-cAMP system. However, Vikse et al. (1985) have provided evidence showing that glucagon, a receptor-mediated agent independent of ß-receptors, which stimulates cAMP production (Exton, 1980; Rodbell, 1983), caused increased $PGE_2$ production and renin secretion. Indomethacin blocked the increased $PGE_2$ production, but only blunted the renin secretion. On the other hand, $(Bu)_2$cAMP caused a

profound increase in renin secretion and had no effect on $PGE_2$ production (Vikse et al., 1985). Vikse and coworkers (1985) have suggested that cAMP may be the final signal for the major prostaglandin-dependent and the minor prostaglandin-independent stimulation of renin secretion by glucagon. Since Ca efflux from the kidney and presumably from the JG cells is also associated with glucagon-induced renin secretion (Harada & Rubin, 1978), cytosolic Ca must also be involved in the final signaling of this pathway.

In summary, four lines of evidence link PG biosynthesis to the control of renin release: (1) inhibiting PG biosynthesis prevents renin release; (2) arachidonic acid stimulates renin release by a PG biosynthesis-dependent mechanism; (3) $PGI_2$ and $PGE_2$ are synthesized by the kidney and are renin secretogogues; and (4) several experimental maneuvers that stimulate renin secretion also activate PG biosynthesis (Jackson et al., 1982). While irrefutable proof that prostaglandins stimulate or inhibit renin release has not been forthcoming to date, the available evidence is consistent with the hypothesis that PGs have some role in at least modulating renin release.

The simplest model compatible with the data is that a stimulus activates a phospholipase which releases arachidonic acid from phospholipid storage pools. At present little is known about the coupling between the stimulus and the precursor release. Cyclooxygenase (prostaglandin endoperoxide synthetase) converts arachidonic acid into the unstable PG endoperoxides that are in turn enzymatically converted into $PGE_2$, $PGI_2$, $PGF_{2\partial}$, $PGD_2$, and thromboxane $A_2$ (McGiff, 1981). Immunohistochemical techniques have localized cyclooxygenase-containing cells in the renal vascular epithelial cells and glomerulus (Smith and Bell, 1978). Hence, the JG cell is implicated as a site of PG synthesis. Furthermore, several investigators have suggested that Ca may be involved since indomethacin is a Ca antagonist in several systems (Northover, 1977). Knapp and coworkers (1977) used Ca ionophores to show that prostaglandin production was stimulated by Ca influx and Zenser and Davis (1978) demonstrated that adding Ca stimulated $PGE_2$ production and verapamil blocked the effect. Linus (1984) has suggested that prostaglandins may stimulate renin secretion by preventing Ca influx. The mechanism whereby prostaglandins transduce their signal to the JG cell may be similar to the mechanism whereby low

pressure transduces its signal, i.e. by lowering Ca influx and thereby effecting a fall in cytosolic Ca (Fray et al., 1987).

It has been known for the past two decades that vasopressin is an inhibitory signal for renin release (Bunag et al., 1967); however, the precise mechanism of this inhibition is not well understood. This is a complicated question because in addition to increasing renal water reabsorption, vasopressin also causes vasoconstriction, increased Na excretion and reflex suppression of sympathetic neural activity ( Reid et al., 1984). Therefore, the inhibition of renin secretion could be a secondary ex post facto response. In support of this hypothesis, the current evidence (Reid et al., 1984) indicates that vasopressin alteration of renin secretion can occur by several different mechanisms, for example, that which occurs during long-term administration of vasopressin is prevented when fluid intake is restricted and therefore appears to be secondary to retention of water and expansion of body fluids. On the other hand, the inhibition produced by short-term infusion of vasopressin in conscious dogs appears to be related to vasoconstrictor activity and can be blocked by renal denervation, suggesting that it's a reflex response mediated by the renal nerves. In contrast, the inhibition which occurs in anaesthetized water-loaded dogs is related to the antidiuretic action of vasopressin and may be mediated through the inositol-lipid pathway and subsequent mobilization of intracellular Ca. Evidence in support of this hypothesis has been reported using Quin-2 to measure changes in cytosolic Ca (Thomas et al., 1984) and $^{3}$H-labeled $PIP_2$ to follow its hydrolysis (Rhodes et al.,1983) on exposure to vasopressin. In high concentrations, vasopressin can inhibit renin release by perfused kidneys and renal slice preparations, possibly by a direct action on the JG cells, though the physiological relevance of this action is questionable. Thus, in conclusion, the effects of vasopressin receptor activation appear to be determined qualitatively and quantitatively by the particular renal hormonal mechanism that is predominating at any given time.

Recent investigations have led to the discovery and characterization of a hormone secreted by contractile atrial myocytes which has natriuretic, diuretic, and vasodilatory actions (DeBold, 1985; Cantin and Genest, 1985; Needleman et al., 1985). Atrial natriuretic peptide (ANP) blocks renin release, opposes angiotensin's vasoconstrictive actions on the vasculature and the adrenal cortex, and by its

natriuretic activity, mitigates the Na-retaining effects of aldosterone (Laragh, 1986). This hormone appears to be a cardiovascular regulatory agent that serves as a natural safety valve and opposes every phase of the activities of the renin system.

The molecular mechanisms of ANP action are not well understood. ANP has been shown to activate membrane-bound guanylate cyclase (Hamet et al., 1984) and to inhibit adenylate cyclase (Anand-Srivastava et al., 1984), thus resulting in an increase in intracellular cGMP and a decrease in cAMP in the kidney, adrenal medulla, pituitary, salivary glands, anterior uvea and retina, brain (Nemer et al., 1986), and vascular smooth muscle cells (Winquist, 1984).

The mechanism(s) involved in ANP-inhibition of renin release is also unresolved. However, the ANP-induced natriuresis would be expected to result in inhibition of the macula densa pathway to renin release. In this regard, Opgennorth and coworkers (1986), using the nonfiltering kidney model, recently demonstrated that intrarenal infusion of ANP in the absence of a functional macula densa, failed to inhibit renin release. In vivo studies using normotensive humans have shown that plasma levels of ANP increase significantly with a concomitant decrease in plasma renin activity with increasing dietary Na intake (Sagnella et al., 1987). On the other hand, in Na-depleted rats, intravenously infused ANP had no significant effect on the elevated plasma renin activity (Chartier and Schiffrin, 1987). Thus one of the the mechanisms of ANP-inhibited renin suppression appears to be mediated by increased delivery of NaCl to the macula densa.

Given the ability of ANP to exert direct effects on other tissues, a primary inhibition of renin release also seems plausible. In support of this hypothesis, Kurtz and coworkers (1986) using cell cultures enriched in JG cells demonstrated that ANP strongly inhibited renin release from the cells in a dose-dependent, calcium-independent fashion apparently mediated by a concomitant increase in cGMP. On the other hand, Henrich and coworkers (1986) using the Ca channel blocker, diltiazen, demonstrated that ANP had no inhibitory effect on renin secretion stimulated by lowered cytosolic Ca. They provide evidence which implicates the cAMP pathway as the site of ANP's direct action on the JG cell rather than cGMP. Furthermore, others have failed to demonstrate an effect of cGMP or its derivatives on renin release (Hofbauer et al., 1978; Obana et al., 1985). Thus a link between changes in cGMP and

renin release is speculative, though still possible. Studies which focus on the exact sequence of intracellular events produced by ANP are needed before the precise mechanisms responsible for ANP inhibition of renin release from the JG cell can be elucidated. A pure culture of JG cells would greatly facilitate such a study.

## 5. ELECTROLYTES

Despite a growing literature, the role of ions in the control of hormone release remains a controversial subject. Indeed for most ions so far investigated, both positive and negative influences upon hormone secretion have been reported. One of the reasons which could explain some of these contradictory findings is that ion-induced changes in hormone secretion and in particular, renin secretion, might depend critically on the environment offered to the secreting cell during ionic studies (a multi-factorial aspect difficult to control in whole animal, isolated kidney, or renal cortical tissue slice studies). In addition, considerable evidence is accumulating for the presence of a proposed membrane defect in the pathogenesis of hypertension which manifests itself particularly in defective membrane control over intracellular calcium and in alterations of membrane permeability to monovalent cations (Postnov and Orlov, 1985). This membrane defect does not appear to be limited to a single cell type, but has been demonstrated in vascular smooth muscle cells (Orlov and Postnov, 1980), cardiomyocytes (Pernollet et al., 1981), erythrocytes (Orlov et al., 1984), adipocytes (Postnov and Orlov, 1980), hepatocytes (Devynck et al., 1982), and synaptosomes (Nunez et al., 1982).

Potassium loading has been reported to suppress renin release (Brunner et al., 1970) and to lower blood pressure (Paller et al., 1984; Tannen, 1983) and K depletion to increase renin release (Abbrecht and Vander, 1970). In contrast, others have reported that K in a broad range of concentrations had no effect on renin release from isolated rat kidney slices (Aoi et al., 1974), but Witzgall and Behr (1986) recently reported that basal PRA increased significantly under K loading with constant Na ingestion in normal men. In contrast to the earlier studies, Witzgall and Behr (1986) used K-citrate rather than KCl. This suggests that chloride may be the important factor in suppressing PRA after salt infusion.

Despite the extensive amount of work that has been done, the mechanism through which K

lowers blood pressure or influences renin release remains uncertain. It should be emphasized that changes in PRA in the earlier experiments were not associated sine qua non with alterations in either aldosterone secretion or in Na balance, and do not necessarily reflect renin secretion per se given the multifactorial influences predominating in whole organism studies. Changes in PRA could also reflect changes in renin synthesis, proportionate release of inactive versus active renin, renin uptake and renin degradation. All of these factors could account for the disparate results.

At the cellular and/or membrane level, a key observation is that high extracellular K concentrations which inhibit renin release (Davis and Freeman, 1976) also depolarize the cell membrane (Fishman, 1976). In addition, elevated extracellular K suppresses renin release induced by low renal perfusion pressure, renal vasoconstriction and catecholamine infusion (Fray, 1978). Numerous studies have shown that infusion of K produces vasodilatation in vascular smooth muscle (Murray and Sparks, 1978; Haddy, 1975; Emanuel et al., 1959; Overbeck, 1972) and the finding of a decreased peripheral vascular resistence in K-supplemented SHR is consistent with a direct vasodilatory effect of K (Workman and Paller, 1985). According to this latter report, vascular ANG II receptors, plasma norepinephrine and plasma renin activity were not affected by K supplementation.

Tobian (1986) recently reported that high K diets allow cerebral arteries to carry very high blood pressures without sustaining either a thickening or damage to the artery wall or without any significant change in the arteriolar lumenal diameter. Rats on a high NaCl diet without K supplementation showed a significant thickening of the arteriolar wall and subsequent decrease of the intralumenal diameter; while rats on the same high NaCl diet supplemented with either 2.6% KCl or 3.8% K citrate, showed no change in the thickness of the arteriolar wall or the intraluminal diameter compared to the controls. Thus, the reported vasodilatory effect of K remains controversial.

Presumably suppression of renin secretion with depolarization of granular JG cells is via an increase in Ca influx. This was evidenced by Park and coworkers (1981) who reported that the Ca antagonist , verapamil, prevented the suppression of renin secretion caused by K-induced depolarization. Verapamil blocks voltage sensitive Ca channels and thus prevents depolarization-induced Ca influx and thereby prevents K inhibition of renin release. Potentiating this Ca influx in primary hypertension is a

reduced Capump efficiency and a decreased Ca-binding ability of the inner surface of the plasma membrane--two of the most common features of the hypothesized membrane defect (Postnov and Orlov, 1985).

Ca, in contrast to being a stimulatory signal in most glandular cells (Rubin, 1982), is an inhibitory signal for renin secretion as evidenced by the infusion of a Ca ionophore (Baumbach and Leyssac, 1977) and the use of Na/K-ATPase inhibitors such as ouabain (Churchill and Churchill, 1979) and vanadate (Churchill & Churchill, 1980); both of which are alleged to cause an increase in both intracellular Na and Ca concentrations. Conversely, maneuvers such as lowering the concentration of Ca in the incubation media, the use of Ca channel blocking agents, or the use of calmodulin antagonists have been shown to stimulate renin secretion powerfully (Vandongen & Peart, 1974; Baumbach & Leyssac, 1977; Park & Malvin, 1978; Churchill and Churchill, 1980; Fray, 1980b; Cohen et al., 1983; Kawamura and Inagami, 1983; Churchill and Churchill, 1985).

Ca ions and cAMP are thought to serve as intracellular signals or second messengers in JG cells, as in many other secretory cells (Rubin, 1982) in the regulation of renin secretion (Churchill and Churchill, 1985). However, the relative importance of these two signals in mediating renin release remains unresolved. It is conceivable that the two systems are linked such that an increase in $[Ca]_i$ leads to a reduction in cAMP, a theory with some support in other experimental systems (Meisheri & McNeill, 1979). On the other hand, cAMP may be the primary messenger by increasing Na/K-ATPase or Ca-ATPase leading to decreased $[Ca]_i$ and increased renin release. Both isoproterenol and $(Bu)_2$cAMP effects on renin release can be blocked by high K, ouabain or vanadate (Churchill & Churchill,1980; Fray, 1980a; Churchill & Churchill, 1982a). Since all of these agents increase intracellular Ca, cAMP may also reduce intracellular Ca.

In search of a clarification of the role of cAMP and Ca in renin release, Antonipillai and Horton (1985) reported that isoproterenol stimulated renin release in the presence of trifluoperazine and calmidazolium, while low extracellular Ca or nifedipine (a specific Ca channel blocker) did not alter isoproterenol-induced renin release. Thus, acute ß-adrenergic stimulation of renin secretion via cAMP

may be independent of changes in the levels of both extracellular and intracellular Ca and calmodulin. Park and coworkers (1986) recently added support to the hypothesis that control of renin secretion by Ca and cAMP is mediated by two parallel but independent pathways. They demonstrated that forskolin ($10^{-5}$M), a potent adenyl cyclase agonist, increased renin secretion by a fixed amount independent of medium (by inference, intracellular) Ca concentration in a high-K medium (Park et al., 1986). Furthermore, the potency of forskolin-stimulated renin release was directly proportional to the removal of the Ca-calmodulin inhibitory pathway with the calmodulin antagonists: W-5, W-7, W-13, TFP and calmidazolium (Park et al., 1986).

In summary, when considering the role of Ca in renin release, it is apparent that intracellular Ca rather than extracellular Ca is the most important determinant since renin release is stimulated in the presence of Ca chelators or the absence of extracellular Ca. In contrast, if the intracellular levels of Ca are elevated by decreasing Ca efflux with lanthanum (Baumbech and Leyssac, 1977) or by increasing Ca influx with high K, ouabain, ANG II, or Ca ionophores, renin release is inhibited (Keeton and Campbell, 1981). This inverse relationship between intracellular Ca concentration and renin release suggests that a mechanism other than exocytosis may be involved in the release of renin from the JG cell. Furthermore, cAMP may elicit renin release in vivo and in vitro by a direct action on the JG cell that is independent of the Ca inhibitory action.

Raising extracellular concentrations of Mg has been shown to stimulate renin secretion (Churchill & Lyons, 1976, Fray, 1977, Wilcox, 1978, Ettienne & Fray, 1979) presumably by hyperpolarizing the cell membrane and inhibiting net Ca influx. It's been demonstrated that depolarizing concentrations of extracellular K inhibited the stimulatory effect of high Mg and a Mg/Na exchange mechanism appears to be involved (Ettienne & Fray, 1979).

More recent investigations of renal handling of Mg indicate that the effects of this divalent cation may be far more complex. Mg is the fourth most abundant cation in the human body and the second most common cation in the intracellular fluid (Quamme, 1986). This opulence and distribution implies an essential role of Mg in cellular metabolism.

Mg, a cofactor for 300 enzyme systems (Ferment and Touitou, 1985), is an essential activator

of membrane bound Na/K-ATPase and Ca-ATPase (Sweadner & Goldin, 1980; Tada et al., 1978), it stabilizes the structures of ribosomes and membranes and may be necessary for the insertion of proteins into membranes (Flatman, 1984). Its important and wide-ranging effects within the cell have led to the suggestion that Mg may be a second messenger coordinating cellular responses to changes in the environment (Rubin, 1977). The chemistry of Mg coupled with its intracellular and extracellular concentrations make it unlikely that Mg can have a "trigger" function like Ca, but slow small magnitude changes in concentration could be important in fine control and coordination of cell activity (Flatman, 1984). The regulation of external Mg balance is primarily via the kidneys and renal conservation is very efficient during periods of depletion (Levine & Coburn, 1984), though the ultimate control of Mg across cell membranes is poorly understood. It is well established that extracellular Mg plays a critical role in the regulation of peripheral vascular tone (Altura et al., 1984) and induces vasodilatation comparable to that induced by Ca entry blockade (Ji et al., 1983). In recent studies, Mg has been implicated in ß-adrenergic stimulation (Whyte et al., 1987); in hyperactivity of platelets from SHR (Baudouin-Legros et al., 1986) and modulation of ionic permeability of synaptosomal plasma membranes (Kauppinen et al., 1986); in marked hyperglyceridemia and reduced insulin response as well as marked changes in the fatty acid pattern of total plasma lipids shown by decreased levels of stearic acid, increased oleic and linoleic acids, and decreased levels of arachidonic acid (Rayssiguier, 1986); and in regulation of anterior pituitary $D_2$ Dopamine receptors (Watanabe et al., 1985). Of special significance in this thesis is the observation that all of these studies point to a plasma membrane-localized role for Mg. The available data appears to support a stimulatory role for Mg in renin secretion with effects similar to those described for Ca antagonists.

As discussed earlier in this chapter under the subtitle, "The Sodium Signal" (cf. pp. 21-29), Na and Cl ions have been implicated for some time in the control of renin release. Intracellular Ca is involved in the regulation of membrane transport of Na (Windhager and Taylor, 1983) and in the function of the gating system of the Na channels of excitable membranes (Hille, 1984) as well as nonexcitable membranes (Cuthbert and Wong, 1972). A decrease in Na concentration may stimulate renin secretion by lowering cytosolic Ca through the macula densa or may decrease the Ca permeability of the cell membrane (Fray et al., 1983a). A decrease in Cl may hyperpolarize the JG cell membrane and decrease

Ca permeability as shown in other cell types (Natochin et al., 1986). Conversely Na or Cl loading may both increase the permeability of the cell membrane to Ca and simultaneously inhibit Ca transport (Natochin et al., 1986) resulting in an increase in cytosolic Ca concentration and an inhibition of renin secretion. In support of an inverse relationship between extracellular Cl concentration and renin release, Rostand and coworkers (1985) using the isolated perfused rat kidney model demonstrated that renin release increased as perfusate Cl was decreased.

In conclusion, it is now clear that changes in the extracellular ionic milieu alter renin secretion. Since Ca plays a key role in stimulus-secretion coupling of hormones and in excitation- contraction coupling of muscle (Rubin et al., 1985), it may also play a key role in the stimulus-secretion coupling of renin release. Whether or not ions which modulate renin release act physiologically as either agonists or antagonists of Ca action in renin secreting cells and/or as modifiers of JG cell membrane Ca interactions remains to be elucidated.

**D. Other determinants of plasma renin activity.**

Plasma renin activity represents a balance between the differential secretion of multiple renin forms and their subsequent equally heterogeneous removal (Shier and Malvin, 1985). Although the mechanism is unknown, it is well established that the liver is the principle site of renin clearance (Heacox et al., 1967; Schneider et al., 1970; Hesse et al., 1978). However, it is less clear whether changes in hepatic renin clearance are important in the physiological control of plasma renin levels. Following hemorrhage (Johnson et al., 1971), hepatic artery and portal vein constriction (Schneider et al., 1970), or chronic Na depletion (Echtankamp et al., 1983), renin clearance decreases with the reduced hepatic plasma flow suggesting a slow-regulated mechanism for the removal of renin. However, during exercise, hepatic plasma flow is similarly decreased, but renin clearance doesn't change, due to an increased renin extraction ratio [i.e. the fraction of renin removed by the liver in a single pass] (Hesse et al., 1978). In contrast, a reduced extraction ratio is associated with the reduced hepatic plasma flow observed in chronic sodium depletion (Echtankamp et al., 1983). The cause of these changes in renin

extraction ratio have not been determined, although Shier and Malvin (1985) recently reported evidence in support of the hypothesis that different forms of renin are cleared by the liver at different rates and that this preference may reflect a differential secretion by the kidney depending on the type and duration of the secretory stimulus. More specifically, the secretory rate as well as the extraction ratio for basic renin forms 10 minutes after aortic constriction was found to be significantly greater than that for acidic forms of renin; whereas the percentages of basic and acidic forms of renin, 60 minutes after constriction were not significantly different from control values. The observed correlation between secretory rate and extraction ratio is consistent with the hypothesis that the increased extraction ratio during the first 10 minutes following imposition of the secretory stimulus is due, at least in part, to an initial preferential secretion of renin forms that are more rapidly removed by the liver (Shier and Malvin, 1985). Thus there appear to be at least two pools of renin available for secretion. Release from one pool comprising mostly basic forms of renin occurs during the initial period of stimulation, whereas, if stimulation is maintained, secretion from another pool, made up of the less rapidly removed acidic forms of renin, predominates. Such a preferential release and removal of renin would contribute to the reduced renin clearance reported with chronic stimulation of renin secretion (Echtenkamp et al., 1983; Schneider et al.,1970).

Sessler and coworkers (1986) recently presented evidence suggesting that the rat kidney secretes a profile of six renin forms directly mirroring a profile of stored renins which in turn, is a function of the background conditions of the animal and reflects the factors influencing synthesis, storage, secretion and uptake. Thus, in acute experiments, the profile secreted contains fixed proportions of the six forms; whereas, in chronic experiments, the secreted profile changes depending on the experimental conditions and how they affect the four factors listed above. The precise reasons for such modifications are still unclear, but this report, which includes the finding that the six forms have different half-lives, coupled with earlier reports that the different forms also have different isoelectric points dependent on the mode of stimulation ( Katz and Malvin, 1982a; Sessler and Malvin, 1985), suggests a coupling of the form of secreted renin with the type of secretory stimulus as well as a subsequent form-specified preferential removal of renin by the liver. The molecular basis for this heterogeneity is unknown, but since rat renin

is known to be glycosylated (Matoba et al., 1978) and glycosylation differences have been reported (Marshall and Malvin, 1978; Schwarz and Datema, 1980), it seems possible that such differences might at least partially explain the differential hepatic removal of different renin forms. Precedence for this hypothesis is found in other molecular species such as pancreatic ribonuclease A and human serum albumen, both of which demonstrate a high hepatic extraction ratio as a function of glycosylation (Wilson, 1979).

The kidney apparently secretes only 2-5% of the total renal renin content each hour (Yamamoto et al., 1967; Fray, 1978; Park et al., 1978). With such a small amount of total renal renin being secreted per hour, it is commonly assumed that a large portion of renal renin is stored in the JG cell (Edelman and Hartroft, 1961; Davis and Freeman, 1976; DeSenarclens et al., 1977; Park et al., 1978; Katz and Malvin, 1982b) in different pools solicited variformly by acute and long-term stimuli (Fray, 1978; Park et al., 1978). Granules of the JG cell consititute one of the renal renin storage pools (Cook, 1968; DeSenarclens et al., 1977; Tanaka et al., 1980; Taugner, 1986), but since granules only store about 30% of cellular renin (Morimoto et al., 1979; Sagnella and Peart, 1979), other pools must be present.

Several lines of evidence suggest that the cytoplasmic space and the plasma membrane are possibly important storage sites for the remaining 70% of cellular renin (Park and Malvin, 1978; Takaori et al., 1979; Nishimura et al., 1980b; Katz and Malvin, 1982a; Zavagli et al., 1983). Takaori and coworkers (1979) demonstrated that dog renin granules contain only renin molecules of 43,000 molecular weight; whereas the cytosolic fraction had a 60,000 d. renin. This big renin is cited as evidence for a cytosolic renin binding protein. Using dog renal cortical slices, Katz and Malvin (1982a) reported that newly synthesized renin appeared to be secreted along with older, possibly stored renin. They postulated the existence of multiple cellular pools of readily releasable renin with one such pool being composed of newly synthesized renin. Nishimura and coworkers (1980a; 1980b) were the first to show that renin is found in the plasma membrane and endoplasmic reticulum fractions of rat renal cortical cells. They also demonstrated that this membrane-bound renin is only released by harsh detergent solubilization of the membrane fraction or melittin dissociation from the lipid bilayer and therefore it was most likely not an artifact of the isolation procedure. Furthermore, the relative rate of release of

renin in the ER-membrane fraction was similar to that of the PM- membrane fraction, but the specific activity of the ER-renin was about one-third that of the PM-renin, even though the endoplasmic reticulum represents a larger surface area (Nishimura et al., 1980a). The three-fold greater specific activity of the PM-fraction suggests that the plasma membrane serves as another storage pool for readily releasable renin. The most recent report of membrane localized renin appeared in August, 1986, when Kawamura and coworkers found an inactive renin (48,000 d.) in the membrane fraction of their rat renin granule isolation procedure (Kawamura et al., 1986). All renin within the primary storage granules was either 36,000 or 38,000 molecular weight.

Sessler and Malvin (1985) studied the storage and secretion of multiple renin forms in the two-kidney, one-clip Goldblatt hypertensive rat (GHR). With this model, it was possible to monitor an increase in renin synthesis and secretion in the clipped kidney and a decrease in synthesis and secretion in the contralateral control kidney. Renin forms stored and secreted were compared and the profile of secreted forms in the clipped kidney of the GHR model were found to be significantly different from the control kidney in the same rat; i.e. there was a shift in the clipped kidney toward the secretion of more acidic forms of renin. These changes were correlated with time and blood pressure and were not seen in the contralateral nonclipped kidneys. Sessler and Malvin (1985)postulate that clipping the kidney induces changes in the renin-synthesizing cells in the partitioning of renin molecules and in their carbohydrate residues. Since the liver degrades preferentially certain forms, it seems possible that the kidney could retain one form more than another. With time this could change the proportions of renin forms stored.

In view of the evidence currently available, a working hypothesis seems to be that plasma renin concentration levels are set by the kidney and the liver, adjusting both the heterogeneous secretion and degradation rates for an appropriate match. In GHR there is an abnormal increase in renin forms focusing at acidic pI, forms which are degraded more slowly by the liver. Perhaps these forms participate more than others in the pathogenesis of certain types of hypertension. In support of this hypothesis, several laboratories from the mid 1970s have reported widespread (i.e. not limited to one cell type) alteration of cell membrane function of various tissues underlying the causes of primary hypertension (for an extensive review see, Postnov and Orlov,1985). Abnormalities of membrane transport (Haddy,

1983; Skrabal et al., 1985), membrane structure and protein composition (Gulak et al., 1986), and phosphoinositide metabolism as an intrinsic membrane deficiency (Marche et al., 1985; 1986) have all been implicated in the pathogenesis of hypertension.

Previous investigations have assumed that the plasma membrane merely served as the site of the exocytosis of renin, the secretory mechanism almost always employed by eucaryotic cells. However, the fact that renin is taken up by the kidney (Meignan et al., 1980), presumably by the JG cells, at a rate proportional to the plasma renin concentration in the normotensive kidney, coupled with the growing evidence of a membrane defect in the hypertensive kidney, suggests that a transport mechanism, perhaps carrier-mediated, is present for renin in the JG cell membrane. Hence, renin secretion may occur by a mechanism quite different from exocytosis (Fray et al., 1983a). Alternatively, renin secretion may occur by more than one pathway as suggested by several groups of investigators (Rouiller & Orci, 1971; Park et al., 1978; Fray, 1978; Katz and Malvin, 1982b; Pratt et al., 1983; Fray et al., 1987). Rouiller and Orci (1971) suggested at least three modes of secretion for renin--granular exocytosis, intraplasmic solubilization, and a lysosomal type (granule-granule fusion?).

Four distinct events have been postulated for the exocytotic cascade. The first is a stimulus-evoked rise in cytosolic Ca as has been demonstrated in a wide variety of secretory cells including chromaffin cells (Douglas, 1968), nerve cells (Rubin, 1970), adrenal medullary cells (Baker and Knight, 1978), and pancreatic acini (Ochs et al., 1985). The second is a Ca-dependent fusion of granules with the plasma membrane (Davis & Lazarus, 1976; Zimmerberg et al., 1980). Exactly how Ca promotes fusion is unclear, but it's been suggested that Ca binds to the granular surface and the cytosolic face of the plasma membrane, thereby diminishing the energy barrier and facilitating fusion (Dean, 1975; Zimmerberg et al., 1980). In support of this hypothesis, Ca-binding proteins [including calmodulin-binding proteins (Chenoufi et al., 1986)] have been identified in both the granular membrane (Creutz et al., 1978; 1983) and the plasma membrane (Meyer & Burger, 1979). The third event is the swelling of granules immediately after fusion perhaps mediated by a postulated osmotic gradient (coupled with the rise in intracellular Ca) which must exist before fusion will occur (Cohen et al., 1980). The granule swells as the hyperosmotic cytosol draws water into the cell and subsequently into the granule (Cohen et al., 1980; Zimmer-

berg et al., 1980; Akabus et al., 1984). Ca has been shown to induce swelling of granules but the precise mechanism of its interaction with the granule is unknown (Vander et al., 1982; Zimmerberg & Whitaker, 1985). Flocculent areas have been identified in the core of the granule and are assumed to be water pockets resulting from osmotic swelling (Ornberg & Reese, 1981). The final event in the exocytotic cascade is the fission or expulsion of granular contents into the extracellular space as evidenced by electron microscopy (Palade, 1975; Kelly, 1985).

The latter two events of the granular exocytotic cascade have been confirmed for renin secretion. Granular swelling (Rouiller & Orci, 1971; Taugner et al., 1984a; 1985) and fission (Rouiller & Orci, 1971; Taugner et al., 1984a) have both been observed at least under basal conditions. The first two events are more difficult to establish since renin secretion has been shown to be inversely, not directly related to cytosolic Ca (Park et al., 1986) and therefore Ca-mediated granule-plasma membrane fusion events are impossible to demonstrate (Barajas & Powers, 1984). The possibility that fusion may occur by some Ca-independent mechanism remains open. A direct relationship between intracellular H ion concentration and renin secretion has been demonstrated (Yamamoto et al., 1967; Morita, 1976; Anderson et al., 1980)and a high H ion concentration has been shown to induce granule-granule fusion (Bondeson et al., 1984). A similar relationship between Mg and renin secretion has also been reported (Churchill & Lyons, 1976; Fray, 1977; Wilcox, 1978; Ettienne & Fray, 1979) and between Mg and granule-granule fusion (Miller et al., 1976; Papahadjopoulos et al., 1976; Holtz & Stratford, 1979), but not between Mg and granule-plasma membrane fusion (Zimmerberg et al., 1980).

To account for these differences, Peter (1976) has suggested a modified version of exocytotic secretion for the JG cell. During stimulation, Peter observed marked invaginations of the plasma membrane associated with granules. He therefore suggested that instead of granules moving to the periphery to fuse with the plasma membrane as in other systems, in the JG cell, the plasma membrane moves inward to the granule (Peter, 1976). Ryan et al. (1982) and Hill et al. (1983) have confirmed the observations of Peter (1976) and have suggested that invagination of the plasma membrane may be a step in the cascade of the renin secretory process. The precise mechanism of plasma membrane invagination as well as the interaction between the invaginated plasma membrane and the associated granules resulting in the

discharge of renin from the granule has not been resolved.

The reported lysosomal nature of renin granules based on the presence of acid phosphatase (Fisher, 1966; Taugner et al., 1985a), cathepsin B (Taugner et al.,1985b), and ∂-glucosidase (Fransen, 1987) suggests the possibility of a lysosomal related secretory pathway for renin similar to that proposed for transport of other lysosomal enzymes such as cathepsin D (Hasilik & Neufeld, 1980). While not much is known about the cellular mechanisms of release of lysosomal enzymes, stimulated neutrophils show lysosome-lysosome fusion (Korchak et al., 1980) and stimulated JG cells show granule-granule fusion (Ryan et al., 1982; Zavagli et al., 1983). This suggests some similarity between the two processes, but since Ca influx by the ionophore A23187 stimulates lysosomal secretion (Naccache et al., 1977; Korchak et al., 1980) but inhibits renin secretion (Baumbach & Leyssac, 1977; Flynn et al., 1977), they are not identical.

Cytoplasmic solubilization as a mode of renin secretion was first suggested by Lee and coworkers (1966) who showed that after blood volume depletion, granules coalesced and released their contents into the cytoplasmic space. Zavagli et al. (1983) have observed that when granules fuse they lose their limiting membrane and dissolve in the cytoplasm releasing their contents into the cytosolic space. In support of this view, renin activity has been demonstrated in the cytoplasmic soluble space (Morimoto et al., 1979; Sagnella et al., 1980; Fray et al., 1983a; Fray & Lush, 1984) and it may be enhanced by lowering extracellular Ca (Fray & Lush, 1984), by trifluoperazine (Fray et al.,1983b), and by Na depletion (DeSenarclens et al., 1977; Morimoto et al., 1979). Furthermore, a renin-binding protein has been demonstrated exclusively in the cytosolic soluble space in a concentration greater than renin itself (Ueno et al., 1981) suggesting that a significant amount of renin is expected to leak out of the granules and therefore, the cytoplasmic solubilization pathway may be an important mode of renin secretion (Fray et al., 1987).

Still another possibility is a phenomon known as membrane shedding (Dean et al., 1984). Shedding is the process in which macromolecules have a finite stay at the plasma membrane before being released into the extracellular fluid either by outward blebbing of micelles or vesicles, or by proteolytic separation of the secretory component of a protein from a hydrophobic anchor which remains

in the membrane (for review see Black, 1980). Membrane shedding is an active process and appears to be initiated by limited proteolysis of a membrane component. Sabatini and coworkers (1982) argue that shedding may have been an evolutionary progenitor of the secretory pathway in which the process was moved to the interior of the cell in a vesicle that could fuse with the plasma membrane subsequent to the limited proteolytic event, thus initiating release of soluble products to the exterior. Whether membrane-bound renin represents an evolutionary atavus or a highly efficient means of storing a readily releasable pool of renin remains to be elucidated.

## E. Overview and critical assessment of renin release studies.

From the early in vivo studies of experimental hypertension (Goldblatt et al., 1934) it soon became obvious that the control of renin release by the kidney was a complex process. In many physiologic conditions several signals act simultaneously on the JG cells, which must process the information and produce an integrated response. As is true of many biological systems, the net response to simultaneous stimulatory and/or inhibitory signals is not always predictable or indeed, even capable of being sorted out in the whole animal system. Two positive signals impinging on the JG cell, for example, may initiate an additive response, a synergistic response, or no net gain over a single response.

A moot point, for example, is that the proposed stretch receptor functions independently of the other intrarenal receptors, such as the macula densa and neurogenic receptors, and is influenced by hemodynamic, elastic, myogenic, hormonal, and ionic factors (Freeman and Davis, 1983). Early theories focused on renal ischemia and decreased pulse pressure as possible hemodynamic stimuli for renin release, but today there is little evidence to support either theory. One of the difficulties encountered in the early experiments on the renal vascular receptor was the inability to control other intrarenal vascular factors, such as renal blood flow and glomerular filtration rate, both of which can influence renin release (Davis and Freeman, 1976). In the early experiments of Skinner and coworkers (1964), not only did mean renal perfusion pressure fall and correlate with increased renin release, but it is likely that GFR and the filtered Na load decreased as well; under these conditions, the influence of the macula densa cannot be

excluded. Likewise, the classical experiments of Vander and Miller (1964) and Vander and Carlson (1969) in which diuretics such as furosemide were used to stimulate renin release, presumably via the macula densa, could not conclusively rule out vascular effects, and hence a role for the baroreceptor as well. With the development of the canine non-filtering kidney model (Blaine et al., 1970), the macula densa influence was excluded, but the intact kidney still represented a complex system influenced by multiple neurohumoral signals. For several years, such technical difficulties limited the process in understanding the intrarenal baroreceptor mechanism for renin release.

In vitro models for studying renin biosynthesis and release are needed because of the multiple factors that influence renin production and secretion in vivo. Most in vitro studies using the isolated perfused kidney or renal cortical slices have been impaired by an inability to isolate JG cells from the influences of other cell types and structures within the renal cortex such as the microvessels and glomeruli embedded in a vast mass of tubular epithelia (Buhrle et al., 1986a). Kidney slices contain nearly all cell types of the kidney, including the renin-producing cells and since JG cells represent only a small fraction (<0.03%) of all kidney cells (Taugner and Hackenthal, 1981), agents applied to kidney slices are prone to produce indirect as well as direct effects in the JG cells. The possibility remains that sympathetic axon terminals adhering to afferent vessels may modulate renin secretion by spontaneous liberation of transmitter and that granular epithelioid cells may be influenced by neighboring cells via gap junctional coupling (Taugner et al., 1984a). Furthermore, the concentration of endogenous and exogenous substances reaching the JG cells cannot be controlled or effectively monitored, and destroyed tissue in the region of the slice surface, may release proteases mimiking renin activity (Kurtz et al., 1986b). This is especially true for thin slices, where a greater percentage of tissue can be assumed to be damaged than in thicker slices. On the other hand, thick slices might be compromised by a less than optimal energy supply resulting from long diffusional pathways.

In the cell culture milieu, neither the presence in explants, nor the addition to monolayers of extracellular matrix proteins and the addition of various growth factors alone, can stabilize renin synthesis under long-term culture conditions (Minuth et al., 1986). In all cases, renin secretion stops after a few days. It is unclear whether renin-containing cells lose their capability to synthesize renin entirely,

or whether they simply switch off their synthesis as a result of the lack of some unknown stimulus. JG cells have been reported to be modified vascular smooth muscle cells that differentiate during stimulation and dedifferentiate during inhibition of the renin-angiotensin system (Minuth et al., 1986).

A human JG cell tumor has been used to immortalize renin-secreting cells by transfection with simian virus 40 mutants (Pinet et al., 1985). However, 95% of the renin produced was released in an inactive form evidencing a dysfunction in the renin-storing capability of the cells. Only 2.5% of active renin production was localized within the cells. After activation, the secreted prorenin had characterisitics similar to those of pure human standard renin as to its enzymatic, immunologic, and biochemical properties, except that it was less glycosylated. Interestingly, carbohydrates of transmembrane glycoproteins have been reported to be important constitutents in orienting and anchoring protein in the lipid bilayer (Sharon, 1984). Additional studies characterizing membrane-bound renin, which is also a glycoprotein, are needed as it is currently unclear whether the transformation from the nonsecretory to the secretory state in JG cells involves alterations of their membrane properties (Buhrle et al., 1985). Hence, a purified JG plasma membrane vesicle preparation capable of giving a secretory response to environmental stimuli, would be a valuable contribution to the field of renin research. There is still no general agreement regarding the optimal conditions for studying in vitro renin release at either the tissue, cellular or subcellular levels. Nor have there been adequate explanations for the large variability in the response of renin release to various agents in the earlier investigations. The present studies probe the nature of in vitro renin secretion and reveal some factors that might be responsible for the variability of renin secretion under different experimental conditions.

# MATERIALS AND METHODS

## A. Experimental animals.

Male Sprague-Dawley rats, both normal controls (150-250g) and hypophysectomized (100-150g) were obtained from Charles River Breeding Laboratories (Wilmington, MA). The rats were maintained on either a normal diet of Purina Laboratory rat chow or a low sodium diet as previously described (Fray, 1978), and tap water ad libitum. Evidence of the completeness of hypophysectomy was established by monitoring the rat's body weight (Simon et al, 1984). It has been shown that an increase in body weight correlates inversely with the degree of hypophysectomy (Mayberry et al, 1971).

## B. Materials and chemicals.

The [$\gamma$-$^{32}$P]ATP (2-10 Ci/mmol) and the angiotensin I RIA kit [$^{125}$I] were obtained from New England Nuclear (North Billerica, MA). Percoll and density marker beads were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) and trypsin, sodium dodecyl sulfate, marker enzyme assay kits and all other chemicals were obtained from Sigma Chemical (St. Louis, MO).

## C. Renin radioimmunoassay.

Renin is an aspartyl peptidase whose function appears to be highly specific in the formation of the decapeptide angiotensin I from its substrate, angiotensinogen, by the cleavage of the singular Leu-Leu (Skeggs et al., 1957) or Leu-Val (Tewksbury et al., 1981) peptide bond located in the amino-terminal region of the substrate molecule. Angiotensin I is is now recognized as the first in a series of active peptides generated by a cascade of enzymes working in concert as diagramed in Figure 2.

Dogs were nephrectomized 24 hours prior to bleeding. As a result, the blood plasma subsequently collected was essentially renin-free and substrate enriched. All assay samples were either sonicated for 10 sec at 4°C using an ultrasonics microtip probe ultrasonicator, or solubilized on ice with

0.1% Triton X-100 for 30 minutes with brief (5s) vortexing every 10 minutes. Aliquots (50µl) of each sample were incubated at 37°C for one hour in a constant temperature oscillating water bath with 50µl of renin-free dog substrate containing 8 mg/ml ethylenediamine tetraacetic acid (EDTA) and 200 µl of 2M maleate buffer (pH 6) supplemented with the inhibitors, 0.02% 8-hydroxyquinoline (1µl) and 0.0056% dimercaprol and 3.3% benzyl benzoate in peanut oil (1µl). Under these conditions, the generation and accumulation of angiotensin I is favored by allowing the endogenous renin from the assay samples and the exogenous but compatible dog substrate to react in the presence of reagents which inhibit both plasma converting enzyme and proteolysis by angiotensinases (Cohen et al., 1971). Maleate buffer assures a constant pH at the optimum for rat renin activity, pH 6-6.5 (Sen et al., 1971), during the generation step. EDTA serves as an angiotensinase inhibitor, an angiotensin converting enzyme (ACE) inhibitor (Cohen et al., 1971), and as most recently reported by Lanzillo et al. (1986), a peptidyl dipeptidase (PDP-4) inhibitor. PDP-4 activity was recently unmasked during work with cultured bovine pulmonary artery endothelial cells and while functionally similar to ACE and other peptidyl dipeptidases, is clearly a distinct molecular species with unique properties toward substrates and inhibitors. Both 8-hydroxyquinoline and dimercaptol, preserve angiotensin I by effectively blocking proteolytic destruction. These enzyme inhibitors act by binding various divalent cations thereby inhibiting endogenous enzymes in the plasma suspended substrate (40 ng angiotensinogen /ml plasma) which degrade angiotensin I (Haber et al., 1969). Renin is not a metal dependent enzyme (Boucher et al., 1964) and its activity is apparently unimpaired by the inhibitor mix (Ryan et al., 1968). To correct for any nonspecific activity of the dog substrate itself, a blank substrate tube containing the same amount of maleate buffer, inhibitor mix, and dog substrate as used in the sample tubes was incubated simultaneously with the experimental tubes and served as a substrate control.

The radioimmunoassay is designed to measure renin activity indirectly by quantitation of generated angiotensin I. At the conclusion of the generation step, all incubated samples are quickly buried in an ice bath to stop the enzymatic reaction and 50µl of each incubated sample is transferred to a clean, disposable binding tube (10 X 75mm borosilicate test tube). A fixed amount (100µl, approximately 0.026µCi) of labelled antigen {angiotensin I (5-L-isoleucine) [tyrosyl-$^{125}$I], monoiodinated} tracer is

added to each incubated sample aliquot. The labelled antigen is supplied by New England Nuclear and was synthesized as described by Haber and coworkers, 1969. Briefly, one of four lysine-succinate residues or 160 moles of peptide/mole of poly-L-lysine are substituted in coupling angiotensin I to succinylated poly-L-lysine. This antigen was injected into New Zealand white rabbits and plasma from the animals with the highest antibody binding activity was selected. Angiotensin I was iodinated and isolated by high voltage paper electrophoresis. The separation is probably the result of a change in the pK of tyrosine on iodination. The hydroxyl pK of tyrosine is 10.07, of diiodotyrosine, 6.48 and of iodotyrosine intermediate between these values (Greenstein and Winitz, 1961). Consequently at the pH of electrophoresis (6.5) the native peptide will be less negatively charged than the diiodo derivative, while the monoiodo derivative will have an intermediate charge (Berson and Yallow, 1966). Monoiodotyrosine angiotensin I had an average specific activity of 100mCi/mg as determined by tracer displacement from antibody (Haber et al., 1969). The antiserum, supplied in Tris-acetate buffer with sodium azide (0.005%), is added to all sample tubes (250µl antiserum/sample tube).

Standards are supplied lyophilized and are reconstituted as directed to produce solutions containing 0, 0.1, 0.25, 0.5, 1.0, 2.5, and 5.0 ng/ml of angiotensin I in TRIS-acetate buffer containing bovine serum albumin. The effect of protein concentration on the radioimmunoassay are minimized by the addition of BSA to the standards. This reduces the occurrence of "less than zero standard values" for low samples. A 50µl aliquot of each standard solution is dispensed to borosilicate binding tubes labeled 0 through 7 and the same amount of tracer and antibody as used for the sample tubes is added. A total tube containing TRIS-acetate buffer (0.8ml) and tracer (100µl) is incubated with the other standard tubes to give an estimate of the total amount of tracer available for binding in the assay. The zero standard tube serves as a control for nonspecific binding (i.e. all binding other than $^{125}$I-ANG I to ANG I antibody) and the #1 standard tube serves as a control for specific binding (i.e. binding of $^{125}$I-ANG I to ANG I antibody in the absence of unlabeled ANG I binding).

All sample and standard binding tubes are incubated for a minimum of 18 hours at 4°C, the time required for the radioimmunoassay to reach equilibrium. The basic principle of this procedure is the competition between radioactive and non-radioactive antigen for a fixed number of antibody binding sites

as represented schematically in diagram 1 (Yalow and Berson, 1971).

Labeled Antigen + Specific Antibody → Labeled Antigen
Ag* Ab Antibody Complex
Ag*Ab

(1)

+

Unlabeled Antigen (In standard solutions
Ag or unknown samples)

↓↑

Unlabeled Antigen-Antibody Complex
AgAb

If increasing amounts of non-radioactive antigen (i.e., standards or unknowns) and a fixed amount of tracer are allowed to react with a constant and limiting amount of antibody, a decreasing quantity of radioactive antigen is bound to the antibody.

The separation of bound from free antigen is accomplished by differential adsorption of the free material onto activated charcoal and the concentration of antibody-bound antigen in the supernatant is determined by gamma counting. The relationship of bound to free is expressed as a standard curve from which the values of the unknowns may be obtained by interpolation. A copy of the computer program used to generate these values is included in Appendix I. Renin activity is expressed as ng ANG I generated/h/ml of incubated sample. Renin specific activity is expressed as ng ANG I/h/mg protein in the assayed sample.

**D. Juxtaglomerular cell isolation.**

Male Sprague-Dawley rats (150-250 gm) were killed by cervical dislocation without anesthesia. Both ether and pentobarbital anesthesia have been reported to increase plasma renin activity (Pettinger et al., 1971; Iversen and Andersen, 1983) and it is well documented that anesthetics influence plasma membrane function (Seeman, 1972; Pang et al., 1979; Roth,1980). Since a significant fraction of the renin of the JG cell is localized in the plasma membrane (Nishimura et al., 1980) a local renin releasing effect at the plasma membrane niveau during anesthesia is therefore to be prevented.

The kidneys were removed and immediately placed on ice. The capsule was carefully peeled away to avoid any damage to the underlying cortical tissue and 2-3 cortical slices (0.5mm thick) per side per kidney were excised with a manually operated specially designed kidney microtome. Following excision, the cortical slices were placed in a small preweighed plastic beaker (25ml capacity) containing 15 ml of cold isotonic Krebs-Ringer buffer (KRB) of the following composition (mM): 118 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 25 $NaHCO_3$, 1.2 $KH_2PO_4$, 11.1 glucose; 1.5% bovine serum albumin (BSA), pH 7.4. This buffer, a modified Tyrode's solution (Buhrle et al., 1985), is a calculated ideal isoosmotic buffer for renal cell isolation (Frederiksen et al., 1975). A high K, low Mg modified Krebs-Henseleit buffer (KHB) was also used where indicated. KHB had the following ionic composition (mM): 75 Na, 50 K, 5 Ca, 0.1 Mg, 1 $PO_4$, 1 $HCO_3$, 1 $SO_4$, 123 Cl. A particular problem encountered in this project in working with isolated cells from sodium-depleted rats was a marked loss of cell viability as reported by Khayat and coworkers (1981). This problem was circumvented by adding 1.5% BSA to the isolation medium to serve as a competitive substrate for destructive proteases released from damaged cells and present in the collagenase itself (Waymouth, 1982). Other advantages of BSA supplemented media include a reduction of cellular aggregation (Greig and Jones, 1977) and a tendency to stabilize the digestive enzymes employed (Schlondorff, 1986). Glucose is included in the buffer as a cellular substrate to improve viability of the isolated cells devoid of the normal substrates found in the extracellular fluid (Klein et al., 1981). Unless otherwise stated all tissue was kept on ice at approximately 0-4º C. Low temperatures considerably lessen cell damage and thus improve viability of the disaggregated cells (Schlondorff, 1986; Waymouth, 1982).

The cortical slices were washed in the same buffer, weighed and harvested to yield 6 gm of tissue slices from 7-10 rats. The slices were then transferred to a sterile plastic petri dish prechilled on an ice-plate and minced into pieces (c.1x1x1 $mm^3$) using a single edged razor blade. The minced tissue was washed with 30ml of KRB and suspended in 30 ml of fresh KRB supplemented with 0.1% collagenase (Type III or IV, Sigma), and 100µg/ml DNAse (Type I, Sigma). The digest media was equilibrated for 20 minutes to 37º C before adding the tissue.

Collagenases include a group of about 20 enzymes (Seifter and Harper, 1971) that hydrolyze

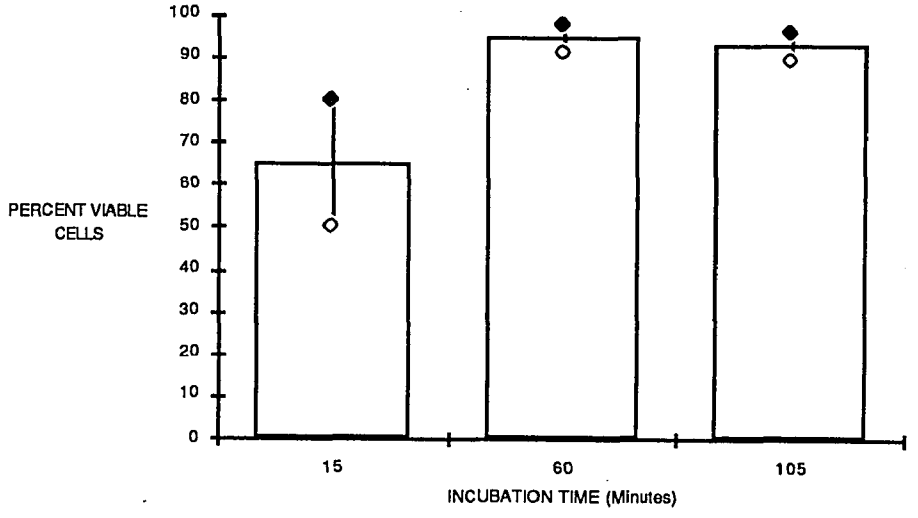
proline-containing peptides, including collagen and gelatin. Our increasing knowledge of specific types of collagen present in the kidney (for epithelia mostly type IV collagen) allows the selection of more specific collagenases (Schlondorff, 1986). Collagenases have been reported to digest portions of the junctional extracellular matrix without altering the general appearance of muscle fibers or causing extensive contraction. The enzyme effectively separates the nerve terminal from the muscle fiber, causing denervation (Bloch et al., 1986). All of these significant properties of collagenase digestion were considered in the selection of this group of enzymes for juxtaglomerular cell isolation.

The minced tissue was then incubated in 30 ml of this enzyme solution for a 15 minute predigestion period at 37º C. In a series of preliminary experiments, this initial tissue digest was found to contain a high percentage of damaged cells (≈35%) as assessed by trypan blue inclusion (one drop of 0.4% trypan blue in 0.9% saline was added to one drop of cell suspension). After five minutes the cells were examined by phase contrast microscopy and those cells that were refractile and excluded the trypan blue dye were considered to be viable cells and were counted with a hemocytometer. All other cells were enumerated as nonviable cells. In view of the potential damage of released proteases on the succeeding cell digests, this initial digest was discarded. The low viability of this preliminary digest was most likely the result of the mincing action on the cells in contact with the razor blade and the subsequent release of destructive proteases from injured cells. By trial and error, a 15 minute predigestion period proved to be adequate to remove most of these surface localized damaged cells. Cells dispersed with proteases also tend to release a hydrated DNA-protein and where a significant number of cells are killed or damaged, this nuclear material forms a gel within which separated cells may be trapped. DNAase prevents or overcomes this aggregation (Price, 1979). Maximal activation of DNAase requires Mg and Ca ions. This technique has been used successfully in conjunction with collagenase for dispersion of kidney cells (Camazine et al., 1976; Price, 1979).

The remaining tissue was resuspended in fresh enzyme media (30 ml) and incubated for two successive 45 minute periods. The release of viable cells (monitored by the exclusion of trypan blue dye), as a function of incubation time is plotted in Figure 5. Cellular viability declined slowly over 105 minutes of collagenase digestion. The average decrease in percent viability was < 10%. This data

**Figure 5.** Release of viable cells as a function of incubation time. The cell digesting media was removed at the indicated times during the digesting period and the minced tissue was immediately resuspended in fresh enzyme-supplemented media and incubated for the remaining periods at 37°C. Aliquots of the cell digest were mixed with trypan blue (an equal volume of 0.4% trypan blue in 0.9% NaCl). The cells were examined aftrer 5 minutes and counted with the aid of a hemocytometer. Nonviable cells were identified by the inability to exclude the trypan blue dye. Percent viable cells = number of viable cells/total number of cells x 100. n = 4.

PERCENT VIABILITY AS DETERMINED BY TRYPAN BLUE EXCLUSION

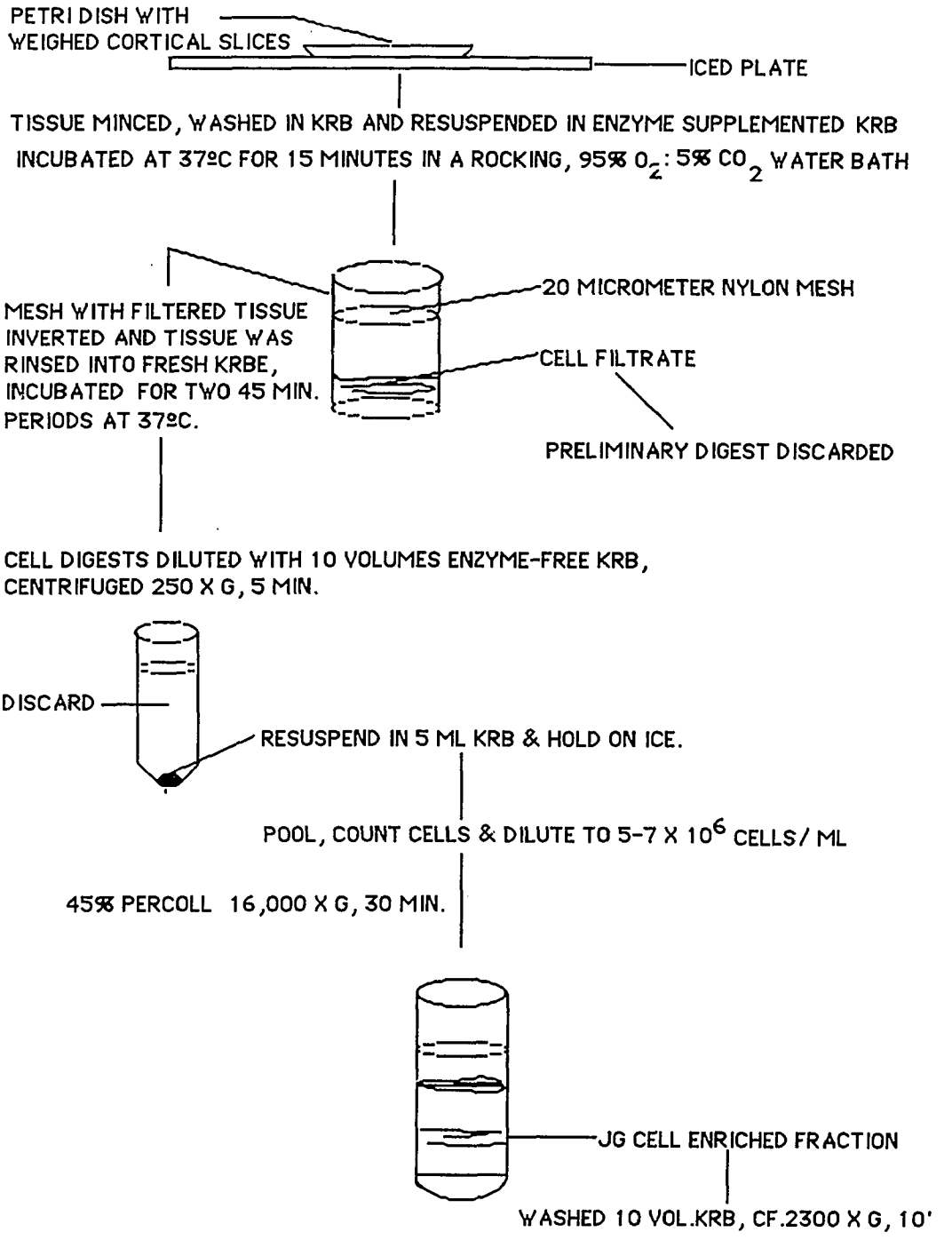


demonstrates that the digesting enzyme activity remained essentially unchanged throughout the period of incubation and suggests the cell accumulation did not inhibit the enzyme within this time period. Schlondorff (1986) has reported that incubation with 0.05% to 0.15% collagenase at 37°C for up to one hour results in minimal cell damage of kidney cells and recommends removing cells at shorter time intervals to maximize viability by decreasing time of exposure to collagenase (Schlondorff, 1986). On the basis of these results, a series of two 45 minute cellular digestion periods were chosen for all subsequent experiments.

The resulting cell digest at the conclusion of each incubation period was poured through a 20μm nylon mesh and the cell filtrate diluted with 10 volumes of enzyme-free KRB and centrifuged at 250 x g for 5 minutes. The speed and time of this initial centrifugation was determined by several trials to be the amount required to just pellet the cells without risking damage. A centrifugal force slightly greater than that generally used was required due to the small size of the JG cells. The time of centrifugation was kept as short as possible within the constraint of not using excessive centrifugal force. This was particularly important for kidney cells which are relatively active cells whose metabolic activity could cause significant alteration to the pH and oxygen content of the medium trapped in the pellet (Elliott, 1979). Undigested tissue, intact glomeruli, tubules, and large cellular aggregates were retained on the mesh and the resulting filtrate contained a mixture of dispersed cells representative of the major cell types of the kidney cortex. The cell pellet was resuspended, using a gentle shaking and swirling motion, in 5 ml of fresh KRB and held on ice. Both 45 minute washed cell digests were pooled, counted with a hemocytometer, and diluted to give a final cell count of 5 - 7 X $10^6$ cells/ ml of suspension media (KRB). This cell isolation procedure is outlined in Figure 6.

The cell suspension was mixed with standard iso-osmotic Percoll (Percoll: Methodology and Application, 1980) to form a range of Percoll solutions from 30% to 60% Percoll and centrifuged at 16,000 x g for 30 minutes in a fixed-angle rotor (Ti 60, Beckman Instruments). This speed and time of centrifugation resulted in a Percoll self-formed gradient optimized for minimal aggregation and maximal band/gradient capacity. The use of 1 x 3.5 inch polycarbonate centrifuge tubes served to minimize the loss of cells due to the wall effect encountered in sedimentation in parallel-walled tubes as they allowed

**Figure 6.** Experimental protocol utilized in the enzymatic dispersion and Percoll density gradient sedimentation of a population of cells enriched in juxtaglomerular cells from rat renal cortex.


PETRI DISH WITH
WEIGHED CORTICAL SLICES
ICED PLATE
TISSUE MINCED, WASHED IN KRB AND RESUSPENDED IN ENZYME SUPPLEMENTED KRB
INCUBATED AT 37ºC FOR 15 MINUTES IN A ROCKING, 95% $O_2$: 5% $CO_2$ WATER BATH
20 MICROMETER NYLON MESH
MESH WITH FILTERED TISSUE
INVERTED AND TISSUE WAS
RINSED INTO FRESH KRBE,
INCUBATED FOR TWO 45 MIN.
PERIODS AT 37ºC.
CELL FILTRATE
PRELIMINARY DIGEST DISCARDED
CELL DIGESTS DILUTED WITH 10 VOLUMES ENZYME-FREE KRB,
CENTRIFUGED 250 X G, 5 MIN.
DISCARD
RESUSPEND IN 5 ML KRB & HOLD ON ICE.
POOL, COUNT CELLS & DILUTE TO 5-7 X $10^6$ CELLS/ ML
45% PERCOLL 16,000 X G, 30 MIN.
JG CELL ENRICHED FRACTION
WASHED 10 VOL.KRB, CF.2300 X G, 10'

separation on gradients removed as far as possible from the center of revolution. Swirling, which tends to cause mixing of different zones in gradients (Pretlow and Pretlow, 1983), was minimized by using the heavy Ti60 fixed-angle rotor and by manually controlling acceleration and deceleration to insure a slow rate of change in angular velocity. Diffusion, electrical charge, degree of hydration, and many of the other factors that influence the sedimentation of some molecules can be ignored in predicting the sedimentation rates of cells (Pretolow and Pretlow, 1983) and therefore did not have to be considered in developing gradient media. The sedimentation of cells in a centrifugal field is described by equation 1 in which "r" is the distance of the cell from the center of revolution; "t" is the time of centrifugation; "a" is the diameter or radius (depending on the value of k) of the cell; "$D_c$" is the density of the cell; "$D_m$" is the density of the gradient at the location of the cell; "w" is the angular velocity (speed of centrifugation); "n" is the viscosity of the gradient at the location of the cell, and "k" is a constant (Pretlow and Pretlow, 1983):

$$dr/dt = a^2(D_c-D_m)\omega^2 r/k\eta \qquad (1)$$

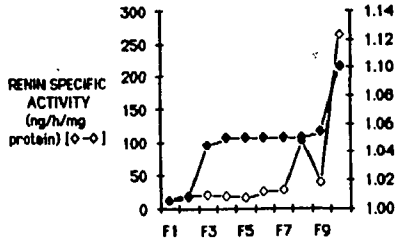
It is important to recognize that both the diameter (a) and density of the cells ($D_c$) are important in determining their velocity of sedimentation. For the separation of most cell mixtures, the optimum experimental design will be such that "a" will be more important than "$D_c$" in dictating its velocity of sedimentation. Usually cells of a single kind are sufficiently heterogeneous from other cell types with respect to density in Percoll to allow a definitive separation. Percoll is a polydisperse colloidal silica coated with polyvinylpyrolidone ( Pertoft et al., 1978). It is completely non-toxic to cells (Pertoft et al., 1977; Kurnick et al., 1979) and has a very low osmotic pressure itself, enabling it to form density gradients which are virtually iso-osmotic throughout (Pertoft and Laurent, 1982). Thus, the velocities of sedimentation for the most dense and least dense cells of one kind tend to differ almost twofold (Pretlow and Pretlow, 1983) often allowing a separation even within one cell type. Dissolving Percoll, BSA or other solutes in the isolation media, will increase "$D_m$" and therefore decrease effective densities ($D_c$-$D_m$). Thus, the velocities of sedimentation of cells with the same diameters but different densities will

become even more heterogeneous with the increased density of the gradient medium. However, the gradient medium chosen should be of sufficient molecular weight to impart the required density without altering the osmolarity of the gradient. Percoll meets all of these requirements and is therefore an effective gradient medium for the iso-osmotic separation of JG cells as demonstrated by Kurtz et al. (1986).

The potential effect of Percoll on renin release from the crude cell suspension was tested and found to have no significant effect within the concentration range utilized in all experiments; i.e. the percent change in total renin activity of the suspension media was <3% from 0 - 60% Percoll. The 45% Percoll gradient was found to be most suitable for the isolation of a JG cell enriched fraction (Figure 7). The resulting gradient formed in situ was calibrated with density marker beads supplied by Pharmacia Fine Chemicals (Upsalla, Sweden) having a density range from 1.016 to 1.139. To localize the JG cells on the gradient, each Percoll fraction following ultracentrifugation was removed and washed free of Percoll with 10 volumes of enzyme-free KRB and centrifugation at 2300 x g for 10 minutes. The resulting supernatant was assayed for lactate dehydrogenase specific activity, a cytosolic marker enzyme and an indicator of cellular damage (Elliott, 1979). This assay was repeated during subsequent washing and incubation of the cells as a means of following cell integrity throughout the isolation and experimental periods.The cell pellet was resuspended in 5 ml KRB and a cell count was done using a hemocytometer and trypan blue exclusion as an additional indicator of cell viability. The remaining cell suspension was sonicated and assayed for renin specific activity, and tubular contamination via the tubular marker enzyme, gamma-glutamyltranspeptidase (Tate and Meister, 1974). In succeeding experiments, a sample of the Percoll fraction determined to have the lowest tubular marker enzyme activity and the highest renin activity and percent viability from these initial tests (Figure 8) was sonicated and reserved for assay of renin and protein, while the remainder of the renin-enriched fraction was washed with KRB (10 volumes of KRB to one volume of cell suspension) and gently pelleted at 2300 x g for 10 minutes. The washed cell pellet from the F3 fraction of the Percoll gradient was gently resuspended in fresh KRB and used in all subsequent whole cell experiments.

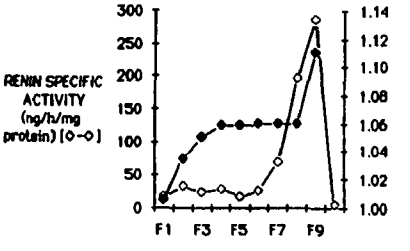
**Figure 7.** Distribution profile of JG cells on Percoll gradients using renin specific activity (ng ANG I/h/mg protein) to localize the renin-containing cells and colored density marker beads (Parmacia) to calibrate the gradient. The renal cortical cells were isolated as outlined in Figure 6 and mixed with solutions of Percoll varying from 30% to 60% of stock iso-osmotic Percoll in 0.15 M NaCl as described in the text. Calibration tubes containing density marker beads were centrifuged with each cell suspension. All tubes were centrifuged at 16,000 x g for 30 minutes at 4°C in a Beckman Ti 60 rotor. The JG cells band at 1.06 g/ml in the 45% Percoll gradient. n = 10.

30% PERCOLL GRADIENT PROFILE



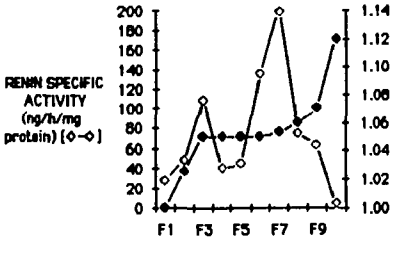

FRACTION NUMBER, TOP TO BOTTOM
(2ml/fraction)

35% PERCOLL GRADIENT PROFILE



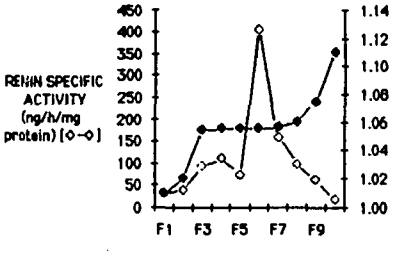

FRACTION NUMBER, TOP TO BOTTOM
(2ml/fraction)

40% PERCOLL GRADIENT PROFILE



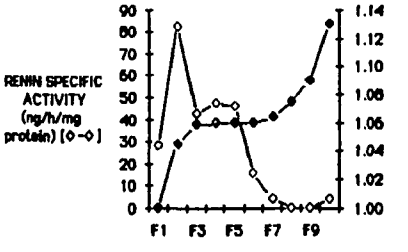

FRACTION NUMBER, TOP TO BOTTOM
(2ml/fraction)

45% PERCOLL GRADIENT PROFILE



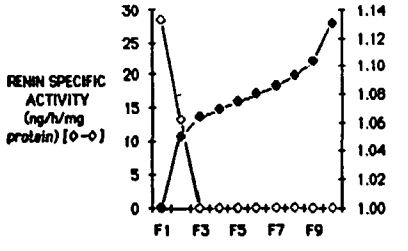

FRACTION NUMBER, TOP TO BOTTOM
(2ml/fraction)

50% PERCOLL GRADIENT PROFILE



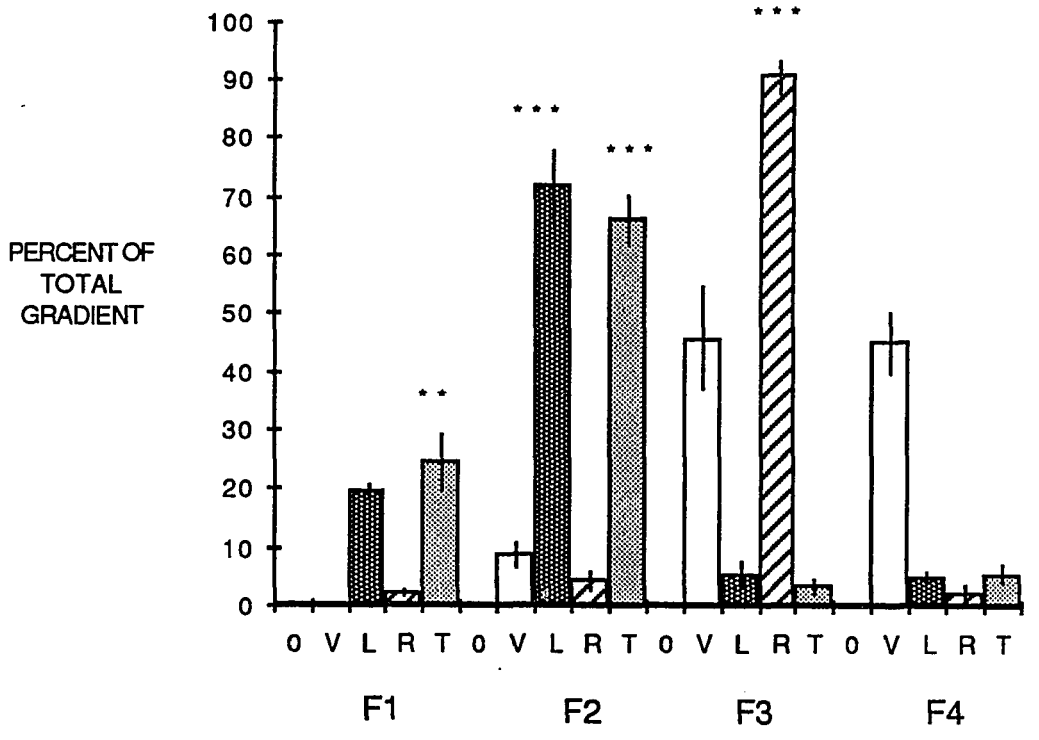

FRACTION NUMBER, TOP TO BOTTOM
(2ml/fraction)

60% PERCOLL GRADIENT PROFILE




FRACTION NUMBER, TOP TO BOTTOM
(2ml/fraction)

**Figure 8.** Characterization of the 45% Percoll gradient separation of JG cells. Viability with trypan blue exclusion (V) and lactate dehydrogenase activity (L), renin distribution (R) and tubular cell distribution with gamma-glutamyl transpeptidase activity (T). F1: top layer of the gradient to the visible damaged cell layer. F2: damaged cell layer. F3: area below F2 to the visible RBC band. F4: RBC band to the bottom of the gradient centrifuge tube. **($p < 01$), ***($p < 001$). $n = 5$.


PERCENT OF TOTAL GRADIENT
100
90
80
70
60
50
40
30
20
10
0
* * *
* * *
* * *
* *
O V L R T O V L R T O V L R T O V L R T
F1
F2
F3
F4

E. Test for the physiological responsiveness of enriched JG cell suspensions.

To demonstrate that the JG cells isolated by this method were physiologically responsive, the purified cell suspensions were incubated for one hour at 37º C in an oscillating water bath gased with 95% $O_2$: 5% $CO_2$ in the presence or absence of 20µl forskolin (2 x $10^{-5}$ M), 20µl cAMP (1 x $10^{-4}$ M), and 10µl ionomycin (2 x $10^{-5}$ M). These agents are known to alter renin secretion in several physiological preparations (Fray & Park, 1986; Park et al., 1986; Baumbach and Leyssac, 1977). At the end of the incubation time, the cells were removed by centrifugation at 2300 x g for 10 minutes and the resulting supernatant was assayed for renin specific activity (ng/h/mg protein) as described below.

F. Renal cortical plasma membrane isolation.

Two approaches, differing in their starting material, were used to purify JG cell plasma membranes. One method began with the foregoing JG cell-enriched suspension and the second began with a renal cortical slice preparation. Since juxtaglomerular cells represent only a small fraction (<0.03%) of all kidney cells (Taugner et al., 1981); the former method should result in less contamination by other renal cortical cell types. As it turned out, the two methods yielded comparable results and the first method did not appear to enhance significantly the isolation of JG plasma membranes. Therefore in all subsequent experiments with isolated PMV, the PMV were purified from a renal cortical slice preparation.

In the first method (Figure 9), a modification of the procedure used by Prpic and coworkers (1984) in the isolation of rat liver plasma membrane vesicles (PMVs), the Percoll-derived suspension of intact renal cortical cells enriched in JG cells was homogenized in a Waring blender for 20 seconds at low speed and the resulting homogenate was diluted to give a 6% (w/v) homogenate in KRB media supplemented with 50mM $K^+$, pH 7.4 or in the same KRB used to isolate the JG cell enriched fraction. This diluted homogenate was centrifuged for 10 min at 4º C and 1400 x g in a Sorvall RC-3 refrigerated centrifuge ($r_{av}$ 14 cm) and the resulting pellet brought to a 6% (w/v) suspension with fresh iso-osmotic

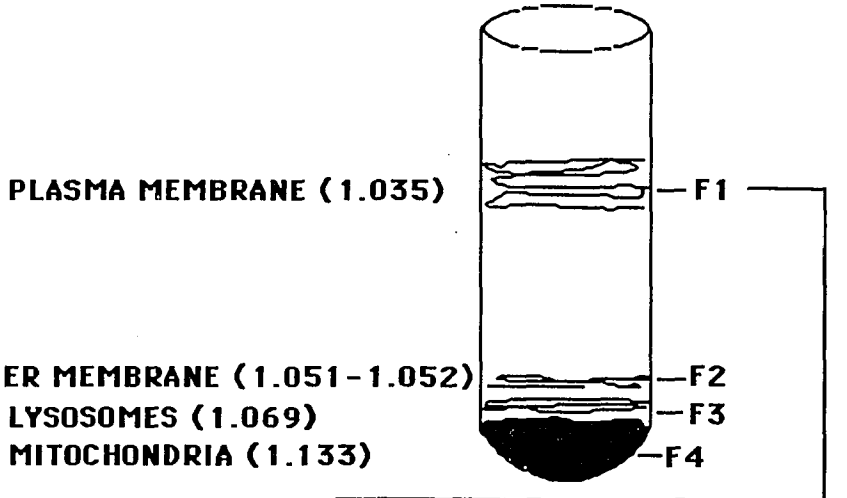
**Figure 9.** Flow diagram for the isolation of plasma membrane vesicles from isolated rat JG cells or renal cortical slices. Iso-osmotic media, either KRB, KHB or 0.3M sucrose in 20 mM Tris-HCl, was used in the purification. The total membrane load for the Percoll gradient was about 1.5 mg protein/ 0.5 ml of gradient material. Subcellular particles and membranes were identified with appropriate marker enzyme analysis of each Percoll fraction. The gradient was calibrated with density marker beads (Pharmacia).

HOMOGENIZATION OF ISOLATED JG CELLS OR RENAL CORTICAL SLICES IN ISO-OSMOTIC MEDIA & CENTRIFUGATION AT 1400g, 10'

SN1
(Discard)

P1

RESUSPEND IN PERCOLL (12% FINAL PERCOLL CONC.) AND CENTRIFUGE 28,195g, 20'




REMOVE F1, WASH IN 5 VOLUMES ISOTONIC MEDIA AND CENTRIFUGE AT 123,400 g, 20 MINUTES

SN2

P2

RESUSPEND IN 20 MM TRIS-HCL (5ML) AND CENTRIFUGE AT 123,400 g, 20 MINUTES

F-SN

F-P

media (KRB, KHB or 0.3 M sucrose in 20 mM TRis-HCl, ph 7.4). Cellular aggregation has been shown to be a function of the time during which cells remain at a high concentration (Pretlow and Pretlow, 1983). Therefore, aggregation was reduced by working rapidly and diluting cells to a low concentration as soon as possible following disaggregation. A volume of this cell suspension was mixed with Percoll to yield a 12% final Percoll concentration using a large 30ml syringe fitted with a #18 gauge needle. Centrifugation at 28,195 x g for 20 minutes at 4ºC in a Beckman L-59 Ultra-centrifuge using a Ti 60 fixed-angle rətor resulted in a Percoll self-formed gradient in which a distinctly white plasma membrane band was visible near the top of the centrifuge tube and well removed from the other visible bands of the gradient. The gradient was calibrated with density marker beads (Figure 9). The total density range covered by the marker beads in isotonic salt is 1.035 - 1.136 g/ml. The exact density of each type of bead is specific for each manufactured lot, but the actual buoyant density of the beads has been shown to vary very little with ionic strength. The accuracy of the beads was further checked and confirmed by an appropriate marker enzyme analysis of each resulting Percoll fraction. Four distinct fractions were revealed as illustrated in Figure 9. These were removed and washed in 5 volumes of iso-osmotic medium and centrifuged at 123,400 x g for 20 min at 4º C to separate the Percoll and further purify the fraction. The partially purified soft subcellular material was ultimately vacuumed from the surface of the firm Percoll pellet and resuspended in 5 ml of distilled water by forcefully passing the material several times through a # 26 gauge needle. This technique has been widely used to lyse membrane vesicles and to form inside-out vesicles spontaneously (Steck et al., 1971).

In the preparation of plasma membrane vesicles without prior JG cell isolation, the rats were killed without anesthesia via cervical dislocation and rapid arterial bleeding. Both kidneys were removed and placed on ice and prepared as diagramed in Figure 9. The capsule was carefully removed to avoid damage to the juxtaposed cortical layer immediately beneath the renal capsule. In whole kidney autoradiographs, the highest concentration of renin was observed in the outermost 2mm section of the renal cortex (Brown et al., 1965). Accordingly, 2-4 cortical slices (approximately 0.5mm thick) per side per kidney were excised with a manually operated specially designed kidney microtome. Ten to twelve rat kidneys were routinely used for this cortical slice preparation. The slices were washed in ice cold KRB

or 0.3 M sucrose in 20 mM TRis-HCl (pH 7.4), weighed and adjusted to a total wet weight of 6 g, and homogenized either in a Waring blender as described above or by hand with 6-8 passes of a Thomas 3431-G40 tissue homogenizer with a teflon plunger (clearance ca. 0.15- 0.23mm). The resulting plasma membranes were purified using the same technique described for the JG cell homogenate.

**G. Characterization of purified renal cortical plasma membrane vesicles.**

The protein content of the Percoll-derived whole cell fractions, membranes and other subcellular material was measured according to the method of Bradford (1976) as modified by Chiappelli et al. (1979), using bovine serum albumin (Fraction V, Lot #401, Sigma) as the standard. This Coomassie brilliant blue G250 dye protein assay depends upon the conversion of the dye from the leuko form to an intensely blue color (Reisner et al., 1975) when the dye anion interacts with the $NH_3$ groups on proteins (Fazekas de St. Groth et al., 1963). This interaction appears to be a function of the H ion concentration with dilute perchloric acid and HCl the most effective of the acids tested for use in the assay of proteins in solution (Sedmak and Grossberg, 1977). Not all $NH_3$ groups interact with the G250 dye identically and not all proteins have the same proportion of $NH_3$ groups. Thus the amount of color development in the assay varies with the proteins present (Sedmak and Grossberg, 1977). Proteins in crude tissue extracts are often determined by the sensitive Lowry method (Lowry et al., 1951), but complications often arise due to nonprotein materials that cross-react with the Folin phenol reagent. In such tissues, one must perform a preliminary precipitation of proteins with trichloroacetic acid (Hoffman et al., 1976; Zinder et al., 1976; Nikodijevic et al., 1976). The Bradford assay is reported to be insensitive to such phenolic compounds and to give results with tissue extracts identical to those obtained with the TCA-Lowry procedure (Pollard et al., 1978). Another advantage of this assay is that free amino acids and very small peptides do not react, making it possible to determine protein concentrations of undialyzed crude cell extracts. Both the Lowry and the fluorescamine assays are strongly influenced by free amino acids (Sedmak & Grossberg, 1977). The Bradford assay can detect less than one microgram of protein, as compared to 5-10μg by the Lowry (Schattiner & Weissman, 1973). All samples were read at an

absorbance of 595nm within 5-45 minutes of incubation with the reagent. The dye binding process is virtually complete within two minutes and the resulting color is stable for approximately one hour (Bradford, 1976).

Alkaline phosphatase activity was determined at 37°C by the method of Lowry et al. (1954) and with slight modifications by Amador et al. (1963). Acid phosphatase activity was determined by the method of Andersch and Szczpinski (1947) and later modified by Sommer (1954) and Jacobsson (1960). Acid phosphatase rapidly loses activity at room temperature (Sigma Technical Bulletin No. 104, 1982). Therefore, all samples were kept frozen until assayed and were then maintained on ice prior to their incubation at 37°C. The relative specific enzymatic activity is defined as the ratio between the enzyme specific activity of the purified fractions and the specific activity of the homogenate. The level of purification of the plasma membrane fraction was assessed as the alkaline to acid phosphatase ratio of the final pellet as compared to that of the original homogenate.

For the membrane sidedness studies, isolated PMVs were resuspended in 1 ml Concanavalin A running buffer (CRB) which consisted of 0.02M Tris-HCl, 0.5M NaCl, 0.001M $MnCl_2$ and 0.001M $CaCl_2$, pH < 5 to preserve the binding activity of the Concanavalin A molecule. Six 1 ml columns were prepared using 1 ml hypodermic syringes (volume 0.5 ml). The column was washed with 5 column volumes of Concanavalin A starting buffer (CSB) containing 0.02M Tris-HCl and 0.5M NaCl, pH 7.4. The PMV sample (25µl) was added to the column and allowed to run into the gel. The column was washed with 5 volumes of CSB to remove unbound vesicles prior to elution. This fraction was solubilized with Triton X-100 (0.1%) and assayed for alkaline phosphatase activity. Unretarded PMVs were considered to be of inside-out orientation. The bound PMVs were eluted with ∂-methylmannoside (0.5M) in CSB. The elution volume (2 ml) was allowed to run into the gel and the column was closed for 60 minutes to allow complete dissociation of any bound PMV to occur. The resulting eluent was solubilized with Triton X-100 (0.1%) and assayed for alkaline phosphatase activity. Bound PMVs were considered to be of right-side-out orientation.

The foregoing Concanavalin A column did not allow for the determination of the percent leaky

vesicles in the PMV population. Therefore, a second assay, an ATPase assay, which permitted determination of both orientation and integrity was included in the characterization of the vesicles. In this ATPase assay, isolated PMVs were suspended in 200-400µl of suspension medium containing 100mM KCl, 100mM sucrose and 2mM Tris-HEPES (pH 7.4) and samples with 50-100µg protein in 10µl suspension medium were preincubated for 20 minutes at 37°C with either 800µl control medium (100mM KCl, 100mM sucrose, and 2mM Tris-HEPES, pH 7.4) or 800µl SDS medium (100mM KCl, 100mM sucrose, 2mM Tris-HEPES, and 10-60µg SDS, pH 7.4). To start the reaction for the ATPase assays, 40µl aliquots of the preincubated control samples or the sodium dodecyl sulfate-activated samples, were added to 750µl of temperature equilibrated (37°C) assay medium containing 150mM NaCl, 10mM KCl, 8mM $Na_2ATP$, 8mM $MgCl_2$, 1mM $H_4EDTA$, 30mM Tris-HCl and [$\gamma$-$^{32}P$]ATP in tracer amounts with or without 1mM Ouabain. The pH of these solutions was adjusted to 7.4 by the addition of Tris buffer. After incubation for 20 minutes at 37°C the reaction was stopped by addition of 400µl of a 10% activated charcoal suspension in 1M HCl and by placement on ice. Charcoal was used to absorb non-cleaved ATP (Bais, 1975) and the liberated $^{32}P$ was separated by centrifugation for 5 minutes at 5000 x g in a refrigerated centrifuge. The radioactivity of the supernatants was determined by liquid scintillation counting (Boumendil-Podevin & Podevin, 1983).

Electron microscopy was used for visual quantitation of both the isolated intact cells and PMVs. The samples were fixed in gluteraldehyde$^{2\%}$ Para$^{1\%}$ in 0.1M phosphate, dehydrated in alcohols through propylene oxide, and embedded in LX 112 (Epon) obtained from Ladd Industries (Vermont). Ultrathin sections, stained with Reynolds lead citrate and urinol acetate$^{aqueous}$, were examined with a JEOl 100S electron microscope operating at 60 kv. The magnifications ranged from 75,000 to 125,000 times for purified membranes and 30,000 times for whole cells.

## H. Measurement of trypsin activation of plasma membrane-bound renin.

Immediately before use, bovine trypsin (type III, Sigma) was dissolved in 0.1M sodium acetate buffer (SAB) containing 0.2M $CaCl_2$, pH 4.5. This stock solution (100 mg trypsin/ml SAB) was

diluted with the same SAB to give the following final concentrations in (mg/ml): 10, 8, 6, 4, 2, 0 (SAB without added trypsin). The diluted trypsin solutions were added to measured aliquots of washed plasma membrane vesicles in suspension and buffered at pH 7.4 in a ratio of 1:10 (Barrett et al, 1982) and incubated on ice for 20 minutes. To terminate the action of trypsin, soybean trypsin inhibitor (SBTI), in a stock solution (200 mg SBTI/ml 20mM Tris-HCl, pH 7.4), was diluted to a final concentration of 2mg SBTI/mg trypsin, and added also in a ratio of 1:10. As controls, both the trypsin buffer and the SBTI buffer were incubated without membrane samples, and isolated PMVs were incubated in the SAB and Tris-HCl buffer without trypsin or SBTI. The samples and controls were centrifuged at 125,400 x g for 30 min at 0 time and after 20 min incubation on ice and the renin activity of the resulting supernatant was measured.

**I. Determination of renin specific activity of isolated plasma pembrane vesicles from hypophysectomized and sodium-deprived rats.**

To determine the renin specific activity of washed plasma membrane vesicles of hypophysectomized (Hx) and Na-deprived rats, kidneys were isolated and perfused with a blood- and renin substrate-free, chemically defined medium by a procedure developed by Nishiitsutsuji-Uwo et al. (1967) and modified by Fray (1976). Briefly, the rats were anesthetized with Na pento barbitol (40 mg $Kg^{-1}$ body weight, I.P.) and placed on a plexiglas stand. The right kidney was exposed through a midline abdominal incision with a right lateral extension. The right renal artery was cannulated and all visible branches on and around the cannulated artery were tied and cut between the sutures. The kidney thus isolated was transferred to a perfusion reservoir in a specially designed plexiglass temperature-controlled (37ºC) perfusion chamber and perfused at 100 mmHg mean pressure (pulsatile) using a peristaltic flow inducer (Watson Marlow, Ltd., Cornwall, England). The pump frequency was maintained at 120 pulsations/ minute. The renal vein was not cannulated and the perfusate leaving the renal vein was allowed to bathe the surface of the kidney. The perfusion medium used for all cell isolations was a high potassium (50mM) modified Krebs-Henseleit bicarbonate solution (KHB). The ionic concentrations of the perfusion

medium were (mM): 75 Na, 50 K, 5 Ca, 0.1 Mg, 1 $PO_4$, 1 $HCO_3$, 1 $SO_4$, 123 Cl. The medium was gased with 95% $O_2$:5% $CO_2$ and supplemented with 10mM glucose and the converting enzyme, angiotensinase inhibitors dimercaprol (BAL) and 8-hydroxyquinoline (8% final concentration). The temperature of the medium leaving the renal vein was measured by a thermoprobe placed just beneath the suspended kidney and was controlled at 37º C. Prior to the removal of the organ, the kidney was perfused for an initial 15 minute equilibration period. During this time stabilization of the perfusate flow and renin secretion were established (Fray, 1980). For a subsequent 15 minute experimental period the kidney was perfused either with normal medium, medium containing 2.83 μM isoproterenol, or 0 Ca medium. After this perfusion period a 1 ml sample of the perfusate was collected and frozen for later determination of renin activity. The kidney was then removed and cortical slices were excised and PMVs were purified as described above. The renal tissue renin activity as well as the renin activity of the final plasma membrane fractions, were solubilized in 0.1% Triton X-100 and measured. Kidneys from normal rats were isolated and perfused in the same manner and the renin activity of the renal tissue and the purified plasma membrane fractions were measured as controls. To compensate for the differences in kidney weight of hypophysectomized verses Na-deprived and control rats, renin activity was determined per gram of cortex homogenized.

**J. <u>Determination of the effects of ions and calmodulin on renin release from isolated plasma membrane vesicles</u>.**

In each experiment of this series, the JG cell-enriched PMVs were washed and pelleted at 123,400 x g for 20 minutes. The plasma membrane pellets were then resuspended in 3 ml of the ion-supplemented experimental media (0mM, 10mM, and/or 100mM of each ion tested in a low bicarbonate (6 mM) KRB) and immediately divided into three 1ml aliquots. One sample was centrifuged without incubation and the resulting supernatants and pellets were rapidly frozen for assay at a later period to represent the zero incubation time. The remaining two aliquots were incubated at 37º C in an oscillating water bath (120 cycles/min) for either 30 or 60 minutes and centrifuged and stored as the first samples.

In this manner, both dose response and time response curves were determined. The ions investigated incuded: Ca, Ba, Mg, Mn, Na, and Cl. Control incubations consisting of suspension medium with/ without plasma membrane vesicles and with/ without added ions, were routinely included in all experiments. To allow statistical comparisons between individual experiments, the renin specific activity (ng ANG I/h/mg protein in the media) was expressed as percent of control values to correct for variations in baseline renin activity among individual experiments.

The approach used in the study of the effect of K on renin release from isolated PMV (>86% IOV) different from that used in the investigation of the other ions. For K, the dose response curve was developed with the following range of KCl concentrations (mM): 0, 35, 70, 140, 280, 560. All solutions were made up in 20 mM Tris-HCl, pH 7.4, and the PMV were washed and equilibrated in 20 mM Tris-HCl instead of distilled water. All samples were resuspended in the appropriate concentration of experimental media and aincubated for 30 minutes on ice, after which the PMV were removed by centrifugation at 123, 400 x g for 20 minutes. The resulting supernatant (the extravesicular compartment) was assayed for renin and protein as described.

To determine if the presence or absence of Ca would influence the effect of K on renin release from isolated PMV, the same K dose response experiment was repeated with 20 mM Tris-HCl (control), or with 20 mM Tris-HCl supplemented with 2 mM $CaCl_2$ or 1 mM EGTA. All samples were treated exactly as described above for the K dose response series.

To investigate the effects of several Ca-related factors on renin release, isolated PMVs were suspended in 20mM Tris-HCl, pH 7.4 (control), and 20 mM Tris-HCl supplemented with 2mM $CaCl_2$ or 2mM EGTA or 1μg calmodulin, or 2 mM EGTA in combination with 1 μg/ml calmodulin/ ml PMV suspension, and incubated at 37º C for 60 min. The incubated samples were centrifuged at 123,400 x g for 20 min. at 4º C in a Beckman Ti 50 rotor. The resulting plasma membrane pellet was sonicated and assayed for renin activity as previously described. These experiments did not enable us to distinguish between renin released into the intravesicular compartment or into the medium (extravesicular compartment). Thus, additional experiments were conducted to determine quantitatively renin in the extravesi-

cular, membrane and intravesicular compartments. In this latter series of experiments, the PMVs were incubated for 60 minutes at 37ºC. with 20mM Tris-HCl, 2mM $CaCl_2$, 2mM EGTA (Figure 22), or 5mM $CaCl_2$ or 5 mM EGTA in 50mM K supplemented Tris-HCl (20 mM), pH 7.4 (Figure 23). Following incubation, the vesicle suspension was centrifuged at 123,400 x g for 20 min. at 4ºC and the resulting supernatant (the extravesicular compartment) was removed, the pH was verified, and the sample was frozen for later determination of renin and protein. The pellet was resuspended in distilled water, syringed several times using a 26 gauge needle to lyse the membrane vesicles and recentrifuged at 123,400 x g for 20 min at 4º C. The pH of the final supernatant (the intravesicular compartment) was determined and this fraction was also frozen for later determination of renin and protein. The pellet (the membrane compartment) was resuspended in distilled water, sonicated and assayed for renin and protein as previously described.

**K. Isolation of plasma membranes from selected extrarenal organs to measure the occurrance of extrarenal renin.**

To investigate the possibility that renin may be found in extrarenal plasma membrane compartments, homogenates were prepared as described for renal cortex with the exception that the entire organ was homogenized and the resulting whole organ homogenate was used to isolate both renal and extrarenal plasma membranes. In addition to the whole kidney, the rat organs studied included brain, heart, spleen, liver, lung and skeletal muscle.

**L. Biometric analyses.**

All data show the mean ± SEM. Statistical comparisons were made by unpaired "t" test to compare a control sample to an experimental sample derived from pooled initial cell suspensions from several rats or pooled plasma membrane suspensions from several rats and all incubated for the same length of time and under the same conditions. Differences were considered significant when $p < 0.05$.

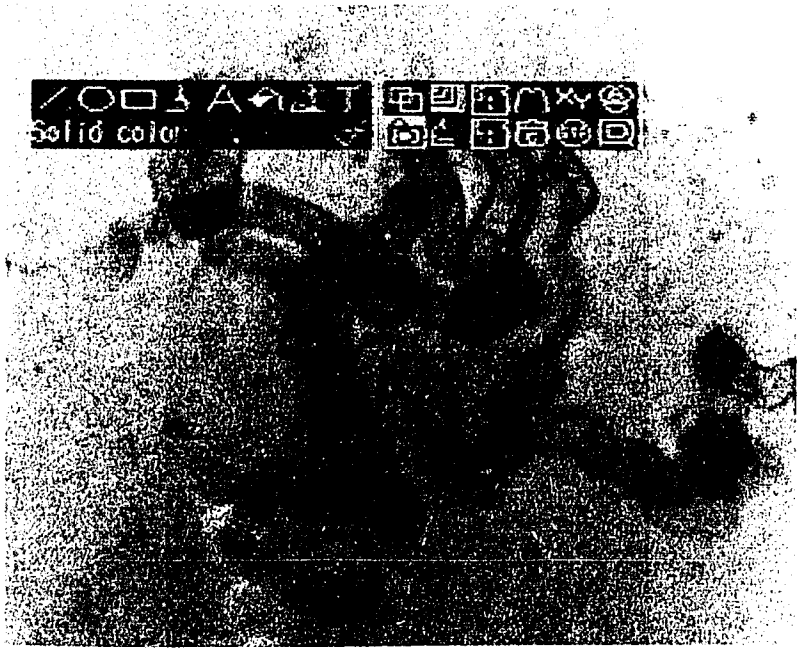
# RESULTS

## A. Renal cortical cell isolation enriched with JG cells.

The total collagenase digestion period resulted in a mixture of partially disaggregated tissue and disaggregated cells. This mixture was separated by pouring the sample through a 20 μm nylon mesh. The material retained by the 20μm mesh consisted of connective tissue, individual tubules and glomeruli (Figure 10). Isolated cells and cell debris passed the sieve. The cellular debris was partially removed by washing in an excess volume of KRB. The final yield was 28.3 x $10^6$ cells per gram of cortex ( n = 6). This represents a 57-fold greater initial yield than that reported by Khayat et al., (1981); a 43-fold greater yield than that reported by Rightsel et al., (1982); and a 4-fold greater yield than that reported by Kurtz and coworkers (1987).

When the disaggregated and washed rat renal cortical cells were separated by ultracentrifugation according to the methodology outlined in Figure 6, greater than 90% of the renin-releasing cells were found in fractions F6-F8 from the top of the 45% Percoll gradient column (Figure 7). Examining the density and renin specific activity profiles of a range of Percoll gradients from 30% Percoll to 60% Percoll (Figure 7) confirmed the selection of the 45% Percoll as the most ideal gradient for the separation of JG cells from rat renal cortical cell digests. Several immunocytochemical studies have confirmed that the JG cell is the site of renin synthesis within the kidney (Lacasse et al., 1985; Faraggiana et al., 1982; Taugner et al., 1979). While it is conceivable that there may be other intrarenal sites of renin synthedid, definitive evidence of this is lacking. It is therefore assumed that renin-secreting cells are JG cells. In subsequent experiments the 10 fractions illustrated in Figure 7 were pooled to yield four fractions as illustrated in Figure 11. Of these four fractions, F3 (d. 1.05 - 1.06 g/ml) consistently yielded a greater than 2,000-fold purification of renin specific activity over the amount measured for the cell suspension applied to the gradient. The buoyancy of these cells in Percoll is due in part to the presence of secretory/storage vacuoles, visible within their cytoplasm under electron microscopy (Figure 12). This cell resembles that reported by Kurtz et al. (1986a) using a similar Percoll gradient to purify JG cells. These

**Figure 10.** Material retained on 20μm mesh. (A) Intact tubules (B) Glomeruli with/without attached vasculature.



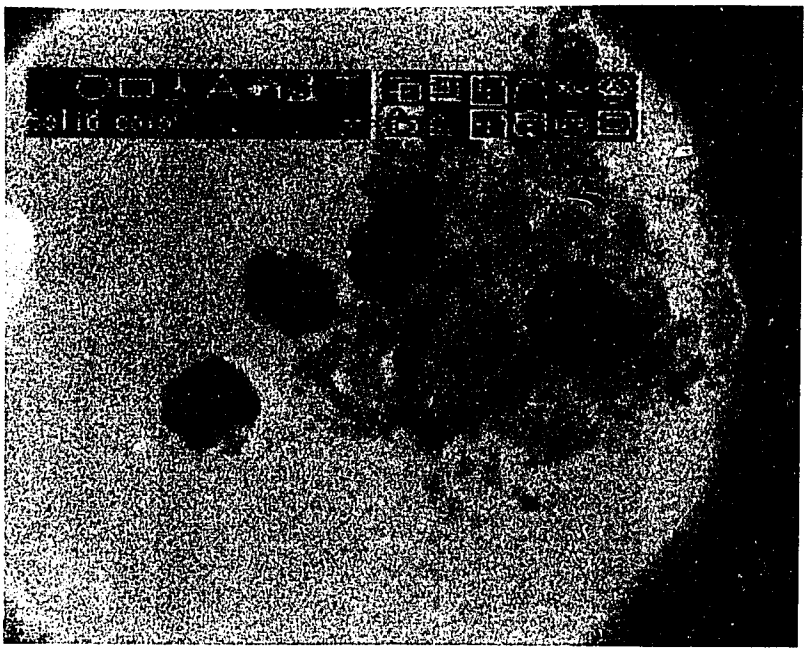


**Figure 11.** Separation of renin-containing cells on a 45% Percoll gradient. Centrifugation at 16,000 x g for 30 minutes. Renin specific activity of the applied cell suspension was 0.02 ng ANG I/h/mg protein. The gradient was calibrated with density marker beads supplied by Pharmacia. n = 5.

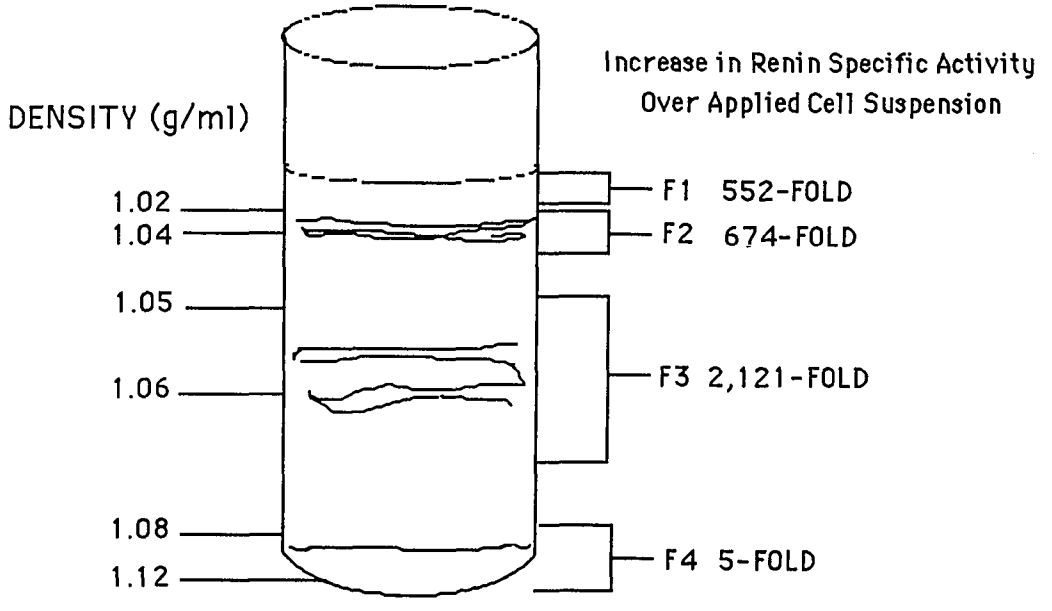
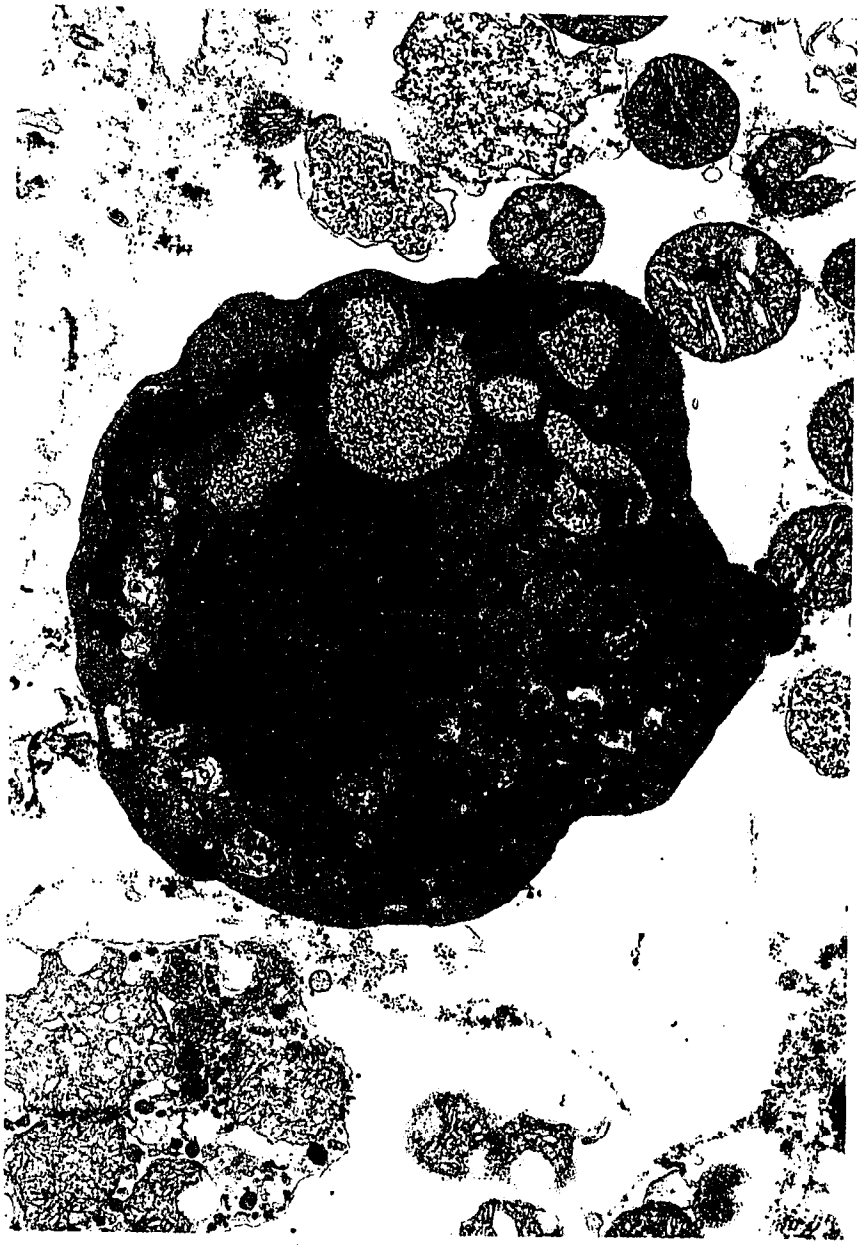

DENSITY (g/ml)
Increase in Renin Specific Activity
Over Applied Cell Suspension
1.02
1.04
1.05
1.06
1.08
1.12
F1 552-FOLD
F2 674-FOLD
F3 2,121-FOLD
F4 5-FOLD

**Figure 12.** Electron micrograph of a typical cell obtained from the 45% Percoll gradient, F3 (density 1.06 g/ml). Note the presence of irregularly shaped granules of varied density and size, some of which have an indistinct membrane border and appear to be dissolving into the cytosolic space. Several membrane-bound vacuoles with electron dense material are also evident and have been reported to be prominant in renin-secreting cells and to contain renin (Lacasse et al., 1985). Magnification is 30,000 X.



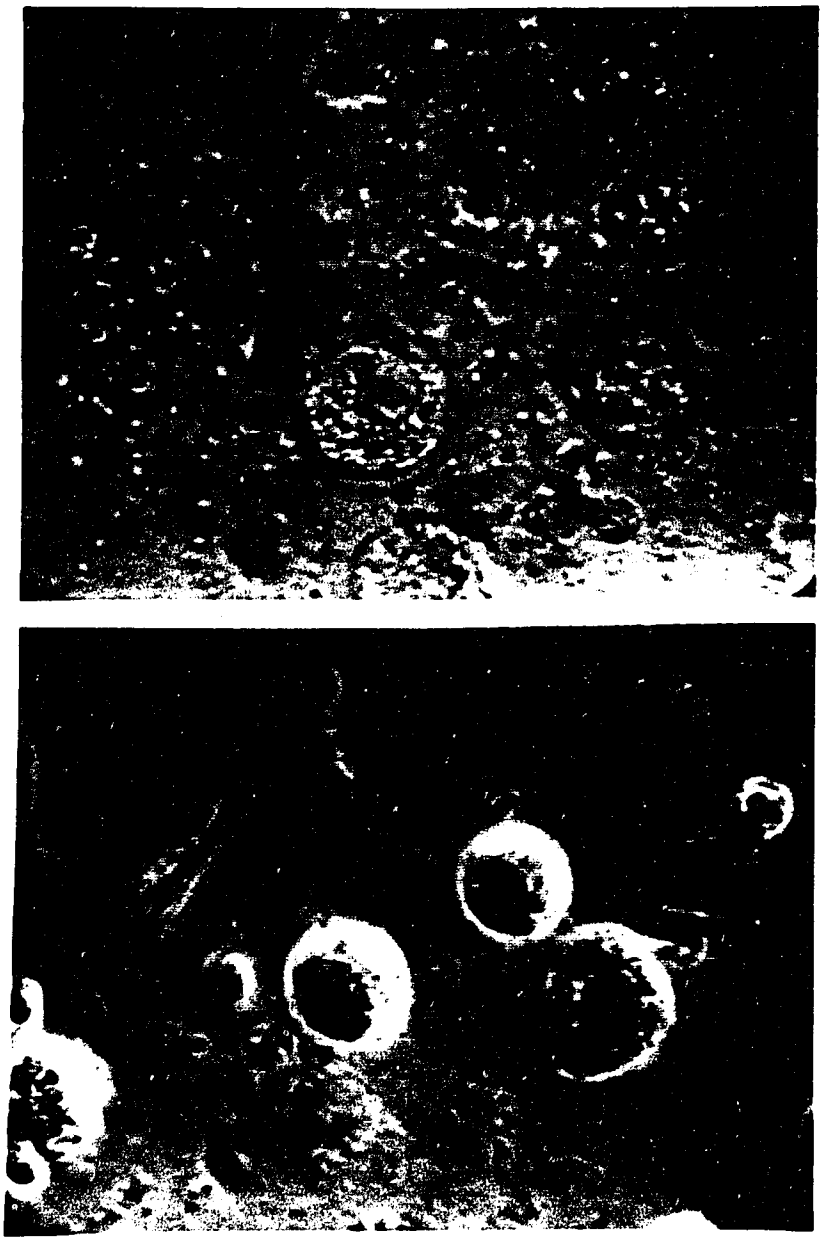
vacuoles were most evident in the cells banding at 1.05 g/ml and were least obvious in cells banding at 1.07-1.08 g/ml. A similar inverse relationship between secretory/storage vacuoles and buoyancy has been reported for isolated rat mammary gland cell separation in Percoll (Raber and D'Ambrosio, 1986). No difference was observed in cell viability between these latter cells and the cells banding at 1.05 - 1.06 g/ml. Viability after sedimentation, as indicated by the low LDH activity of the media, ranged from 87% to 97% in the F3 and F4 fractions from all five trials (Figure 8). Dead or degenerating cells were consistently found in the F2 fraction (density 1.04 g/ml) along with cell debris (Figure 11). Phase contrast photomicrographs of cells obtained from the F3 fraction revealed relatively small cells (5 to 10μm in diameter) with numerous granules (Figure 13). A majority of the cells (>95%) were highly refractile when visualized with phase-contrast optics, although it was not possible to differentiate renin-containing from non-renin-containing cells by this technique.

Electron micrographs of the same fractions clearly demonstrate the absence of a brush border or distinct microvilli suggesting that these cells are not proximal tubule cells (Figure12). Brush borders from renal tissue are derived exclusively from the plasma membrane fragments of the proximal convoluted tubule cell (Wilfong and Neville, 1970). This morphological feature is considered to be characteristic of the proximal tubule cell, reflective of its reabsorptive function, since microvilli greatly increase the luminal absorptive surface area of the cell (George and Kinny, 1973). Granules as well as several large vacuoles containing electron-dense material are visible in the cytoplasm. Numerous mitochondria and a large prominant nucleus are also clearly evident within the cell (Figure 12). These characteristics suggest an actively secreting cell and are consistent with those described for JG cells (Kurtz et al., 1986; Barajas, 1985; Hartroft, 1961).

**B. <u>Physiological responsiveness of purified juxtaglomerular cell suspension</u>.**

Forskolin and cAMP are known to stimulate renin secretion in the isolated rat kidney (Fray and Park, 1986); rat renal cortical slices (Henrich and Campbell, 1986) and isolated JG cells (Kurtz et al., 1984). Renin secretion has also been shown to be inversely related to intracellular Ca concentration

**Figure 13.** Phase contrast photomicrographs of juxtaglomerular cells from Fraction 3 of the 45% Percoll gradient. Centrifugation at 16,000 x g for 30 minutes at 4°C in a Ti 60 rotor. Fraction density is 1.06 g/ml. Images are polaroid photographs reproduced from a Sony CCD XC 37 video screen. Magnification is 1000 X.



(Park et al., 1986). Thus, the enriched JG cell suspension was incubated for one hour at 37°C in the absence (control) or presence of forskolin ($10^{-5}$M) or cAMP ($10^{-4}$M), known stimulators of renin secretion, or ionomycin ($2 \times 10^{-5}$M) a known inhibitor of renin secretion by raising cytosolic Ca. These experiments are iancluded in this study to serve as a Percoll-derived whole cell model in the characterization of isolated JG cell membranes on this gradient media. If whole cells exhibited normal responsiveness to the presence of known renin secretogogues following Percoll isolation, it could be inferred that JG cell membranes may also be functionally competent following Percoll purification. The rate of renin secretion of the control cells was 58.0± 12.4 ng ANG I/mg protein/hr. Figure 14 shows that forskolin and cAMP each significantly stimulated renin secretion ($p<0.01$) and that the magnitude of stimulation was comparable to results reported from rabbit renal cortical slices (Park et al., 1986). The calcium ionophore, ionomycin, which bypasses cell surface receptors and increases intracellular Ca levels (Pozzan et al., 1983), inhibited renin secretion. All experiments were done with cells in a medium containing 2mM Ca. These results demonstrate that JG cells prepared by Percoll density gradient maintain normal secretory responses to forskolin, cAMP and ionomycin and by inference to other known renin secretory modulators.

C. <u>Plasma membrane isolation and characterization.</u>

Figure 9 outlines the procedure developed to isolate plasma membrane vesicles simply and rapidly from rat renal cortical cells enriched with JG cells. Purity was assessed by the determination of the relative specific activities of the appropriate marker enzymes (Figure 15) . Sucrose is commonly used as a solute in homogenization media since it is inert, does not depress the activities of most enzymes, and does not permeate most cells (Robinson, 1975). In order to preserve the morphological integrity of the membranes, and specifically the loss of peripheral proteins, an isotonic isolation and separation medium was used throughout the procedure. In differential centrifugation, an homogenate is successively centrifuged for longer times at higher centrifugal forces to sediment the organelles on the basis of their size. Nuclei and any unbroken cells are sedimented first, followed by the mitochondria, lysosomes and

**Figure 14.** Physiological responsiveness of purified juxtaglomerular cell suspen- sions. JG cells were incubated for 1 h. in the presence or absence of forskolin ($2 \times 10^{-5}$M), cAMP ($1 \times 10^{-4}$M) and ionomycin ($2 \times 10^{-5}$M). Renin secretion was significantly stimulated in the presence of forskolin and cAMP ($p < 0.001$) and inhibited in the presence of ionomycin. $n = 4$.

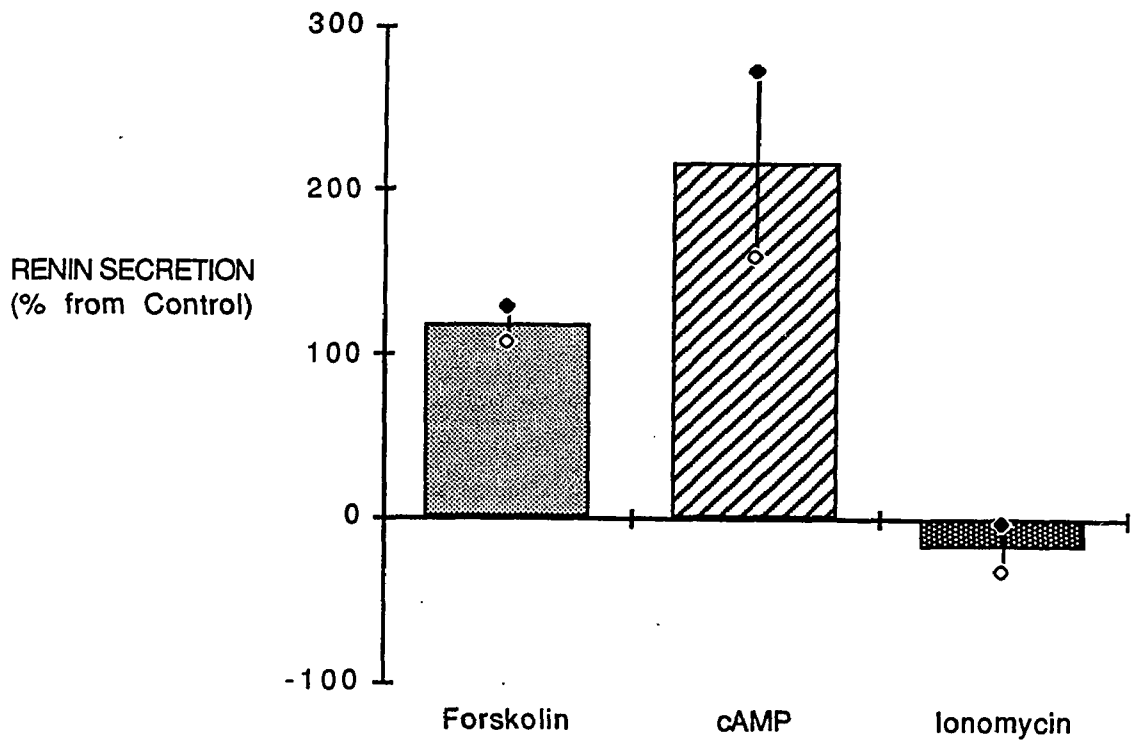
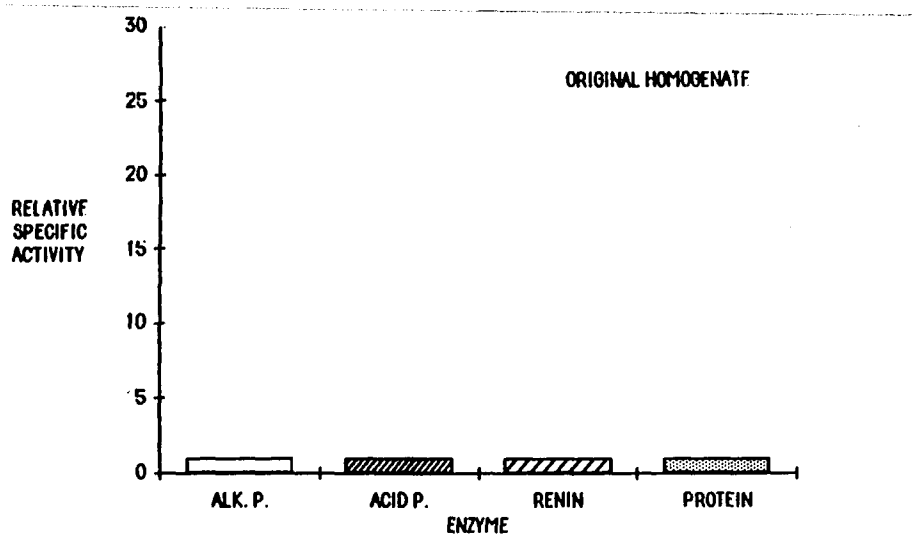
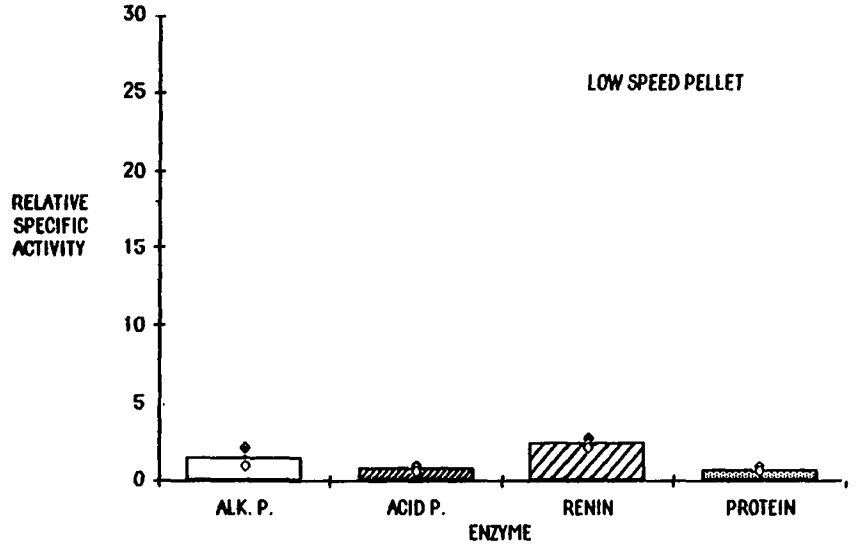
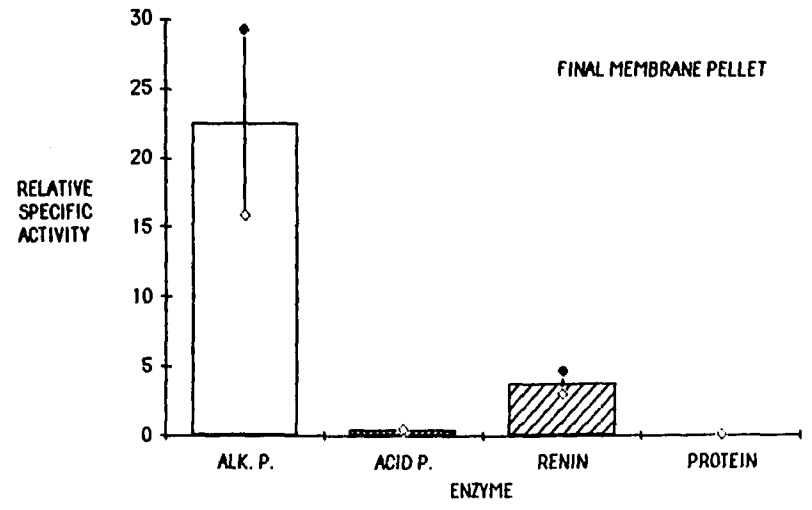

RENIN SECRETION
(% from Control)
300
200
100
0
-100
Forskolin
cAMP
Ionomycin

**Figure 15.** Distribution of marker enzymes and renin specific activities and protein in purification fractions isolated from rat renal cortex. Original homogenate was diluted to give a 6% (w/v) homogenate in iso-osmotic media and centrifuged at 1400 x g, 10 min. (low speed pellet). This low speed pellet was resuspended in 0.3M sucrose in 20 mM Tris-HCl to give a 6% (w/v) suspension and mixed with Percoll (12% final Percoll concentration) and centrifuged at 28,195 x g, 20 min. Plasma membrane fraction was removed and washed in 0.3M sucrose in 20 mM Tris-HCl followed by a second wash in distilled water and centrifuged 123,400 x g, 20 min. (Final Pellet). Relative specific activity = enzyme specific activity of fraction/enzyme specific activity of homogenate. Protein is illustrated as concentration in mg/ml. n = 6.


ORIGINAL HOMOGENATE
RELATIVE SPECIFIC ACTIVITY
30
25
20
15
10
5
0
ALK. P.
ACID P.
RENIN
PROTEIN
ENZYME


LOW SPEED PELLET
RELATIVE SPECIFIC ACTIVITY
30
25
20
15
10
5
0
ALK. P.
ACID P.
RENIN
PROTEIN
ENZYME


FINAL MEMBRANE PELLET
RELATIVE SPECIFIC ACTIVITY
30
25
20
15
10
5
0
ALK. P.
ACID P.
RENIN
PROTEIN
ENZYME

microsomes in that order (Robinson, 1975). These fractions are always impure since plasma membranes may disrupt to give fragments and vesicles of varying size which contaminate all fractions; however, preliminary studies using differential centrifugation of rat renal cortical homogenates showed that a significant quantity of the alkaline phosphatase activity, with minimal acid phosphatase activity, sedimented at a low speed (1400 x g for 10 min). Alkaline phosphatase is a classic plasma membrane marker enzyme (Robinson, 1975). For an enzyme to serve as a marker, ideally it must be confined to one class of subcellular organelles and be present in every organelle of that class. In practice it is difficult to demonstrate that these ideal conditions are fulfilled by putative markers. Alkaline phosphatase was chosen because of its abundance in renal tissue (Reynolds et al., 1980). The alkaline phosphatase activity of this initial low speed pellet is probably indicative of the presence of large membrane sheets resulting from the relatively mild shearing forces of the homogenization procedure (Robinson, 1975).

Given the proposed lysosomal nature of renin granules (Fransen, 1987; Taugner et al., 1985a), acid phosphatase activity was considered to be a marker enzyme for renin granules as well as lysosomes (Appelmans et al., 1955). The initial low-speed supernatant fraction had a 10-fold enrichment in acid phosphatase specific activity and a nearly 3-fold enrichment in renin specific activity compared to the homogenate (Table 1). The corresponding low-speed pellet exhibited a decreased acid phosphatase specific activity and a 2-fold increased renin specific activity (Figure 15). Since acid phosphatase is located inside lysosomes and renin granules, all samples were solubilized with Triton X-100 or sonicated prior to assay to disrupt and release the enzyme from the organelles. Thus, the primary advantage of this initial low-speed centrifugation was the separation of most of the renin granules, which remained in the supernatant fraction. Most of the glucose-6-phosphatase activity has been reported to sediment at higher g forces than the 1400 g force of the low-speed centrifugation (Prpic et al., 1984). Glucose-6-phosphatase is considered to be an endoplasmic reticulum marker enzyme (DeDuve et al., 1955). Therefore, the low speed pellet was not highly contaminated by granules, lysosomes, or endoplasmic reticulum membranes and was a highly concentrated, though admittedly impure, plasma membrane fraction (Table 1). This low speed crude plasma membrane pellet was resuspended and mixed with Percoll (12% final concentration) and centrifuged at 28,195 x g for 20 min to separate plasma membranes from most

**Table 1.** Enzyme activities and recoveries in rat kidney cortex homogenate and subcellular fractions. Values are given as means ± S.E.M. Abbreviations: S.A. = specific activity, defined as μmol product or substrate/min./mg protein for alkaline, acid, and glucose-6-phosphatases and as ng ANG I/h/mg protein for renin. T.A. = total activity in μmol/min/sample volume for alkaline, acid and glucose-6-phosphatases and as ng ANG I/h/sample volume for renin. R.S.A. = relative specific activity, defined as the ratio of specific activity of a fraction to that of the homogenate. Recovery (%) represents the percentage of total activity of the homogenate in the fraction. n = 6.

Table 1. Enzyme activity and percent recovery in rat kidney cortex homogenate and subcellular fractions.

| | HOMOGENATE | LOW SPEED | PELLET | | PERCOLL | GRADIENT | | ISOTONIC | WASH | HYPOTONIC | WASH | TOTAL RECOVERY |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | SN | P | F1 | F2 | F3 | F4 | ISO-SN | ISO-P | F-SN | F-P | % |
| PROTEIN (mg/ml) | 5.77 ± 0.49 | 3.75 ± 0.27 | 3.81 ±0.28 | 1.79 ± 0.14 | 1.40 ± 0.20 | 1.60 ± 0.03 | 13.51 ± 0.68 | 0.14 ± 0.01 | 0.57 ± 0.01 | 0.36 ± 0.02 | 0.52 ± 0.14 | |
| TOTAL PROTEIN (mg) | 230.80±19.78 | 150 ± 10.7 | 38.1 ± 2.81 | 3.58 ± 0.27 | 2.8 ± 0.04 | 3.20 ± 0.05 | 27.02 ± 1.36 | 0.70 ± 0.03 | 2.85 ± 0.06 | 1.80 ± 0.10 | 0.52 ± 0.14 | |
| RECOVERY (%) | | 67.3 ± 7.1 | | | 1.3 ± 0.1 | 1.5 ± 0.1 | 12.2 ± 1.4 | 0.3 ± 0.04 | | 0.8 ± 0.1 | 0.2 ± 0.02 | 83.6 ± 8.6 |
| | | | | | | | | | | | | |
| RENIN S.A. | 1.42 ± 0.22 | 3.54 ± 0.28 | 3.35 ± 0.39 | 12.11 ± 1.0 | 2.18 ± 0.09 | 1.41 ± 0.06 | 0.54 ± 0.05 | 1.67 ± 0.14 | 3.02 ± 0.14 | 1.8 ± 0.06 | 5.42 ± 0.13 | |
| T.A. | 56.8 ± 4.37 | 21.24 ± 1.69 | 33.5 ± 3.92 | 24.22 ± 2.01 | 4.36 ± 0.17 | 2.82 ± 0.12 | 1.08 ± 0.11 | 8.35 ± 0.69 | 15.10 ± 0.72 | 9.00 ± 0.31 | 5.42 ± 0.13 | |
| R.S.A. | 100 | 2.57 ± 0.26 | 2.48 ± 0.41 | 8.81 ± 0.99 | 1.56 ± 0.17 | 0.93 ± 0.12 | 0.39 ± 0.04 | 1.19 ± 0.10 | 2.16 ± 0.12 | 1.37 ± 0.14 | 3.94 ± 0.34 | |
| RECOVERY (%) | 100 | 38.5 ± 3.9 | | | 8.0 ± 8.8 | 5.2 ± 0.4 | 1.9 ± 0.2 | 14.9 ± 1.2 | | 16.5 ± 1.7 | 9.9 ± 0.8 | 97.2 ± 6.3 |
| | | | | | | | | | | | | |
| ALP S.A. | 0.25 ± 0.02 | 0.07 ± 0.01 | 0.35 ± 0.02 | 9.90 ± 0.72 | 4.00 ± 0.25 | 1.05 ± 0.02 | 1.51 ± 0.06 | 0.63 ± 0.05 | 3.24 ± 0.21 | 1.92 ± 0.10 | 5.74 ± 0.21 | |
| T.A. | 47.5 ± 3.86 | 11.06 ± 1.53 | 35.00 ± 1.71 | 19.80 ± 1.45 | 8.00 ± 0.51 | 2.10 ± 0.05 | 3.02 ± 0.12 | 3.15 ± 0.26 | 16.18 ± 1.06 | 9.60 ± 0.49 | 5.74 ± 0.21 | |
| R.S.A. | 100 | 0.28 ± 0.03 | 1.44 ± 0.13 | 41.77 ± 5.76 | 16.61 ± 1.87 | 4.37 ± 0.41 | 6.29 ± 0.63 | 2.52 ± 0.22 | 13.42 ± 1.45 | 8.02 ± 0.94 | 23.91 ± 2.46 | |
| RECOVERY (%) | 100 | 23.2 ± 2.4 | | | 14.2 ± 3.4 | 4.5 ± 0.4 | 6.6 ± 0.7 | 6.8 ± 0.6 | | 21.1 ± 2.5 | 12.6 ± 1.3 | 89.0 ± 6.3 |
| | | | | | | | | | | | | |
| ACID. P. S.A. | 0.09 ± 0.01 | 0.93 ± 0.12 | 0.07 ± 0.01 | 0.18 ± 0.01 | 0.04 ± 0.01 | 0.05 ± 0.01 | 0.28 ± 0.02 | 0.01 ± 0.001 | 0.06 ± 0.01 | 0.04 ± 0.03 | 0.05 ± 0.02 | |
| T.A. | 14.85 ± 2.04 | 13.02 ± 1.64 | 1.12 ± 0.17 | 0.36 ± 0.02 | 0.08 ± 0.02 | 0.10 ± 0.02 | 0.56 ± 0.03 | 0.05 ± 0.01 | 0.30 ± 0.4 | 0.20 ± 0.16 | 0.05 ± 0.02 | |
| R.S.A. | 100 | 12.65 ± 3.45 | 0.90 ± 0.19 | 2.18 ± 0.30 | 0.55 ± 0.19 | 0.66 ± 0.25 | 3.62 ± 0.77 | 0.12 ± 0.03 | 0.81 ± 0.26 | 0.33 ± 0.24 | 0.51 ± 0.12 | |
| RECOVERY (%) | 100 | 87.7 ± 10.9 | | | 0.7 ± 0.2 | 0.8 ± 0.3 | 4.4 ± 0.9 | 0.4 ± 0.1 | | 1.3 | 0.3 ± 0.1 | 94.7 ± 5.8 |
| | | | | | | | | | | | | |
| GLUC.-6-P. S.A. | 0.33 ± 0.02 | 0.3 ± 0.02 | 0.18 ± 0.03 | 0.19 ± 0.01 | 0.67 ± 0.03 | 0.02 ± 0.01 | 0.01 ± 0.001 | 0.02 ± 0.01 | 0.05 ± 0.01 | 0.03 ± 0.01 | 0.04 ± 0.01 | |
| T.A. | 13.20 ± 6.90 | 11.40 ± 1.23 | 1.8 ± 0.25 | 0.38 ± 0.03 | 1.34 ± 0.06 | 0.04 ± 0.01 | 0.02 ± 0.001 | 0.10 ± 0.07 | 0.25 ± 0.06 | 0.15 ± 0.06 | 0.04 ± 0.01 | |
| R.S.A. | 100 | 0.90 ± 0.07 | 0.54 ± 0.06 | 0.58 ± 0.02 | 2.05 ± 0.12 | 0.06 ± 0.02 | 0.03 ± 0.01 | 0.05 ± 0.03 | 0.15 ± 0.03 | 0.09 ± 0.04 | 0.13 ± 0.03 | |
| RECOVERY (%) | 100 | 85.8 ± 6.7 | | | 10.2 ± 0.6 | 0.3 ± 0.1 | 0.2 ± 0.0 | 0.7 ± 0.4 | | 1.2 ± 0.5 | 0.3 ± 0.1 | 98.7 ± 6.7 |

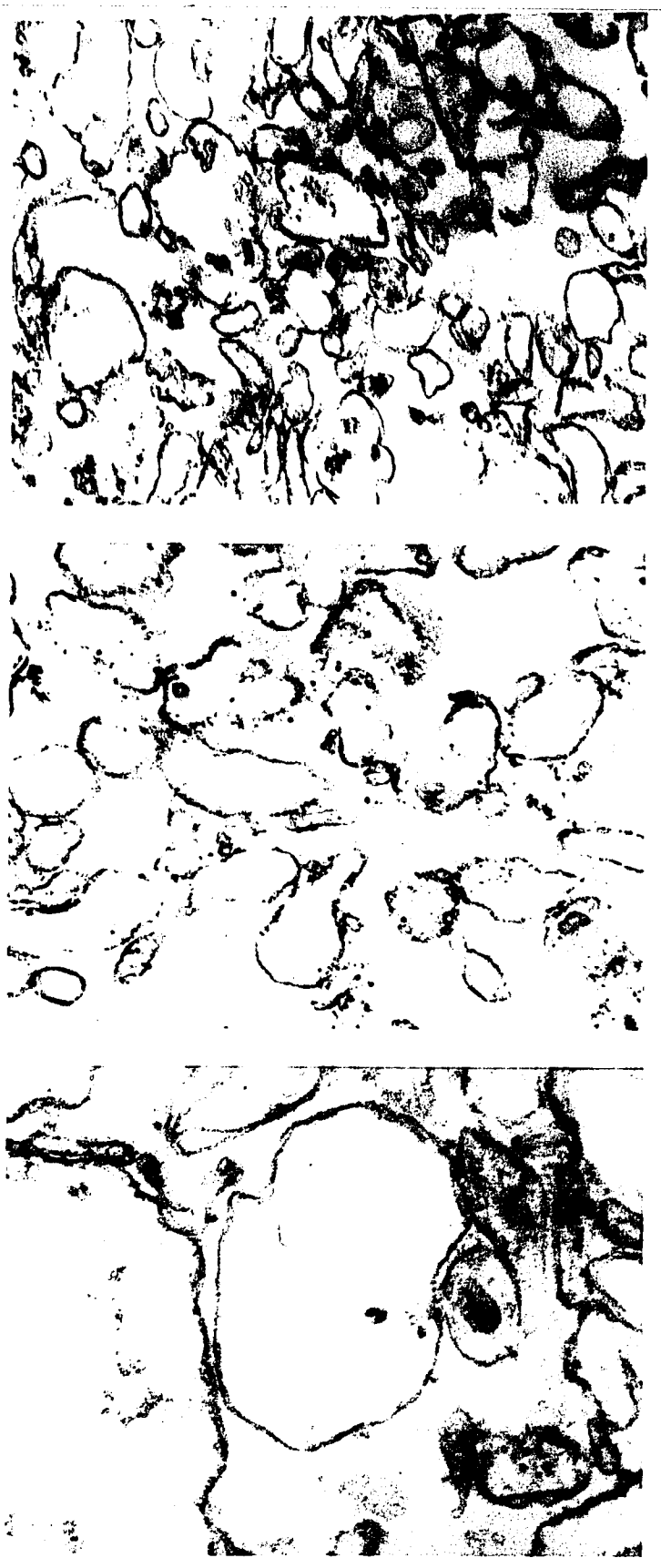
of the remaining contaminating subcellular material. Four distinct layers were observed in the resulting Percoll self-forming gradient tubes with the white, cloudy, F1 fraction located near the top of the tube and well removed from the other three fractions (Figure 9). Sixty-one percent of the total alkaline phosphatase specific activity of the four Percoll fractions was found in the F1 fraction (Table 1). Calibration of the gradient with density marker beads (Figure 9) of known buoyant density in isotonic sucrose indicated that the F1 fraction had a buoyant density range equivalent to that reported for plasma membranes isolated from a variety of mammalian cells (Jenkins et al, 1979; Meyer & Burger, 1979; Perret et al., 1979).

A comparison of the relative specific activities of the marker enzymes, alkaline and acid phosphatase, and renin (Figure 15) showed that the final membrane pellet had a 23-fold enrichment ($p< 0.01$) in alkaline phosphatase specific activity over the crude homogenate; the acid phosphatase specific activity was significantly less ($p < 0.05$) than that measured in the original homogenate. Thus granule/lysosomal contamination of the final membrane pellet was minimal. The alkaline to acid phosphatase ratio of the final membrane pellet showed a significant 41-fold enrichment ($p < 0.01$) over that measured in the original homogenate (Table 1). Others have previously confirmed that these enzyme activities are not influenced by the addition of Percoll solutions under similar assay conditions (Kawamura et al., 1986).

Renin specific activity was also monitored throughout the isolation procedure and the lysed and washed membrane fraction contained a significant amount of renin (Figure 15), suggesting the presence of membrane-bound renin even after a hypotonic wash (Table 1). Most of the renin specific activity was recovered in both the isotonic and hypotonic supernatant fractions suggesting the effectiveness of the washing procedure using an isotonic buffer followed by hypotonic lysis in removing any soluble renin trapped inside the membrane vesicles (Table 1). In addition to trapped renin, the washing procedure was designed to remove both intravesicular and intervesicular soluble proteins; about 70% of the protein in the Percoll F1 fraction was removed, while none of the membrane markers were significantly altered (Table 1).

Electron micrographs of the plasma membrane enriched fraction are shown in Figures 16a-16c.

**Figure 16.** JEO1 100S electron micrographs of purified suspensions of rat renal plasma membrane vesicles (PMVs) containing membrane-bound renin as assessed by radioimmunoassay of washed, solubilized PMVs. Ultrathin sections were stained with Reynolds lead citrate and urinol acetate. Numerous bilaminar vesicles are evident as well as various junctional complexes. The majority of the vesicles appear to be empty and well sealed. Figures 16A and 16B the magnification is 75,000X and Figure 16C, the magnification is 125,000X.



At low magnification (Figures 16a and 16b), a relatively homogenous membrane fraction was seen consisting largely of closed membrane vesicles of different sizes. These demonstrations agree well with those of Matlib et al. (1985) for plasma membrane from mesenteric arteries. At higher magnification the membranes showed the bilayer structure (Figure 16c), as is typical for plasma membranes (Singer and Nicolson, 1972). In addition, junctional complexes, tight junctions or gap junctions were observed (Figure 16c). As expected, there were no identifiable ribosomes, mitochondria, dense microbodies, or granules present. Most of the vesicles appeared to be free of trapped organelles as judged by the virtual absence of visible dense bodies within the PMVs.

The membrane vesicle orientation and integrity were confirmed using concanavalin A affinity chromatography and an ATPase binding assay. The first eluant (unbound volume) of the concanavalin A affinity column contained 86.4± 1.6% (n = 6) of the total alkaline phosphatase activity recovered from the column, whereas the bound volume eluted with ∂-methylmannoside (0.5M) contained the remaining 13.6± 1.6% of the recovered alkaline phosphatase activity (Table 2). Therefore, greater than 86% of the PMVs recovered from the column were oriented inside out. The total percent recovery from the column was > 88% in six trials with a mean recovery of 89.5 ± 0.8%.

The second estimate of sidedness using an ATPase binding assay exploited the fact that ATP and ouabain diffuse poorly across the membrane and have opposite sidedness of action. The ouabain binding site is on the external surface of the membrane whereas the ATP catalytic site is on the cytoplasmic surface (Baker and Willis, 1970; Kyte, 1974). Thus in sealed right-side-out vesicles, the only ATPase activity which could be expressed with added ATP would be due to any ATP trapped inside the vesicle during vesiculation. If ouabain is added, it would have access to its binding site on the external surface and would inhibit the cleavage of trapped ATP. If right-side- out vesicles are rendered permeable to ATP by SDS treatment in the absence of ouabain, ATPase activity would be expressed, since exogenous ATP could easily cross the SDS-activated membrane. In sealed inside-out vesicles, Na/K-ATPase activity would be expressed with added ATP and would be unaffected by added ouabain, since the ouabain binding site would be inaccessible on the inside of the sealed vesicle. In SDS-activated vesicles, Na/K-ATPase activity would be inhibited in the presence of ouabain. Leaky vesicles would be fully permeable to both

**Table 2.** Estimate of rat renal cortical plasma membrane vesicle (PMV) sidedness using Concanavalin A affinity chromatography. Sample: 25µl. Column dimensions 50 x 10 mm (bed volume 500µl). Elution procedure: column washed with 2/5 ml CSB (Con A starting buffer: 20 mM Tris-HCl, 50 mM NaCl, pH 7.4) followed by 2 ml 50 mM AMM (∂-methylmannoside in CSB. Abbreviations: $V_o$ = void volume (no binding to column), $V_e$ = elution volume (bound sample eluted with AMM), IOV = inside out vesicles, ROV = right-side out vesicles. Calculation of percent orientation: % IOV = $V_o/(V_o + V_e) \times 100$; % ROV = $V_e/(V_o + V_e) \times 100$. n = 6.

Table 2. Estimate of rat renal cortical plasma membrane vesicle sidedness using Concanavalin A affinity chromatogrpaphy.

| | ALKALINE | ALKALINE | PHOSPHATASE | SPECIFIC | ACTIVITY | | |
|---|---|---|---|---|---|---|---|
| | | | (µmole/min/ | mg protein | | | |
| | SAMPLE APPLIED | Vo | Ve | (Vo + Ve) | % IOV | % ROV | %RECOVERY |
| Exp. 1 | 0.84 | 0.65 | 0.09 | 0.74 | 88.1 | 11.6 | 88.1 |
| Exp. 2 | 0.71 | 0.57 | 0.70 | 0.64 | 89.6 | 10.4 | 90.0 |
| Exp. 3 | 0.60 | 0.51 | 0.05 | 0.56 | 90.7 | 9.3 | 93.0 |
| Exp. 4 | 1.05 | 0.77 | 0.16 | 0.93 | 82.8 | 17.2 | 88.3 |
| Exp. 5 | 0.73 | 0.56 | 0.09 | 0.65 | 86.3 | 13.7 | 88.6 |
| Exp. 6 | 1.42 | 1.03 | 0.24 | 1.27 | 80.9 | 19.1 | 89.0 |
| MEAN ± SEM | 0.89 ± 0.12 | 0.68 ± 0.08 | 0.12 ± 0.03 | 0.80 ± 0.11 | 86.4 ± 1.6 | 13.6 ± 1.6 | 89.5 ± 0.8 |

ATP and ouabain. Thus Na/K-ATPase activity would be inhibited in the presence of ouabain and uninhibited in its absence. Figure17 demonstrates that plasma membrane vesicles incubated with exogenous ATP and SDS showed maximal ATP binding to the ATPase. This binding decreased only slightly in the absence of SDS. Thus, under both of these experimental conditions, most of the added ATP had access to the inside-surface ATPase indicating that most of the membrane vesicles were oriented inside-out or were leaky. In the presence of ouabain, the Na/K-ATPase activity of both right-side-out and leaky vesicles would be inhibited. Hence, only inside-out vesicles would be unaffected and would show ATPase activity as indicated in Figure 17. With SDS treatment in the presence of ouabain, the added ouabain became fully effective in inhibiting the Na/K-ATPase and no binding was observed. The percentage of sealed right-side out vesicles, sealed inside-out vesicles and leaky vesicles was estimated from the observed $^{32}P$ cpm. The calculations showed that 86.8 ± 2.5% of the vesicles were inside-out, 6.2 ± 1.7% were leaky, and 7.0 ± 1.0% were right-side-out. These results correlated well with those obtained from the concanavalin A affinity column (Table 2).

Since greater than 86% of the isolated PMV were determined to be of inside-out orientation, additional experiments were conducted to determine whether renin may be released by trypsin. The washed plasma membrane fraction was incubated on ice for 20 minutes in the presence or absence of trypsin at various concentrations. This low incubation temperature and very short reaction time was chosen to minimize the potential effect of membrane proteases on renin activation and to partially restrain the trypsin activity. The final pH of the reaction mixture was adjusted to 7.11 with 0.1M Tris-base, which is within trypsin's optimal pH range of 7 - 9 (Merck Index). Neutral pH would also minimize any residual kallikrein activation of renin since the pH optimum of rat tissue kallikrein is pH 8 - 9 (Schachter et al., 1986). After termination of proteolysis by addition of a large excess of SBTI, the incubation mixtures were centrifuged at 123,400 x g to pellet the PMV and aliquots of the incubation media free of PMV were assayed for renin activity. Renin activity increased 2-fold in the presence of 2 mg trypsin/ml of PMV suspension over that measured in the untreated controls (Figure 18). Higher trypsin concentrations failed to result in further renin activation. Additional experiments demonstrated that neither the incubation media, nor any of the reagents used in these experiments significantly

**Figure 17.** Determination of plasma membrane vesicle orientation and integrity. PMVs, purified as described in the Materials and Methods section, were preincubated 20 min. at 37ºC in the presence or absence of 10-60µg SDS in 800µl PMVs suspension medium. To start the reaction, 40µl aliquots of the preincubated control or SDS-activated samples were added to 750µl of temperature-equilibrated ATPase assay medium containing [∂-$^{32}$P]ATP in tracer amounts in the presence or absence of 1mM ouabain and incubated 20 min. at 37ºC. The reaction was stopped by adding 400µl activated charcoal (10%) in 1M HCl and the liberated $^{32}$P was separated by centrifugation and counted via liquid scintillation counting. Values are expressed as means ± S. E. M. (n = 4).

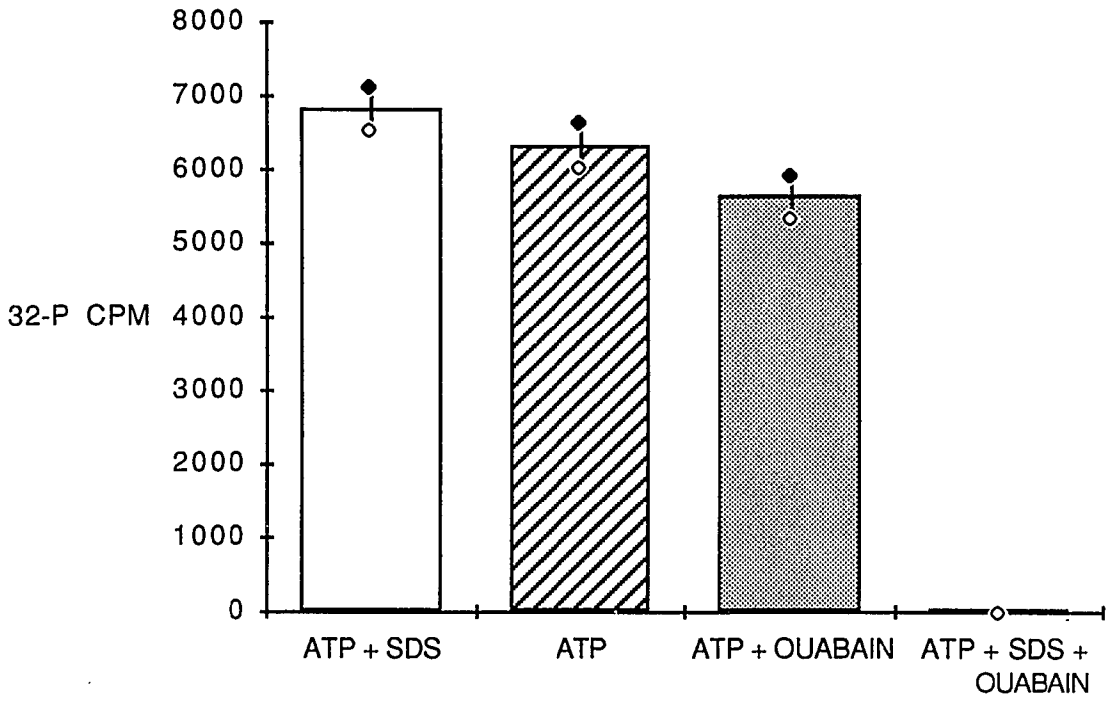
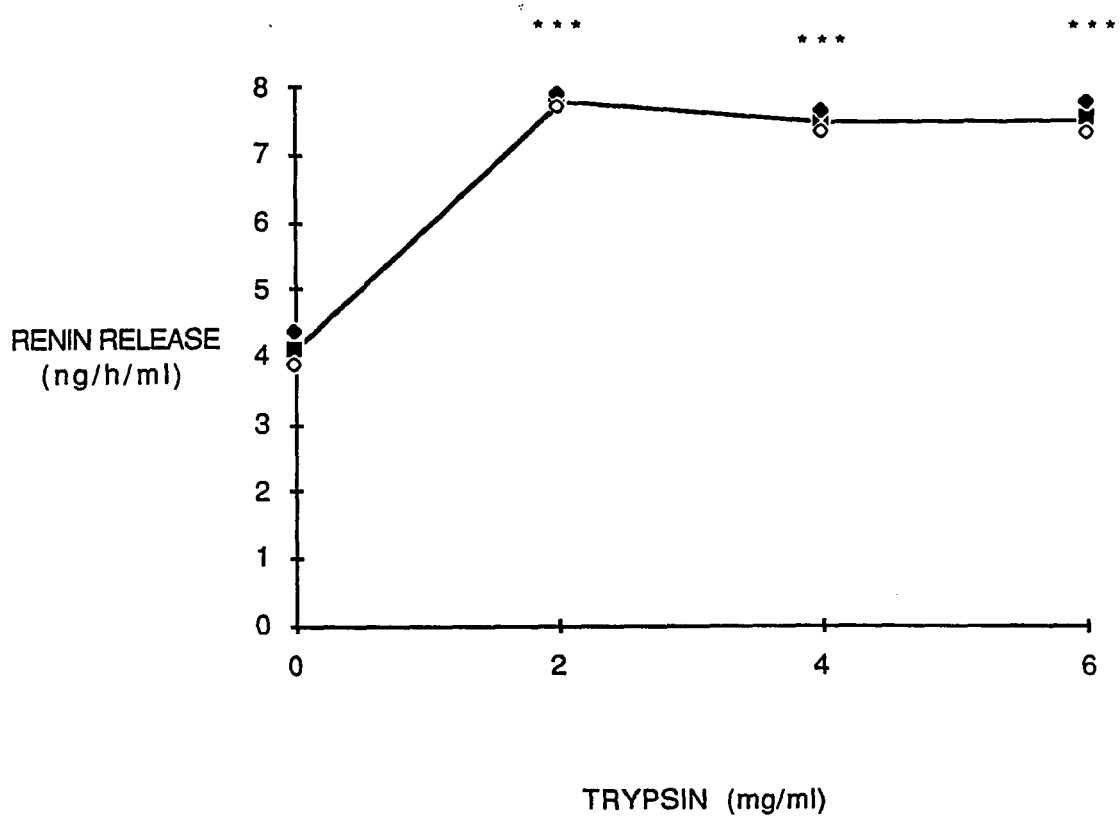

8000
7000
6000
5000
32-P CPM 4000
3000
2000
1000
0
ATP + SDS
ATP
ATP + OUABAIN
ATP + SDS + OUABAIN

**Figure 18.** Effect of trypsin on renin release from isolated JG plasma membrane vesicles (>86% IOV). PMVs were suspended in 20 mM Tris-HCl (pH 7.4) and treated with trypsin dissolved in sodium acetate buffer (pH 4.5) supplemented with 0.2M $CaCl_2$. The ratio of enzyme to PMVs suspension was 1:10. Trypsin action was terminated by adding soybean trypsin inhibitor (2mg SBTI/mg trypsin). Renin activity was measured at $t_0$ and $t_{20}$ minutes incubation on ice. Values are expressed as means ± S. E. M. ***($p < 0.001$). n = 4.


RENIN RELEASE
(ng/h/ml)
8
7
6
5
4
3
2
1
0
0
2
4
6
TRYPSIN (mg/ml)
* * *
* * *
* * *

interfered with the renin assay in the concentrations used. Furthermore, a cytosol-exposed domain could be anticipated to be more trypsin sensitive than a membrane-integrated domain, the latter being more resistant largely by virtue of its inaccessibility to trypsin.

**D. Effect of a low sodium diet and hypophysectomy on renin secretion from isolated perfused kidneys, renin activity of renal tissue and isolated renal cortical plasma membrane vesicles.**

Figure 19 shows the effects of hypophysectomy and Na deprivation on renin in renal tissue and plasma membranes. The renin content measured in the normal rats was very comparable to that previously reported (Honeyman et al., 1983). Renin in the plasma membranes of these freshly isolated kidneys represents 5.8% of the total renin in the tissue (Figure 19). Hypophysectomy increased both renal renin content ($p < 0.05$) and plasma membrane renin ($p < 0.01$) 2- fold. Similarly, Na deprivation increased both renal renin content ($p < 0.001$) and plasma membrane renin ($p < 0.05$) 3-fold. These results suggest that an increased renal renin content is associated with an increased amount of renin in the plasma membrane fraction.

Figure 20 summarizes the effects of stimulation on renin secretion and storage in plasma membranes of kidneys from normal, hypophysectomized, and Na deprived rats. Figure 20 (Panel A) shows that isoproterenol or Ca omission stimulated renin secretion in kidneys from all three sets of rats. However, compared to normal, hypophysectomized rat kidneys secreted 52% ($p < 0.05$), 52% ($p < 0.05$), and 54% ($p < 0.05$), less renin in response to basal conditions, isoproterenol, and 0 Ca, respectively. Similarly, hypophysectomized rat kidneys secrete much less renin than Na deprived rat kidneys (Figure 20). This was surprising considering the observation that hypophysectomized rat kidneys stored more than twice the amount of renin in normal rats and comparable to that in Na deprived rats (Figure 19).

Figure 20 (Panel B) shows the effects of the above maneuvers on renin in the plasma membrane fraction. In normal rats, neither isoproterenol nor 0 Ca altered the quantity of renin in the plasma membrane. In hypophysectomized rats, however, both isoproterenol ( $p < 0.05$) and 0 Ca ($p < 0.05$) caused

**Figure 19. A.** Effect of hypophysectomy and low sodium diet on renal tissue renin content. Rat kidneys were isolated and perfused with a high potassium (50 mM) KHB as described in Materials and Methods. Tissue renin activity was determined per mg of cortex homogenized to compensate for differences in kidney weight. **B.** Effect of hypophysectomy and low Na diet on rat renal plasma membrane renin content. PMV of rat kidneys perfused as described above and PMV were isolated as outlined in Figure 9. Plasma membrane renin specific activity was measured as ng ANG I generated/h/mg protein. Values are given as means ± S.E.M. *($p < 0.05$); **($p < 0.01$; ***($p < 0.001$). $n = 4$.

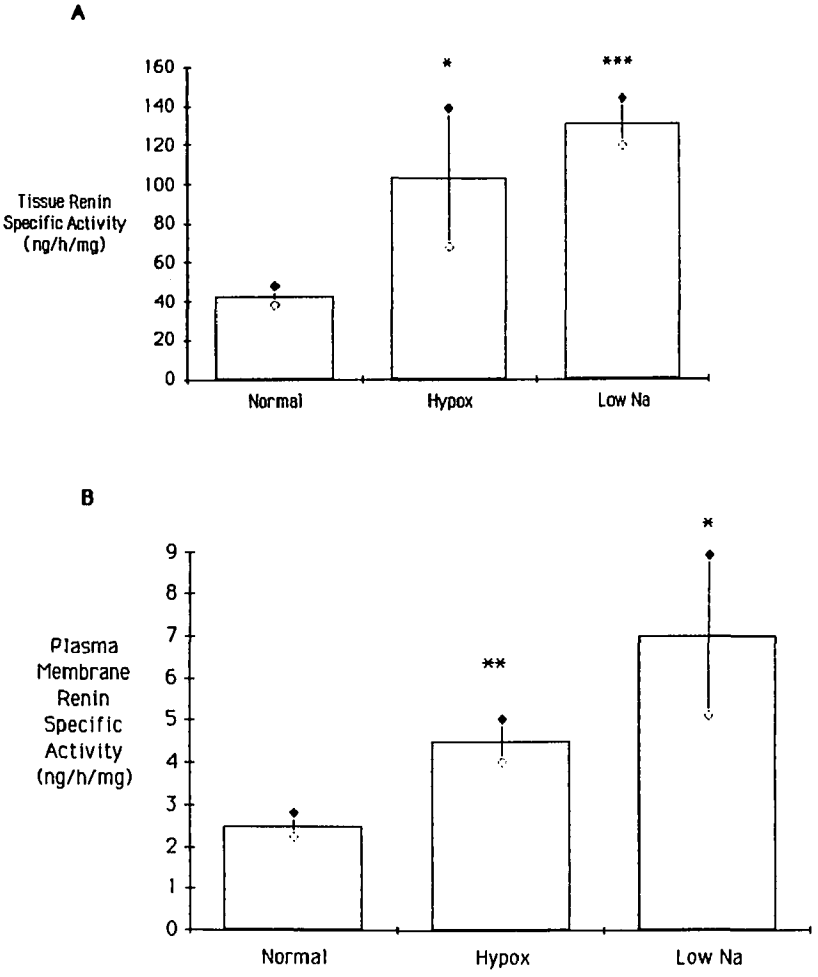
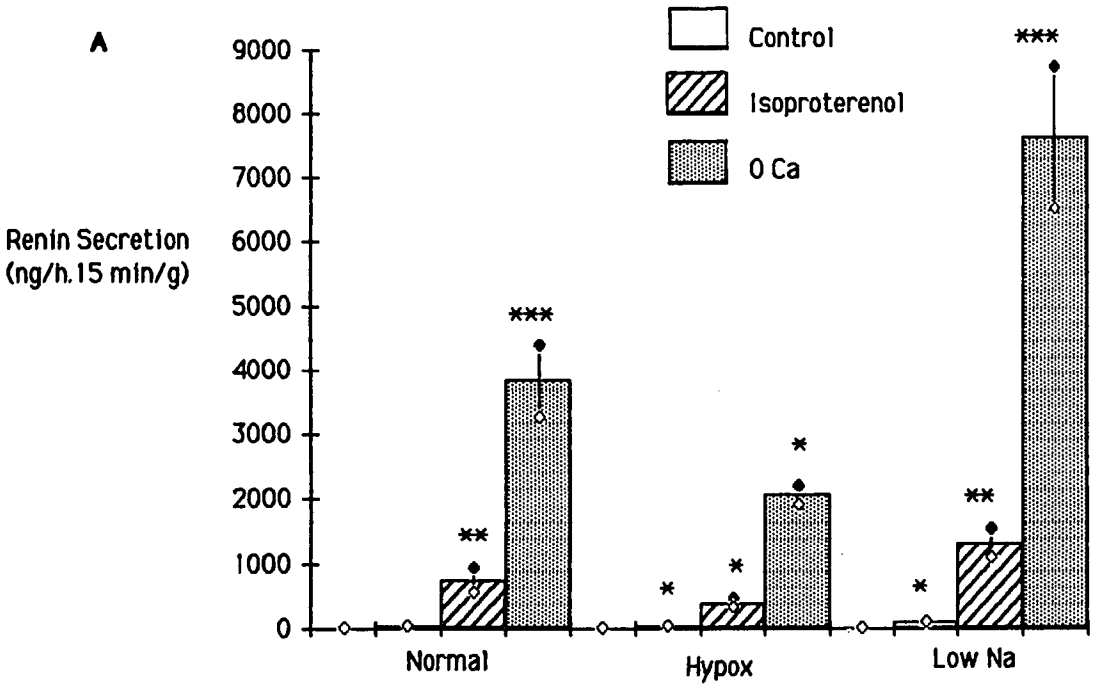
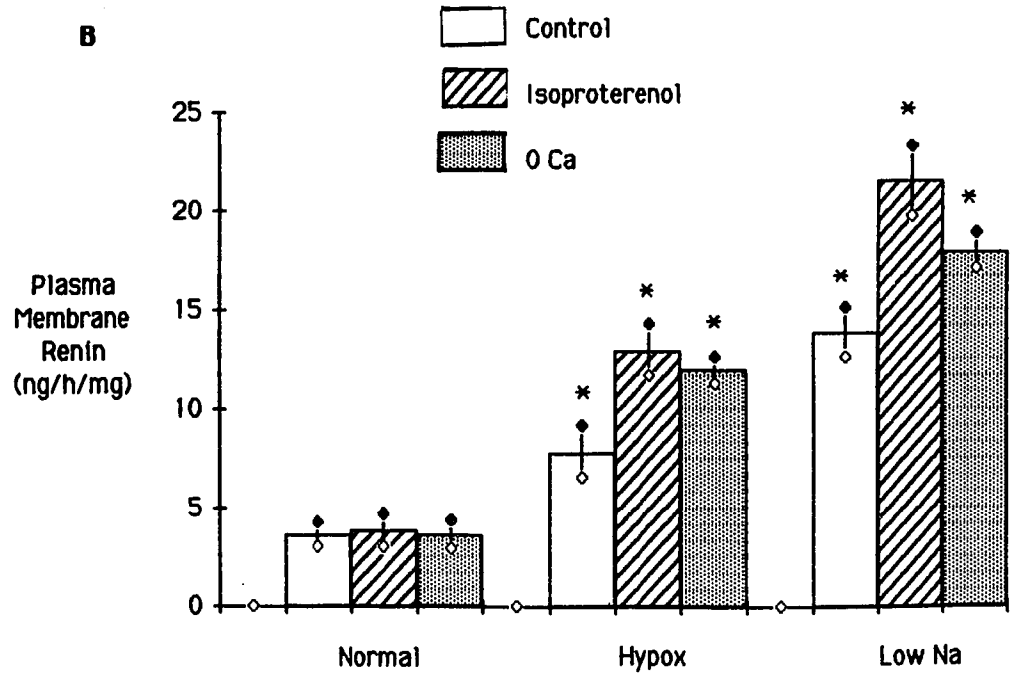

A
Tissue Renin Specific Activity (ng/h/mg)
160
140
120
100
80
60
40
20
0
*
***
Normal
Hypox
Low Na
B
Plasma Membrane Renin Specific Activity (ng/h/mg)
9
8
7
6
5
4
3
2
1
0
**
*
Normal
Hypox
Low Na

**Figure 20.** Effect of 0 Ca and isoproterenol (2.83μM) perfusion of isolated kidneys from normal, hypophysectomized and sodium deprived rats on renin secretion measured as ng ANG I generated/hour of incubation/ 15 minutes perfusion/ gram of tissue (**Panel A**), and plasma membrane renin content measured as ng ANG I generated/h of incubation/ mg of renal cortical tissue(**Panel B**). Values are means ± SEM. Significance was determined by the unpaired student "t" test. *($p<0.05$); **($p<0.01$); ***($P<0.001$). n = 4.


A
Renin Secretion
(ng/h.15 min/g)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Control
Isoproterenol
0 Ca
Normal
Hypox
Low Na


B
Plasma
Membrane
Renin
(ng/h/mg)
25
20
15
10
5
0
Control
Isoproterenol
0 Ca
Normal
Hypox
Low Na

statistically significant increases in plasma membrane renin. Plasma membrane renin in unstimulated (control) hypophysectomized rats was significantly greater ($p < 0.05$) than that found in plasma membranes from unstimulated, normal rats, but was considerably less ( $p < 0.001$) than that measured in plasma membranes from unstimulated, Na deprived rats. These experiments confirm the hypothesis of Simon et al.( 1984) that plasma membranes of hypophysectomized rats may store a large amount of renin; they also provide the additional evidence that plasma membranes of Na deprived rats also store large amounts of renin.

E. Effects of ions and calmodulin on renin release from isolated JG plasma membrane vesicles.

Na and Cl ions have long been implicated in the control of renin secretion (Kotchen et al., 1977; Vander, 1967; Thurau, 1964). However, it is unclear whether the effects of these ions are mediated by a JG cell membrane-localized mechanism or by some other mechanism outside the juxtaglomerular cell, as for example, the macula densa mechanism. Figure 21 shows the effects of $NaCl_2$ and $CoCl_2$ on renin release from isolated renal cortical PMVs. Renin release decreased by 80% and 68% by 30 minutes when PMVs were exposed to 100mM ($p < 0.01$) or 10mM Na ($p < 0.01$), respectively. Figure 21 also shows that at 30 minutes lowering Na caused a nearly 2-fold increase in renin release ($p < 0.05$). Renin release decreased 31% further in 10mM Na medium, but not in 100mM Na, when the incubation time was extended to one hour. Cl, on the other hand, had no effect on renin release from PMVs. Generally, PMVs incubated in medium containing Na released a greater amount of renin than those in Cl containing medium (Figure 21).

Figure 22 summarizes the effects of several Ca related factors on renin release from PMVs. The percentage of renin in the PMVs represents the remainder after exposure to the relevant media. Adding Ca to the incubation medium caused a substantial reduction in renin remaining in the PMVs ( $p < 0.05$). Neither EGTA nor calmodulin significantly affected renin release, but a combination of both caused a substantial release of renin from the vesicles and thereby lowered the amount in the plasma membranes ($p < 0.01$).

**Figure 21.** Effect of the monovalent ions Na and Cl on renin release from isolated JG plasma membranes (> 86% IOV). Experimental ions were superimposed on KRB media and PMV were incubated 0, 30 and 60 minutes at 37°C. At the end of the incubation time, the plasma membranes were removed by centrifuging at 123, 400 x g for 20 minutes and the renin specific activity (ng ANG I/h/mg protein) of the media was determined. The controls consisted of PMV incubated in KRB without Na or Cl supplementation. The basal renin release from these untreated controls was as follows (ng ANG I generated/hour of incubation/mg protein): $t_0$ 17.76 ± 0.28; $t_{30}$ 15.66 ± 2.98; $t_{60}$ 15.54 ± 0.34. Values are means ± S.E.M. n = 5. *($p < 0.05$). It is unclear why the 0 time for Na is higher than control.

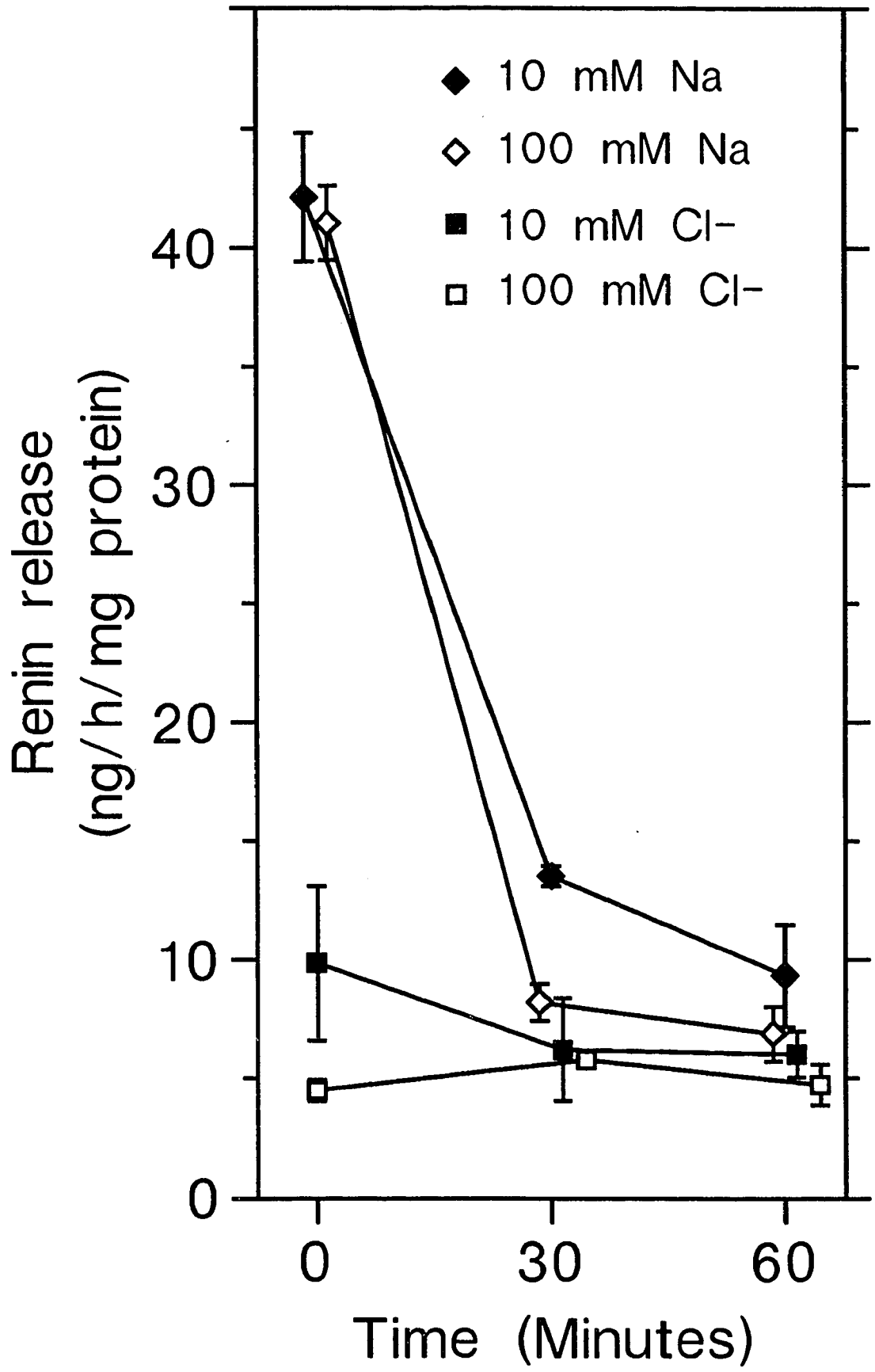

10 mM Na
100 mM Na
10 mM Cl-
100 mM Cl-
Renin release
(ng/h/mg protein)
0
10
20
30
40
0
30
60
Time (Minutes)

**Figure 22.** Effect of calcium, EGTA and calmodulin on plasma membrane renin content. Purified PMVs (>86% IOV) were suspended in 20 mM Tris-HCl (pH 7.4, control) or 20 mM Tris-HCl (pH 7.4) supplemented with 2 mM $CaCl_2$, 2 mM EGTA, 1 μg calmodulin/ml PMVs suspension or 2 mM EGTA + 1 μg calmodulin/ml PMVs suspension. All samples were incubated at 37°C for 60 minutes. The EVC, IVC and PMC were separated as described in Materials and Methods. Results are expressed as the percent of total renin activity [Σ (EVC, IVC, PMC)] found in the PMC. Values are means ± S.E.M. * ( $p < 0.05$); ** ( $p < 0.01$). $n = 4$.

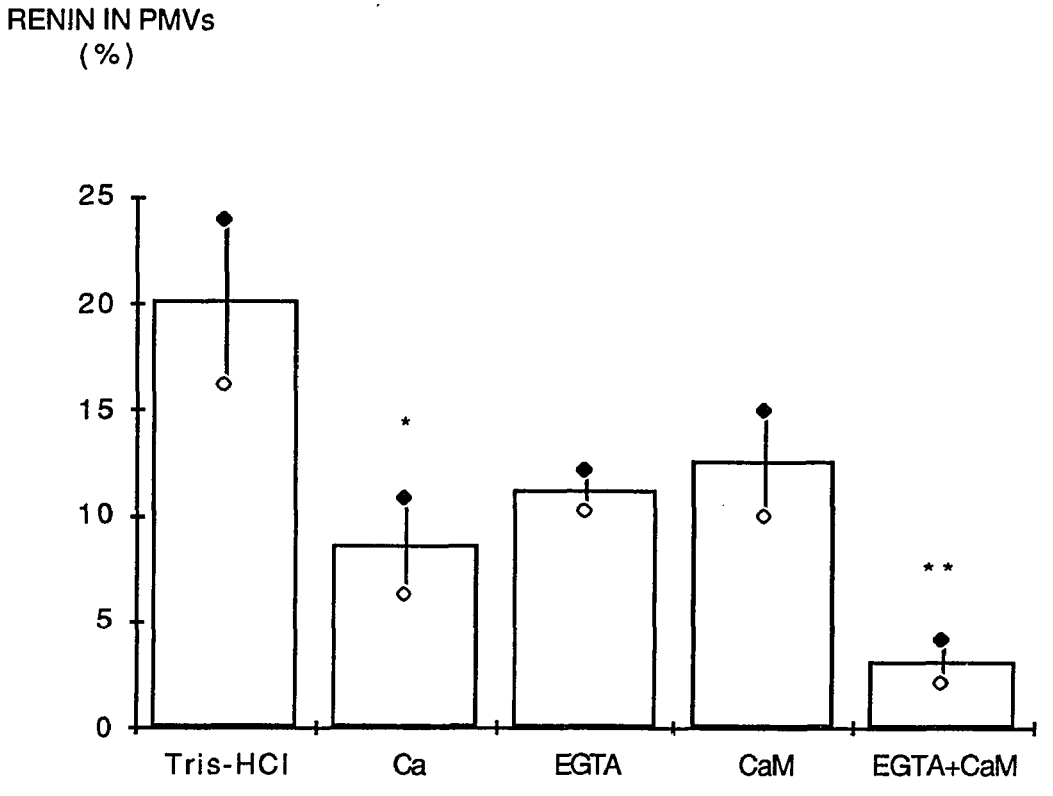
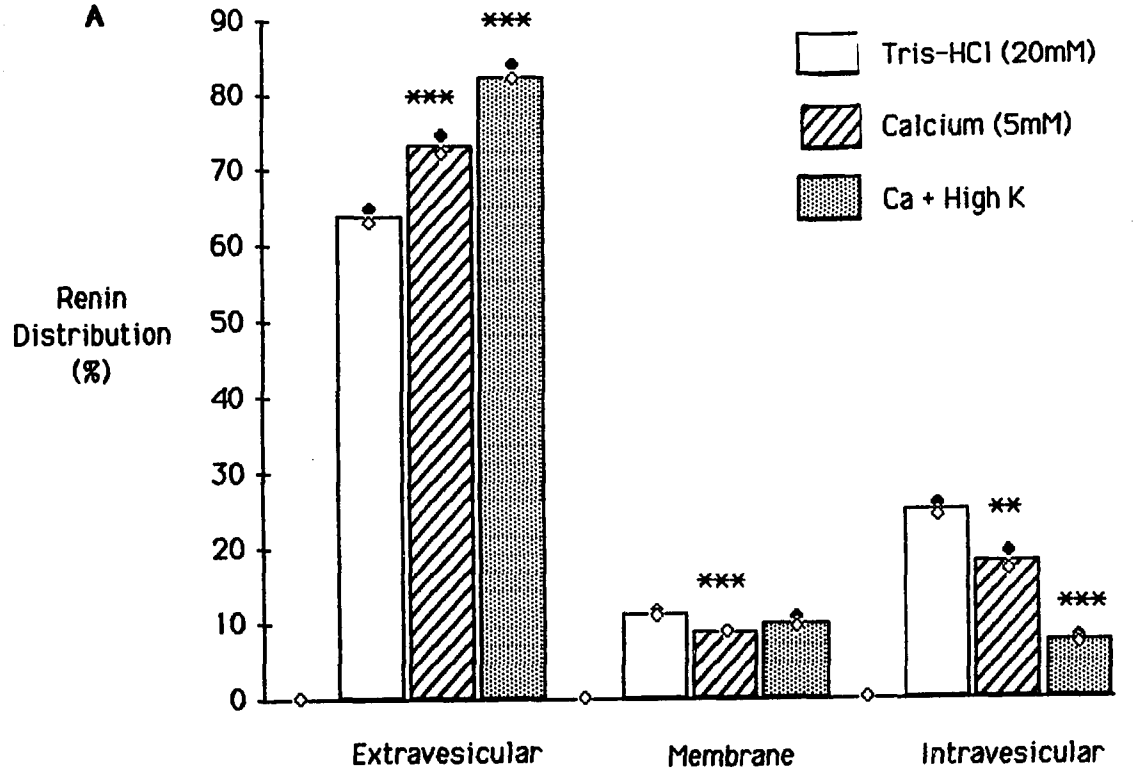

RENIN IN PMVs
(%)
25
20
15
10
5
0
*
* *
Tris-HCl
Ca
EGTA
CaM
EGTA+CaM

Figure 23 (Panel A) shows the results from additional experiments conducted to determine quantitatively the distribution of renin in the extravesicular (EVC), plasma membrane (PMC) , and intravesicular (IVC) compartments. After incubation for 60 minutes in 20mM Tris-HCl, pH 7.4 (control medium), 63.8 ± 1.0% of the renin was released into the EVC, 11.2 ± 0.6% remained in the membrane, and 25.0 ± 0.8% was released into the IVC. Addition of Ca caused a 15% increase in the EVC ($p < 0.001$), 22% decrease in the membrane ($p < 0.001$), and a 28% decrease in the IVC ($p < 0.01$). High K (50mM) in the presence of Ca also caused a substantial increase in the EVC (30%, $p < 0.001$) and a decrease in the IVC (70%, $p < 0.001$), without affecting the renin in the membrane (Figure 23, Panel A). Figure 23, Panel B shows the effect of EGTA on the renin distribution in PMVs also incubated for 60 minutes at 37°C. Adding 5 mM EGTA had no significant effect on the percent distribution of renin in the samples. The percent recovery of total renin activity in this experiment was 98.2 ± 0.5%. Equilibrating the PMVs in high K media in combination with 5mM EGTA, on the other hand, caused a 30% increase in EVC ($p < 0.001$) and a 70% decrease in IVC ($p < 0.01$), without significantly affecting renin in the plasma membrane. The percent recovery in this series of experiments was 96.4 ± 1.5%. It is very interesting that high K caused a substantial increase in renin release into the EVC with or without Ca present, suggesting that the effect of this ion may be mediated by voltage or some other effect of this cation and not by Ca per se.

Figure 24 illustrates the effects of divalent cations on renin release from PMVs. Ca and Ba both caused a significant increase in renin release into the medium ($p < 0.01$ for Ca, $p < 0.05$ for Ba). This increase was most pronounced at 10mM concentrations for both ions. Mg and Mn, on the other hand, caused a significant inhibition of renin release from PMVs ($p < 0.05$).

K has also been shown to affect renin secretion (Fray & Park, 1979; Churchill & Churchill, 1979). Figure 25 shows that when isolated PMV were exposed to concentrations of K there was a dose-dependent loss of renin specific activity in the extravesicular compartment below 140 mM K, but a gain above 140 mM K. At medium concentrations of K greater than normal cytosolic concentrations (≈140mM) up to at least 4-fold greater, the renin specific activity measured in the extravesicular compartment significantly increased ($p < 0.01$). The renin response to extravesicular K was, in part a function

**Figure 23. A.** Effect of Tris-HCl (20 mM), Ca (5 mM) and Ca (5 mM) + high K (50 mM) on renin distribution in isolated PMV. **B.** Effect of Tris-HCl (20 mM), EGTA (5 mM) and EGTA (5 mM) + high K (50 mM) on renin distribution in isolated renal cortical PMV (> 86% IOV). Partially purified PMV were suspended in 20 mM Tris-HCl (pH 7.4, control) or 20 mM Tris-HCl (pH 7.4) supplemented with 5 mM $CaCl_2$ or 5 mM $CaCl_2$ + 50 mM KCl. All samples were iancubated at 37°C for 60 minutes. The EVC, IVC and PMC were separated as described in Materials and Methods. Results are expressed as the percent of total renin activity defined as [$\Sigma$(EVC, IVC, PMC)] found in each compartment. Values are given as means ± S.E.M. ** ($p < 0.01$); *** ($p < 0.001$). n = 10.


A
Renin Distribution (%)
90
80
70
60
50
40
30
20
10
0
***
***
***
***
**
***
Tris-HCl (20mM)
Calcium (5mM)
Ca + High K
Extravesicular
Membrane
Intravesicular

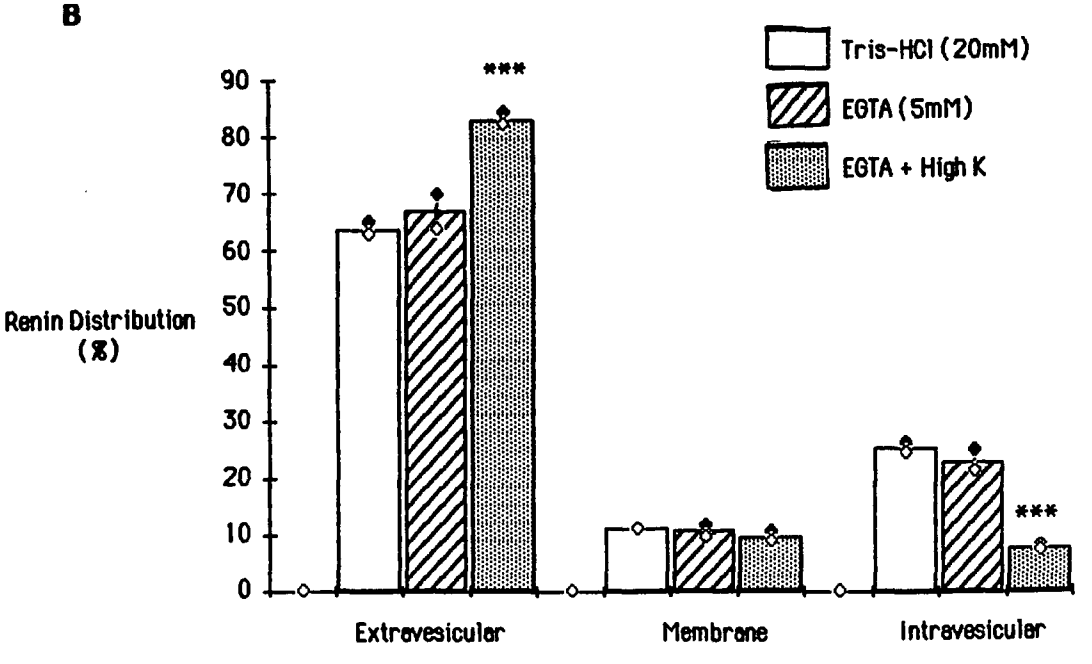

B
Renin Distribution (%)
90
80
70
60
50
40
30
20
10
0
***
***
Tris-HCl (20mM)
EGTA (5mM)
EGTA + High K
Extravesicular
Membrane
Intravesicular

**Figure 24.** Effect of divalent cations on renin release from isolated JG plasma membranes (>86% IOV). Experimental ions were superimposed on KRB medium and PMVs were incubated for 60 minutes at 37º C. At $t_{60}$ minutes, plasma membranes were removed by centrifuging 123,400 x g for 20 minutes and the medium renin specific activity (ng ANG I generated/hour of incubation/mg of protein) was determined. Values are expressed as the percent change in renin specific activity of the incubation medium fromPMVs exposed to the superimposed ion as indicated (abscissa, mM). Percent change = $(C_i - C_o)/C_o$ x 100 where $C_i$ = renin specific activity of the incubation medium at $t_{60}$ min. for PMVs exposed to the experimental ion of a given concentration (abscissa); $C_o$ = renin specific activity of the incubation medium at $t_{60}$ min. of the PMVs exposed only to the incubation medium (controls). n = 6, $Ca^{++}$; n = 4, $Ba^{++}$, $Mn^{++}$, $Mg^{++}$. Values are expressed as means ± S.E.M. * ($p < 0.05$).

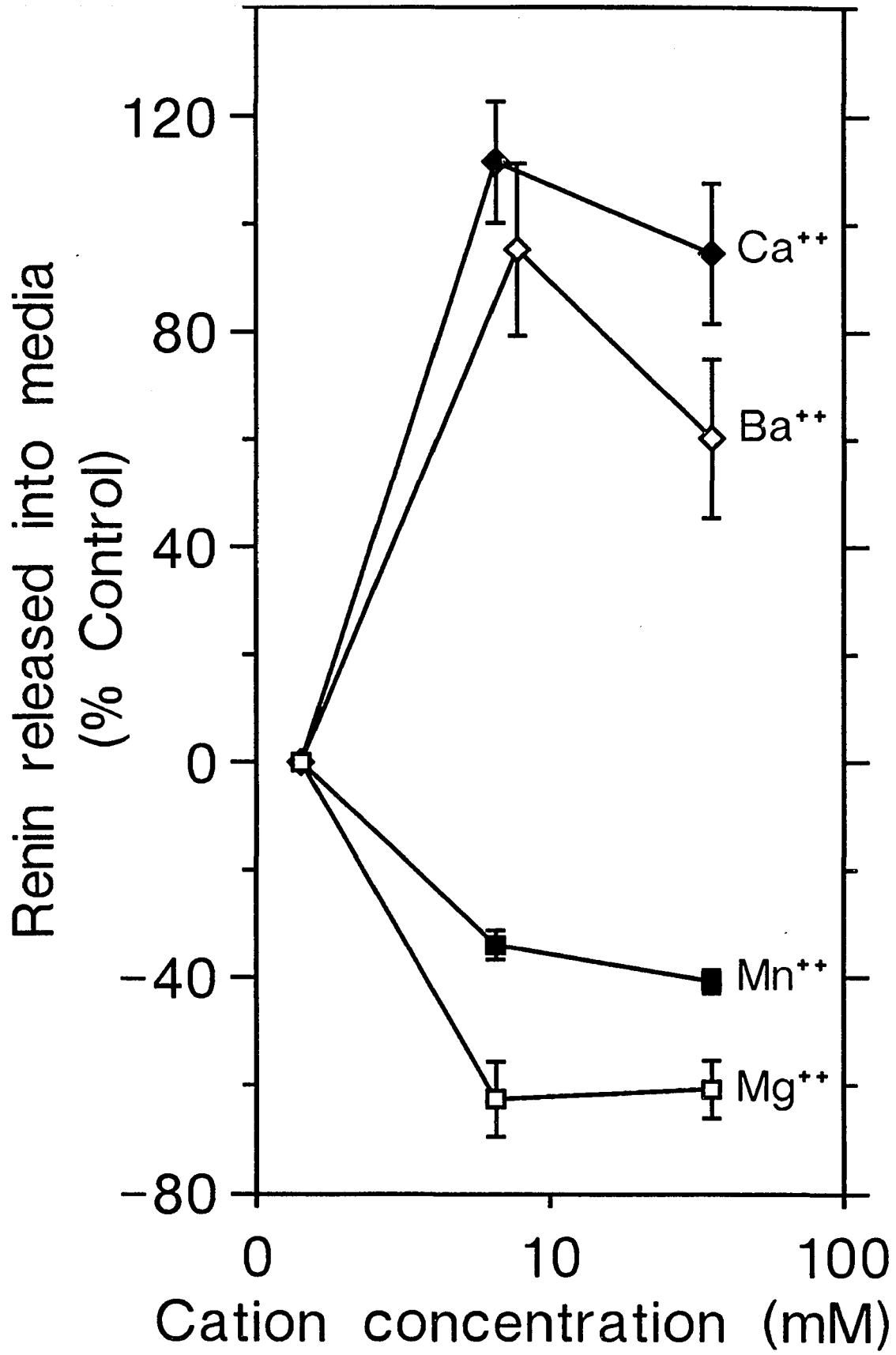
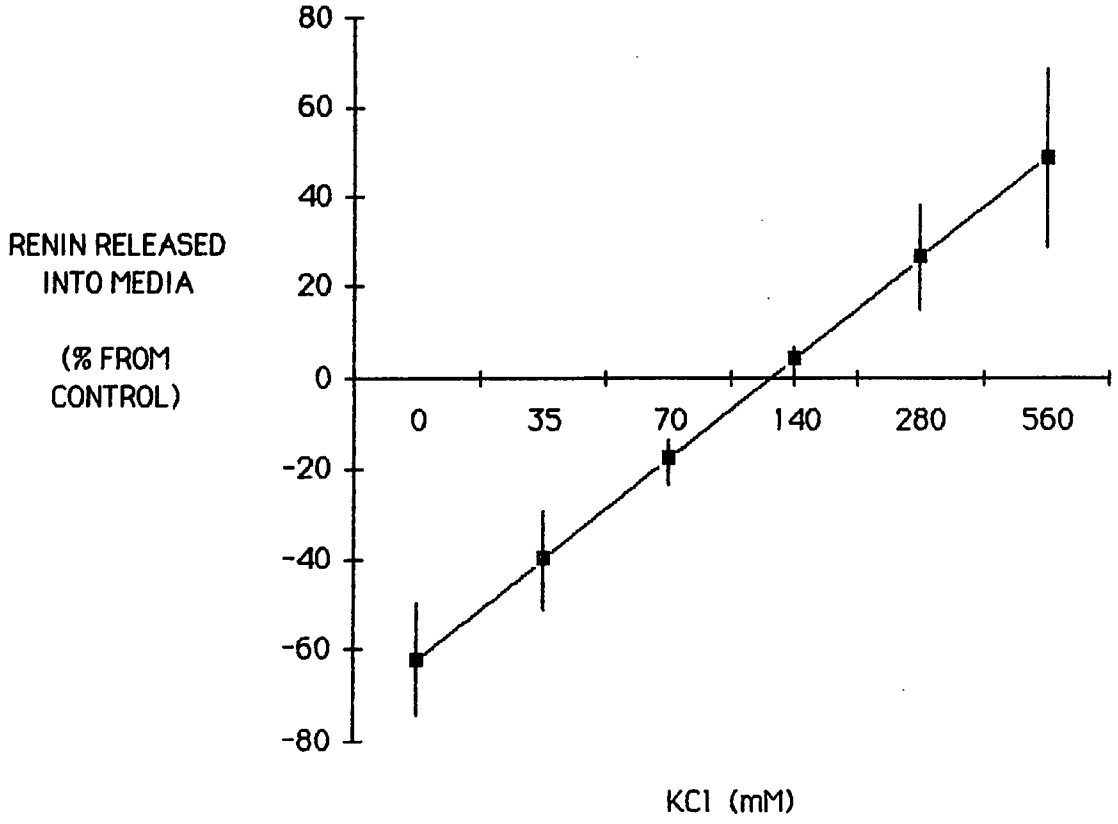

120
80
40
0
-40
-80
Renin released into media
(% Control)
Ca++
Ba++
Mn++
Mg++
0
10
100
Cation concentration (mM)

**Figure 25.** Effect of media $K^+$ on renin release from isolated JG plasma membranes (>86% IOV) incubated 30 minutes on ice. Basal level of renin release (control) from the isolated PMVs in the absence of K supplementation was 12.29 ± 1.32 ng ANG I generated/hour of incubation/mg of protein. The graph is a computer generated straight line fit of the data by least squares approximation. Values are given as the percent change in renin specific activity of the medium in the presence or absence of millimolar concentrations of KCl. *( $p < 0.05$); **( $p < 0.01$). $n = 4$.


RENIN RELEASED
INTO MEDIA
(% FROM
CONTROL)
80
60
40
20
0
-20
-40
-60
-80
0
35
70
140
280
560
KCl (mM)

of Ca in the incubation media, since the dose response curve shifted to the left in the presence of Ca and to the right in the absence of Ca (Figure 26). Thus, more renin accumulated in the extravesicular space (and by inference, less renin was secreted to the intravesicular compartment) in the presence of extravesicular Ca than in its absence. This data lends support to the hypothesis that a rise in K inhibits renin release by a corresponding increase in intracellular Ca through Ca influx (Park et al., 1981).

**F. Extrarenal renin.**

The demonstration of renin in some extrarenal tissues, including brain (Dzau et al., 1986), adrenals (Naruse et al., 1983), reproductive organs (Naruse et al., 1985), heart (Dzau and Re, 1987), lungs (Soubrier et al., 1982), and vascular walls (Ganten et al., 1976) has evoked considerable interest in the possible physiological function of the extrarenal renin-angiotensin system. Figure 27 shows that PMVs isolated from selected organs reported to contain renin, also have a measureable amount of renin, though less than 10% of that found in rat renal PMVs with an average amount of about 3.5 to 6% of rat renal PMV renin. Of the PMVs examined, those isolated from the heart contained the highest percent of renin ($p < 0.001$) compared to the other tissues. Skeletal muscle was at the opposite end of the spectrum and had significantly less PMV renin ($P < 0.001$) in comparison with the other tissues.

**Figure 26.** Effect of medium K on renin release from isolated JG plasma membranes (>86% IOV) resuspended in 20 mM Tris-HCl and incubated 30 minutes on ice in the presence or absence of Ca. Basal level of renin release in (ng ANG I generated/hour of incubation/mg of protein): in the absence of Ca or EGTA supplementation, 9.8 ± 2.2; in the presence of 2 mM $CaCl_2$, 9.5 ± 0.9; and in the presence of 1 mM EGTA, 6.1 ± 1.1. Values are given as the percent change from control (0 mM KCl). Each slope is a computer generated straight line fit of the data by least squares approximation. * ($p < 0.05$). n = 4.

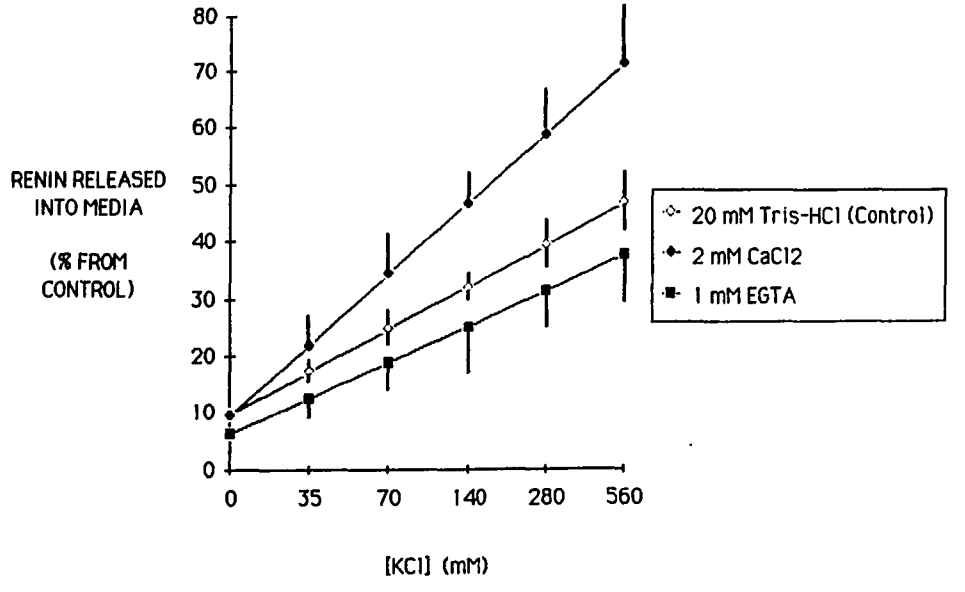
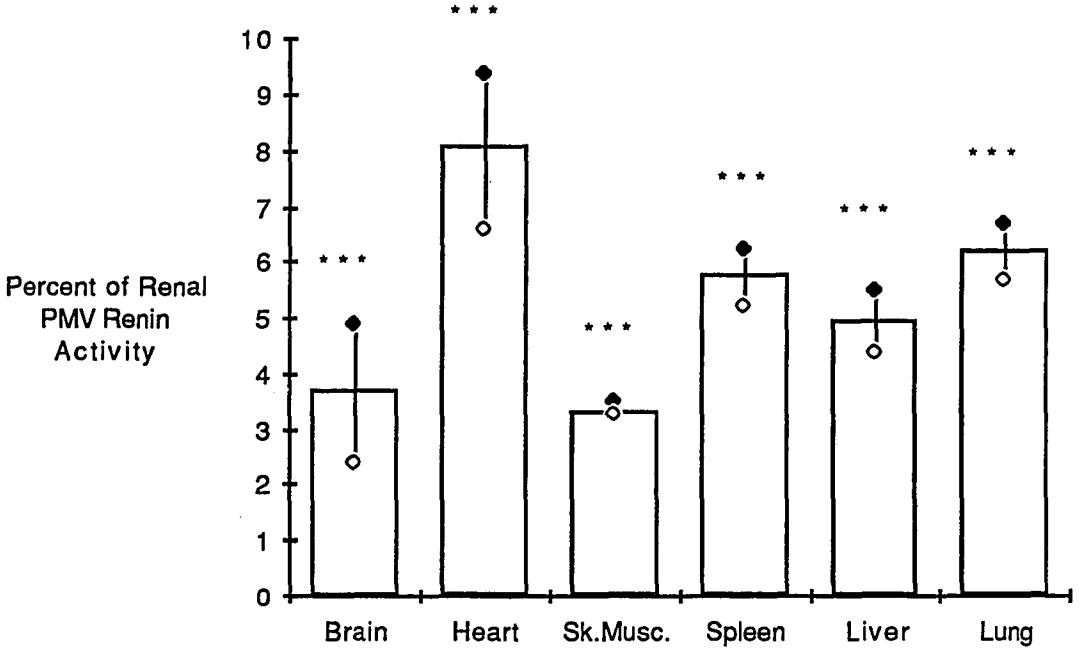

RENIN RELEASED
INTO MEDIA
(% FROM
CONTROL)
80
70
60
50
40
30
20
10
0
0
35
70
140
280
560
[KCl] (mM)
20 mM Tris-HCl (Control)
2 mM CaCl2
1 mM EGTA

**Figure 27.** Extrarenal PMV were isolated from rat organs reported to contain renin (Ganten et al., 1976). Values represent the percent of whole kidney PMVs renin specific activity in ng ANG I generated/hour incubation time/mg protein (100%) found in the extrarenal PMVs ± S.E.M. *** ($p < 0.001$). $n = 7$.


Percent of Renal PMV Renin Activity
10
9
8
7
6
5
4
3
2
1
0
* * *
* * *
* * *
* * *
* * *
* * *
Brain
Heart
Sk.Musc.
Spleen
Liver
Lung
PMV FROM EXTRARENAL ORGANS

# DISCUSSION

## A. Validation of the experimental design.

The secretion of renin from juxtaglomerular cells is regulated by a variety of stimuli that are presumed to be transmitted by stimulatory or inhibitory plasma membrane localized receptors on the juxtaglomerular cell as hypothetically diagramed in Figure 4. It is generally accepted that calcium acts as a negative modulator of renin secretion (Fray, 1983a), as it does in the release of parathyroid hormone ( Patt and Luckhardt, 1942; Copp and Davidson, 1961; Sherwood et al., 1966), glucagon (Leclercg-Meyer and Malaisse, 1983) and human placental lactogen (Choy and Watkins, 1976; Handwerger et al., 1981). However, little is known about the molecular biology of the renin secretory process at the cellular and subcellular levels. The extremely low number of juxtaglomerular cells in the kidney (<0.03%, Skøtt, 1986) and the difficulties encountered in isolating these cells from the other cell types of the renal tissue are significant contributors to the minimal success in this area of research. A quantitative characterization of renin release from juxtaglomerular cells has been hampered by the lack of a JG cell and plasma membrane preparation of sufficient homogeneity, integrity, and concentration to allow such measurements.

The results presented in this thesis demonstrate the feasibility of both cellular isolation and plasma membrane purification technqiues for the in vitro study of renin release by the JG cell of the rat kidney. The cell isolation method permits the quantitative determination of specific factors affecting renin secretion from the smallest intact physiological unit of the kidney, the whole cell; and the plasma membrane purification technique provides a model system for a critical evaluation of the evidence for the existence, localization and physiological role of a possible transport system across this final membrane barrier in the renin secretory pathway. A careful examination of the plasma membrane vesicle data presented here, in concert with that obtained from the disaggregated cell suspension enriched in JG cells, provide an excellent tool to study the molecular physiology of the renin secretory process at both the cell and plasma membrane levels, and offer a means of validating earlier renal cortical slice data as well as

that obtained from whole perfused kidneys. The methods permit determination of both quantitative and qualitative characteristics of renin secretion in a system devoid of extrarenal influences. Analysis is based on morphological, biochemical and physiological criteria affecting the movement of renin from the cytosolic compartment to the membrane compartment and ultimately to the extracellular space. These investigations serve to identify some of the significant effectors and inhibitors of renin release and potential sites of physiological regulation of renin secretion and blood pressure homeostasis. The results are based on recovery of renin immunoactivity and specific activity with appropriate marker enzymes in the cytosolic, plasma membrane and extracellular compartments.

The plasma membrane model provides a basis from which to pursue studies of the regulation of renin release from the plasma membrane compartment in relation to both environmental factors, in particular pH, temperature, osmolarity, and ionic composition; and physiological factors, including hormones, diet, metabolites or disease conditions. The limitations of the model and of in vitro experiments in general, to be discussed in greater detail in the following sections, also point to areas of future research in renin synthesis, storage and release. These include the question of the precise role of Ca in the various subcellular compartments relative to renin storage and release as well as the evolutionary advantage(s) of proposed multiple pathways of renin secretion and the role of the unusually large store of plasma membrane localized renin (i.e. 10% of total cortical homogenate renin). The methods described herein are currently limited to an investigation of the physiological responsiveness of a renal cortical cell suspension enriched in JG cells and the characterization of a renal cortical plasma membrane preparation enriched in JG plasma membrane vesicles and containing a significant amount of membrane-localized renin. This thesis extends the work of Nishimura and coworkers (1980) by allowing plasma membrane isolation in 95% less time than Nishimura with a 200% greater recovery of plasma membrane- bound renin (Table 1). It also demonstrates that the membrane-bound renin, they were the first to identify and activate using selected lipids and the polypeptide, melittin, can be induced to respond to known physiological and environmental effectors of renin secretion. It extends the work of Naruse and coworkers (1983; 1985) as well as Soubrier et al. (1982) by demonstrating that renin is found in the plasma membranes of the extrarenal organs reported by these investigators to contain intracellular renin.

To clarify further the role of plasma membrane renin, future research efforts are needed to elucidate the precise morphological and physiological architecture of the JG plasma membrane itself. Exactly how renin is sequestered by and anchored in the JG plasma membrane with a constant 10% distribution relative to the rest of the cell, ( or $11.2 \pm 0.6\%$ relative to the three membrane compartments, IVC, EVC, PMC) remains unclear. The fact that renin may be cleaved from the plasma membrane by several proteases (Nishimura et al., 1980) and trypsin (this study), suggests anchoring may be by peptide bonds.

Another area in need of additional work is that of the development of a pure culture of proliferating JG cells. Cultured cells provide a model system which can significantly facilitate the dissection of complex cellular and molecular processes. Historically, kidney physiologists have attempted to simplify the study of renal function by employing better defined and better controlled preparations. Important examples include the development of tubular microprofusion methodology by Burg and coworkers (1966) in the mid 1960s and the design of microanalytical methods by Morel et al., (1981) and Schmidt and Guder (1976). However, all these approaches necessitate microdissection and manipulation of individual nephron segments containing only a few hundred cells and therefore severely limits their general application. In the late 1960s, Leighton et al. (1969) realized that cultured renal epithelia can continue to express structural polarity and vectorial transport activity even after maintenance in vitro for over 20 years. These observations received the attention of transport physiologists when it was demonstrated that monolayers of epithelial cells could be analyzed using procedures developed by Ussing and Leaf (Misfeldt et al., 1976). However, despite the extensive successful work done in the isolation and culture of renal tubular cells, JG cell culture has met with minimal success. Primary cultures of renin-secreting cells have been described in the rat by Kurtz et al., (1984), but these cultures contained only 50% JG cells. Until, very recently, the sole attempt to maintain a rat JG cell culture was made by Rightsel et al. (1982) by subculturing and cloning dissociated cortical cells from neonatal kidney.

In 1985, Pinet and coworkers reported the development of a continuous JG cell line, of renin-producing human cells by transfection with three simian virus 40 mutants. To date no such immortalized renin-secreting cell cultures for normal nontumoral JG cells has been reported. The renin

synthesized by the transformed tumoral JG cell was similar to renin extracted from human cadaver kidney by its enzymatic activity and immunological identity but was glycosylated to a lesser degree than tissue renin. This is a particularly interesting observation since carbohydrates of transmembrane glycoproteins have been implicated in the insertion, orientation and anchoring of such proteins in the lipid bilayer (Sharon, 1984). Furthermore, whereas the biological activity of glycoprotein enzymes and lectins is independent of their carbohydrate moiety, this is not the case for glycoprotein hormones, as the hormonal activity of human chorionic gonadotropin and of the pituitary gonadotropins is lost on removal of the carbohydrate moieties (Kalyan and Bahl, 1983). This is an exciting finding, being the first demonstration of a direct role of carbohydrate in the biological activities of the protein (Sharon, 1984). More than 95% of the renin secreted in the medium from Pinet's transformed JG cell cultures was in an inactive form, but it could be fully activated by limited proteolysis, as occurs in plasma (Pinet et al., 1985). This suggests that, contrary to other glycoprotein hormones, the carbohydrate moiety of renin is not necessary for its hormonal response, but rather may serve to protect the renin glycoprotein against proteolytic degradation (Gottschalk and Fazekas de St. Groth, 1960). Such enhanced susceptibility to limited proteolysis has precedent in the finding that the enzymatic removal of the terminal neuraminic acid from ovine submaxillary gland mucoprotein similarly enhanced its susceptibility to limited trypsin digestion (Gottschalk and Fazekas de St. Groth, 1960). Although glycoproteins are present in great abundance in eukaryotic cells, the biological function of the constituent oligosaccharide units generally remain to be elucidated. The wide distribution and conserved structure of the carbohydrate moieties of glycoproteins in animal tissues suggests their importance in an undefined, but universal physiological process (Olden et al., 1982).

**B. <u>Renal cortical cell isolation enriched in JG cells.</u>**

The isolation of a completely pure suspension of JG cells as well as a pure culture of continuously secreting JG cells remain challenging possibilities, not unlike searching for and culturing the perennial "needle in a haystack" given the scarcity of renin-producing cells in the renal cortex (Taugner et

al., 1981), and further complicated by the tendency of JG cells, developed by metaplasia of preexistent smooth muscle cells, to revert to their nonsecretory smooth muscle cell phenotype (Cantin et al., 1977). A number of JG cell isolation procedures have been reported (Lyons and Churchill, 1975; Rightsel et al., 1982; Galen et al., 1984; Kurtz et al., 1984; Pinet et al., 1985; Buhrle et al., 1986a, 1986b), but the methods employed were often long and tedious resulting in low yields of viable cells. More rapid methods usually produced mixed populations of cells with high tubular cell contamination. A recent method using the hydronephrotic kidney (Buhrle et al., 1986) resulted in viable, physiologically competent cells essentially free of tubular contamination, but a major disadvantage of the method was the time required to produce the experimental hydronephrosis, i.e. 8-10 weeks. The method reported here represents an improvement over earlier methods in that it is fast, taking less than two hours; it is a relatively simple procedure characterized by minimal handling of the cortical tissue with a series of short term, less damaging exposures to the dissaggregating enzymes, followed by a gentle low speed wash to remove the enzymes before they can attack important components of the cell surface, as for example, those as yet unknown factors required for cellular proliferation in culture. It is effective with routine renal cortical cell yields at least 4-fold greater , and plasma membrane yields 8-fold greater, than the recently reported methods of Kurtz et al. (1987 ) for JG cell isolation and Maeda et al. (1983) for plasma membrane purification. In addition, the plasma membrane purification procedure results in relative enrichments of the ratio of plasma membrane marker enzyme specific activity to granule/lysosomal marker specific actvity (i.e. alkaline phosphatase/ acid phosphatase) in the range of a 45- to 50-fold increase over that measured in the original homogenate (Table 1).

The buffer in which the JG cells were isolated was a modified Tyrode's solution designed to maintain normal osmolarity at the high end of the normal range (i.e. 318mOsm) to prevent swelling of the cells during isolation. Retention of stored renin by the cells requires energy and renin release may be controlled physiologically by mechanisms which regulate the volume of the JG cells (Frederiksen et al., 1975). Therefore glucose (11.1mM) was included in the medium both to provide an energy source and to raise osmolarity. Glucose was favored over other substrates for energy metabolism because of its ability to depress oxygen consumption slightly (Howard et al., 1973). Thus circumventing, in part, the

potential problem of decreased viability due to hypoxic conditions. A reduction in osmolarity to 285 mosmole/L has been reported to double renin release rate, whereas a rise in osmolarity of 30 mosmole/L, halved the renin release rate (Frederiksen et al., 1975). It was therefore considered advantageous to isolate the renin-containing cells at the high end of the iso-osmotic range.

Bovine serum albumin (BSA) was included in the disaggregation media to coat the cells and prevent them from adhering to each other or to the vessel in which they were contained, and as a competitive substrate for destructive proteases released by damaged cells (Waymouth, 1982). Furthermore, Rubin and coworkers (1977) added BSA to solutions of collagenase and DNAase for disaggregating liver, noting that it also served as a competitive substrate for contaminating proteases present in the crude preparation of the digestive enzyme, the collagenase itself. Albumin has also been reported to bind to the plasma membrane and thus stabilize it and prevent the escape of small molecules and cofactors from the cell as well as the entry of the external medium (Howard et al., 1967). In addition to this contribution to the structural integrity and osmolarity of isolated cells, BSA greatly stimulates the endogenous rate of respiration of cell suspensions up to the same rate attained by malate stimulation and thus contributes to the physiological stability of isolated cells as well (Howard et al., 1967). Of particular significance for this study was the observation that BSA protected purified rat renin from inactivation during purification and storage (Matoba et al., 1978). In light of all of these advantages, BSA was routinely included in all isolation media.

The nature of intercellular matrices that hold cells together is now beginning to be understood, though little is known of the extracellular milieu of the JG cell (Schlondorff, 1986). However, Khayat and coworkers (1981) have shown that collagenase-dispersed rat renal cortical cells synthesize and release renin into the supernatant medium and respond as expected to pharmacological stimuli, while cells dispersed with the other digestive enzymes tested, failed in both regards.

The frequent assessment of cell viability was considered important for these in vitro studies of renin release to exclude passive release of renin due to cellular damage or death. In addition, retention of cellular renin was viewed as added evidence of cell viability. Cell density has been reported by others to be a measure of cellular integrity, since it seems to be a particularly sensitive indicator of cell swelling

(Hefley et al., 1981). In Hefley et al.'s investigations intracellular fluid space was found to correlate well with survival of dispersed cells such that a decline in cell density is associated with an increase in intracellular fluid space and a loss of viability. In our work, the highest percentage of trypan blue dyable cells was consistently found in the lower density region of the Percoll gradient (i.e. d. 1.02 - 1.04 g/ml). Coqnizant of the early reports of the limits of confidence in the use of trypan blue as the sole indicator of viability (Tennant, 1964), all of the foregoing parameters as well as lactate dehydrogenase assays were used as additional assessments of cell viability. This enzyme is considered to be a cytosolic marker enzyme (Elliott, 1979) and therefore serves as an indicator of cellular leakage of cytoplasm to the external media. These additional tests were used to confirm and validate the trypan blue estimates.

One component of crude collagenase that has been found to be particularly destructive to cells has yet to be characterized, but has been named factor CDE (cell damaging enzyme). CDE can be inhibited by increasing the K concentration of the digesting medium and withholding Mg (Hefley et al., 1981). There are precedents for the deletion of Mg in the successful isolation of cardiac myocytes (Pretlow et al., 1972) and epithelial cells from rat palate (Terranova and Brand, 1979), both of which require that Mg be withheld from the digestion medium. The mechanism of the Mg effect is unclear. It is unlikely that Mg is a cofactor for some protease in the enzyme mixture, since this divalent cation rarely serves that function for proteolytic enzymes(Hefley et al., 1981). Mg could possibly alter the availability of a site for attack on the plasma membrane. It is a cofactor for many membrane-bound enzymes and its absence could alter the conformation of a membrane protein (Ferment & Touitou, 1985). There is precedent for the use of a high K medium during the isolation of other cell types, specifically in the isolation of bone cells (Hefley et al., 1981) where there is data suggesting that the extracellular fluid of bone is high in K (Canas et al., 1969), and recently in the isolation and culture of smooth muscle cells from human umbilical cord arteries (Okker-Reitsma et al., 1985). Furthermore, numerous studies have shown that infusion of small quantities of K produces vasodilation in isolated perfused vessels from both normal and hypertensive animals ( Emanuel et al., 1959; Overbeck, 1972; Haddy, 1975). This effect is believed to be mediated by stimulation of membrane Na/K-ATPase by K and hyperpolarization of the cell since its effect was prevented or reversed by ouabain (Chen et al., 1972). In the isolation of JG cells

in this study, a reduced concentration of Mg (0.1mM or tenfold less than normal Tyrode's solution) and an increased concentration of K was found to improve viability and yield of renin-containing cells and was routinely used in all isolation media, except where KRB is specified.

The first report on the use of colloidal silica (Percoll) for cell separation was published by Mateyko and Kopac (1963) and Pertoft (1966) subsequently found that high-speed centrifugation of colloidal silica gave rise to density gradients in the centrifuge and proposed that these self-generated gradients could be used for isopycnic banding of particles. This gradient medium possesses a low osmolality, low viscosity and a high density (Pertoft et al., 1978) and does not seem to penetrate easily through biological membranes (Feucht et al., 1979). Since the buoyant density of cells appears to be a function of both osmolality of the surrounding medium and the real density of cells (Cercek and Cercek, 1978), Percoll must be rendered iso-osmotic before use in cell separation. To make an iso-osmotic stock solution, nine parts Percoll was mixed with one part 1.5M NaCl (Percoll: Methodology and Applications, 1980). Adjustment to appropriate densities to determine the most ideal gradient for the separation of JG cells was achieved by a serial dilution with the cell suspension media and centrifugation at varying centrifugal forces and times with density marker beads.

The effect of various densities of Percoll solutions on the separation of renin-containing cells from disaggregated renal cortical cell suspensions was tested by trypan blue exclusion and assay of renin and lactate dehydrogenase specific activities. By this method a 45% Percoll gradient was selected as the most ideal concentration for the separation of JG cells (Figure 7). Knowledge of the sedimentation density (approximately 1.06 g/ml) for the renin-containing cells allowed the location of such cells with certainty in all future experiments. This density correlates well with that reported by others in similar isolations of renin-containing cells with Percoll gradients (Kurtz et al., 1986a; Johns et al., 1987). In our hands, a 45% Percoll gave a much sharper band of JG cells with more than 92% of the renin-containing cells confined to two adjacent fractions in the gradient (Figure 7) than any of these other reports. The two JG enriched fractions in the 45% gradient had only 7% of the total protein in the gradient indicating that 93% of the renal cortical protein (and by inference most of the other renal cortical cell types) were not present in this JG-enriched fraction. The 40% Percoll, of Johns and coworkers (1987) resulted in two

nonadjacent bands of renin-containing cells dispersed over six of the ten fractions in the gradient (Figure 7). This same group also reported the recovery of two bands of renin-containing cells with the lower band (d.1.067) showing a threefold greater renin specific activity than the upper band (d.1.033). In these experiments, the 40% Percoll showed a very similar threefold greater renin activity in the lower band in comparison with the upper band. However, the 45% Percoll gradient produced a single significant band of renin-containing cells representing 92.4% of the renin specific activity of the total gradient and a 2,121-fold purification over that measured in the applied cell suspension (Figure 11). Kurtz and coworkers (1986) used a 25% Percoll and reported the recovery of two bands of renin-containing cells, an upper band (Band II, d. 1.05 g/ml) with 11% of the total renin specific activity and a lower band (Band III, d. 1.06 g/ml) with 87% of the total renin specific activity. Thus the 45% Percoll gradient gives a significantly greater purification of renin-containing cells than any of these other reports. The possibility that some of these renin-containing cells may be mesangial cells rather than JG cells cannot be absolutely excluded since mesangial cells have been reported to band at a density of 1.048 g/ml in iso-osmotic Percoll (Johns et al., 1987) and the density marker beads for 1.048 and 1.062 formed very close adjacent bands in the 45% gradient. Increasing or decreasing either the centrifugal force or the time of centrifugation did not succeed in a further separation of these beads.

C. <u>Physiological responsiveness of the JG cell-enriched suspension</u>.

Having developed an adequate renin-containing cell isolation procedure, we next sought evidence that the isolated cells had maintained functional ability despite the experimental manipulation required to isolate the cells. Factors which physiologically stimulate renin secretion are proposed to do so by lowering cytosolic Ca, and those which inhibit secretion do so by raising Ca (Fray et al., 1983a). Several reviews have appeared which repeatedly point to Ca as the common signal most likely responsible for controlling renin secretion ( Fray, 1980b; Fray et al., 1983a; Freeman and Davis, 1983; Hackenthal et al., 1983; Fray et al., 1986). Using rabbit and dog renal cortical slices, Park and coworkers (1986) have recently provided direct experimental evidence in support of this proposed inverse relationship

between renin secretion and cytosolic Ca. In the present study very similar results to those of Park et al. (1986) were obtained using the isolated JG cell enriched suspension described above.

Forskolin, a potent activator of adenylate cyclase (Seamon & Daly, 1981) was chosen to avoid the potential influence of the ß-adrenergic receptor responding to the varying ionic composition of the incubation media as expected with actively metabolizing cells, and to circumvent the potential problems of JG cell membrane permeability to cAMP and its derivatives. It was originally proposed that forskolin acts by direct stimulation of the catalytic component of the membrane-bound adenylate cyclase system, because it stimulates adenylate cyclase activity in guanine nucleotide regulatory protein-deficient mutants of S49 murine lymphoma cells (Johnson et al., 1978) and in other cyclase preparations apparently devoid of hormone receptors or guanine nucleotide regulatory proteins (Seamon et al., 1981; Daly, 1984). Other studies have indicated that both the stimulatory and inhibitory forms of the guanine nucleotide regulatory protein that couple receptor and catalytic subunits, as well as more poorly characterized components of the adenylate cyclase system, although probably not essential for the action of forskolin, at least appear to exhibit some modulating effects (Green & Clark, 1982; Stengel et al., 1982; Seamon & Daly, 1982; Morris & Bilezikian, 1983; Barber & Goka, 1985). Hence the exact molecular mechanism of action of forskolin is still obscure.

Seamon and coworkers (1981) were the first to show that forskolin, besides its direct effect on adenylate cyclase, also potentiates the effect of hormones on this enzyme. This "two-site-model" was also described by Green and Clark (1982) for S49 lymphoma cells and Barovsky et al. (1984) for C6-2B rat astrocytoma cells. In addition, forskolin also has the capacity to reverse agonist-dependent desensitization in some types of cells (Darfler et al., 1982; Daly, 1984). Very recently, Krall and Jamgotchian (1987) reported further evidence in support of the "two-site-model", although they pointed out that both sites may reside in the same heterogeneous N-regulatory protein complex. They found that rat aortic endothelial cells in cell culture had significantly higher N-nucleotide-dependent activity when pretreated with forskolin, compared to untreated cells. Furthermore, the effect of forskolin exposure at the level of the N-regulatory protein in these cells did not seem to extend to the coupling of the ß-adrenergic receptor with the catalytic subunit. Therefore, subunits of the N-protein different than those that couple receptors

with the catalytic subunit may function in forskolin activation (Krall & Jamgotchian, 1987). Shi and coworkers (1986) have proposed a working model of forskolin activation of adenylate cyclase readily admitting that it is probably an oversimplication of the true picture and most likely suffers from the limits of the current methodology used in the studies to elucidate the model. However, the model does provide for a low affinity, high capacity forskolin binding site as well as a high affinity, low capacity binding site and illustrates the possibility of forskolin activation of the free catalytic subunit of adenylate cyclase as well as activation of the N-protein-catalytic subsite complex of adenylate cyclase. It thus allows a working hypothesis accounting for the currently apparently conflicting data on forskolin activation of adenylate cyclase (Appendix II).

Ziyadeh et al. (1985) reported that the addition of forskolin to frog skin increased the secretory Ca flux severalfold, inducing net Ca secretion, while the absorptive flux was unchanged. Administration of vasopressin or PTH, agents known to increase intracellular cAMP production in their appropriate target tissues, had no significant effect on Ca transport. This suggests the existence of discrete adenylate cyclases resulting in equally discrete cytosolic pools of cAMP or since cAMP is thought to exert its physiological effects by activating cyclic AMP-kinases (Seamon & Daly, 1981), it may represent the activation of a specific forskolin sensitive cAMP-kinase which mediates Ca efflux. In support of this hypothesis, Leitman and coworkers (1986) recently reported that forskolin increased cAMP-kinase activity ratio in a dose-dependent manner in cultured bovine aortic endothelial cells. A similar dose-dependent inhibition of Ca uptake by forskolin (0-100μM) in rat aortic cells cultured for 20 hours in the presence of 25μM forskolin has also been reported (Krall & Jamgotchian, 1987). At least one form of forskolin-insensitive adenylate cyclase has been reported in mammals (Seamon & Daly, 1981), i.e. sperm adenylate cyclase.

In view of its ability to stimulate net Ca secretion (and by inference a decrease in cytosolic Ca) it is not surprising that forskolin markedly stimulated renin secretion in vitro from rabbit renal cortical slices (Park et al., 1986), from isolated perfused rat kidneys (Fray & Park, 1986), and from isolated JG cells in culture (Kurtz et al., 1984). In the present study forskolin also significantly stimulated renin secretion ($p < 0.01$) from the JG cell-enriched suspension. Since forskolin has been shown to modulate

Ca channels in other cells (Siegelbaum & Tsien, 1983) and to stimulate a net Ca secretion as described above, it seems reasonable to propose that forskolin may have similar effects on JG cells. Furthermore, Kurtz et al., (1984) have shown that forskolin stimulates renin secretion by increasing cAMP production in JG cells and that it decreases Ca influx and Ca permeability of JG cells. Fray and Park (1986) have presented evidence suggestive of a mechanism of forskolin stimulation independent of or noncompetitive with extracellular Ca concentration, since raising intracellular Ca by depolarizing the cells with high K reversed the stimulatory effect of forskolin to the same absolute level regardless of media Ca in the range of 1.25 to 5mM Ca.

As discussed earlier, the intracellular signal of the ß-adrenergic pathway may be cAMP, but it is uncertain whether cAMP is the final signal which couples ß-stimulation with renin secretion. In these experiments, exogenous cAMP strongly ($p < 0.01$) stimulated renin secretion from the cortical cell suspension enriched in JG cells. A first step in assessing the regulatory role of cAMP is the measurement of variations in the intracellular concentration of cAMP in different metabolic situations. Such measurements, however, are not completely straightforward (Ullmann & Danchin, 1983) and reliable data are scarce. Changes in cAMP concentrations are most often produced by signals acting on the plasma membrane (Gancedo et al., 1985). In higher eukaryotes, cAMP changes generally depend on the action of hormones, and extracellular cAMP, itself, acts like a hormone, binding to a specific membrane receptor and stimulating adenylate cyclase (Devreotes, 1983).

For a large group of cAMP actions mediated by protein phosphorylation, the target proteins may not be enzymes but proteins which control processes like transcription, translation, membrane permeability, or hormone secretion. In rat pituitary cells, increased cAMP rapidly stimulates the transcription of the prolactin gene (Murdoch et al., 1982); the possibility that differential phosphorylation by a variety of protein kinases could direct translation of different mRNAs has been proposed and supported by studies in vitro with synthetic messengers (Burkhard & Traugh, 1983); phosphorylation of membrane proteins by a cAMP-dependent protein kinase increases ion transport in higher eukaryotic cells (Greengard, 1978); and cAMP enhances membrane permeability towards a variety of compounds by a mechanism which could involve activation of the plasma membrane ATPase (Foury & Goffeau, 1975).

Finally, the possibility of cAMP-dependent phosphorylation of membrane proteins causing changes in Ca fluxes has been the subject of intense studies (Rasmussen, 1981) that show that phosphorylations triggered by either cAMP or Ca are to some degree interdependent. In view of the strong inverse correlation between cytosolic Ca and renin secretion (Park et al., 1986) and the fact that cAMP lowers cytosolic Ca by promoting Ca efflux and sequestration as well as reducing influx, perhaps by decreasing plasma membrane permeability to Ca (Kurtz et al.,1984; Fray & Park, 1986), it seems likely that changes in intracellular Ca levels assume pivotal regulatory importance in the renin release process. However, the ultimate rate of secretion may be dependent upon the net effect of the strength of the Ca inhibitory and cAMP stimulatory signals as suggested by Fray & Park (1986). On the other hand, Park et al. (1986) recently demonstrated that cAMP may exert a direct stimulatory action on the renin secretory process independent of the Ca inhibitory pathway. Precedence for such distinctive biochemical pathways for regulation of secretory processes has been reported for the secretion of insulin (Schubart et al., 1980), aldosterone (Balla et al., 1982) and pancreatic amylase (Heisler et al, 1981). Direct measurements of cytosolic Ca and cAMP will be necessary to settle the issue of Ca vs. cAMP decisively.

At low concentrations ionomycin, a divalent cation ionophore, has been reported to produce a sustained enhancement in intracellular Ca in the $GH_4C_1$ strain of rat pituitary cells (Albert and Tashjian, 1986). This appears to be due to a small amount of ionomycin which partitions into the plasma membrane to cause Ca influx. Selective partitioning of ionophores into biological membranes has also been described for polyene antibiotics (Lampen et al., 1962). Amphotericin B, for example, permeabilizes membranes to monovalent ions in proportion to membrane cholesterol content. Conversely, the efficacy of valinomycin to transport K ions selectively across human erythrocyte membranes is inversely proportional to cholesterol content (Feingold, 1965). Like ionomycin, valinomycin acts on intracellular membranes rather than the cholesterol-containing plasma membrane (Felber & Brand, 1982; Labelle, 1979). It is unknown whether cholesterol content is the sole or major determinant of membrane-specific partitioning of ionomycin. However, it has been shown to permeabilize plasma membranes to Ca (Vaartjes et al., 1986) causing Ca influx; to act directly as an ionophore to release Ca from intracellular

stores; and to bypass inositol triphosphate as a mediator for release of cellular Ca (Gershengorn et al., 1985).

Furthermore, addition of 1μM ionomycin to platelets caused a small transient drop in labeled $PIP_2$ followed by a 3-fold increase in diacylglycerol levels (Rittenhouse and Horne, 1984), a potent activator of protein kinase C (PKC) (Nishizuka, 1984). There is now growing evidence that stimulation of PKC opens Ca channels (De Reimer et al., 1984). Kurtz et al. (1986b) recently demonstrated that an activation of PKC by the phorbol ester 12-O-tetradec- anoylphorbol-13-acetate (TPA) stimulated Ca influx and inhibited renin release. Exactly which family of Ca channels is being acted on by PKC has not been determined for JG cells. However, in hepatocytes its been shown that the vasoconstrictors, angiotensin II, arginine vasopressin and norepinephrine all open a common pool of Ca channels (Mauger et al., 1984); and Kurtz et al. (1986b) has demonstrated that all of these substances inhibit renin release by the phospholipase C, diacylglycerol, PKC pathway in JG cells. Hence they may act in JG cells via a similar common pool of Ca channels as they do in hepatocytes. It seems reasonable to conclude that the observed inhibition of renin release by ionomycin in these experiments may reflect the activation of the PKC pathway inducing Ca influx through opened Ca channels as well as the release of Ca from intracellular pools. In support of this hypothesis, Pollock and Rink (1986) have recently demonstrated that addition of ionomycin to human platelets elevated intracellular free Ca from less than 100nM to a maximum of greater than 3μM, presumably by discharge of Ca from internal stores. They postulated that the major role of the accompaning Ca influx was to prolong the rise in intracellular Ca rather than to make it larger. The relative importance of the two processes, influx through the plasma membrane and cytosolic redistribution, as well as the precise transmembrane route of the influx has not been elucidated.

**D. <u>Plasma membrane isolation and characterization</u>.**

This study was designed to develop a simple and rapid technique for isolating plasma membranes of JG cells, to characterize the technique, and to show that such a preparation may be useful in the study of renal renin secretion at the plasma membrane level. Development of the technique was facilita-

ted by first harvesting an enriched population of JG cells from the kidney as described earlier. Since the JG cells constitute <0.03% of the total renal cells (Skøtt, 1986), the demonstration in this study of a population of >90% enrichment of JG cells is significant (Figure 8). The Percoll gradient technique used for the preliminary cell isolation is modelled after that recently reported by Kurtz et al. (1986a) in which they obtained >80% enrichment. As described earlier, the JG cell preparation isolated by Kurtz et al.(1987) as well as by the present study responds to renin secretagogues such as forskolin and cAMP as well as to the Ca ionophore, ionomycin (Figure14). The JG cells isolated in the present study (Figure 12) resemble those reported by Kurtz et al. (1986a) and therefore provide confirmation of their studies.

Although the method employed in the present plasma membrane purification technique is a modification of that of Prpic et al. (1984), several features were found to be essential for the successful preparation of highly purified PMVs. The new modification described here has several advantages: it is fast, taking less than 2 hours; it is simple, requiring a single low speed centrifugation, a brief centrifugation in a Percoll self-forming gradient, and two high speed washes; it is effective with routine yields at least 8-fold greater than other reported methods (Maeda et al., 1983), and it results in relative enrichments of the ratio of plasma membrane marker specific activity to granule/ lysosomal marker specific activity (i.e. alkaline phosphatase/acid phosphatase) in the range of a 45- to 50-fold increase over that measured in the original homogenate.

The features that are essential for success with the preparation are: first, to avoid autolysis, the excised kidneys were immediately submerged in ice-cold isotonic medium; cortical slices were removed and washed in the same ice-cold medium and the tissue was homogenized, all within 5 minutes of the initial nephrectomy. Second, to increase the size of the resulting membrane vesicles and/or decrease the production of small membrane fragments, a very gentle manual homogenization was employed, with minimal generation of bubbles or vacuum during either the up or down strokes. Third, to minimize the loss of peripheral proteins and to facilitate the formation of well sealed vesicles (Robinson, 1975), an isotonic medium (0.3M sucrose or KRB) was used throughout the procedure. Fourth, to increase the generation of a relatively homogeneous population of PMVs of predominantely inside-out orientation, the sample was forcefully passed through a #26-gauge 1/2 inch hypodermic needle (Steck et al., 1971).

This procedure was repeated several times in the course of the isolation and washing procedures. In addition, Percoll-derived membranes have been reported to have a higher proportion of inside-out polarity (Prpic et al,1984). Finally, to reduce contamination of endoplasmic reticulum membranes, the 1400 x g pellet was resuspended in a very diluted 6% (w/v) suspension. The technique allowed the less dense plasma membrane fraction to migrate near the top of the Percoll gradient, whereas the heavier endoplasmic reticulum fraction banded near the surface of the bottom pellet, thus well removed from the upper plasma membrane fraction (Figure 9). The renin-containing cells of the renal cortex are known to be modified smooth muscle cells and the sarcoplasmic reticulum of the smooth muscle cell is a convoluted system of tubular membranes located beneath the sarcolemma (Carsten, 1969). Some of these membranes, therefore, might be expected to co-purify readily as a contaminant of the sarcolemma. However, the low glucose-6-phosphatase specific activity of the plasma membrane fraction compared to that of the original homogenate or the location of the endoplasmic reticulum fraction of the Percoll gradient well removed from the plasma membrane fraction, suggests that endoplasmic reticulum contamination of the plasma membrane fraction is minimal (Figure 9).

Monitoring "marker" enzyme specific activities has become a widely used method of following and subsequently characterizing subcellular fractions. However, in recent years the concept of unique "markers", particularly for organelles of the secretory pathway (i.e., RER→ Golgi→ Granules→ PM) has been re-evaluated (Stanley et al, 1980; Muller et al., 1980). As a result, several uncertainties in the use of markers for the plasma membrane of polarized myoepitheliod cells, such as the JG cell of the afferent arteriole, are recognized. The first is that the evidence currently suggests that overlap of markers among the secretory compartments may occur (Hubbard et al., 1983), but the extent of the overlap remains to be elucidated. The second is that the probable functional heterogeneity of the rat renal JG cell membrane may necessitate the use of different plasma membrane markers for the different domains. Since the majority of the JG cells are found in the media of the afferent arteriole (Bing & Kazimierczak, 1962), they may be bordered by a variety of different cell types including lacis cells, macula densa cells, and endothelial cells separating the JG cell from the lumen of the afferent arteriole. This mixed cell popu-

lation may well provide for a variety of intercellular communications resulting in extremely diversified plasma membrane domains on the surface of the JG cell. Evidence for the existence of such specialized domains can be inferred from the frequently observed extensive involution of the JG cell membrane with its folds penetrating deep into the interior of the cell (Peter, 1976; Ryan et al., 1982; Hill et al., 1983) and in some cases appearing to be continuous with the endoplasmic reticulum (Hartroft & Newmark, 1961). Furthermore, the proximity of JG cells to macula densa cells suggested to McManus (1947) the existence of possible cytoplasmic bridges between the JG cells and the macula densa cells requiring additional specialization of the juxtaposed surfaces of the two cells. Subsequent electron microscopic observations support this early hypothesis (Hartroft and Newmark, 1961). The third is that there is some evidence for the existence of internal (recycling?) pools of plasma membrane enzymes (Stanley et al., 1980). The fourth is that JG cells are known to be connected by junctional complexes (Biava & West, 1966) believed to be gap junctions (Rouiller & Orci, 1971; Taugner et al., 1984b), while no gap junctions have ever been observed between the JG cell and the and the adjoining macula densa cell (Fray et al., 1987). The fifth is that the biochemical assays for plasma membrane markers such as alkaline phosphatase may not be specific for one enzyme in one location and are usually substantially different from the cytochemical assays used to localize a particular activity in situ. Nevertheless, alkaline phosphatase activity has been chosen as a plasma membrane marker and this activity was enriched 23-fold in the final plasma membrane fraction (Figure 15). Both the yield and relative enrichment of this traditional marker were comparable to that reported by others for rat renal cortical plasma membrane isolation (Nishimura et al., 1980; Ward et al., 1976).

In addition to the use of conventional biochemical markers to identify and quantitate the various subcellular components of the isolated PMVs, electron microscopy was used (Figures 16a-16c) to assess the relative purity and integrity of the final PMV fraction. This evidence, coupled with an ATPase binding assay (Figure 17) confirmed that more than 87% of the vesicles were sealed and were of inside-out orientation. Results presented in this study demonstrate that the final PMV preparation consists of 6% leaky vesicles, 7% right-side-out vesicles and 87% sealed inside-out vesicles. The membrane orientation has been evaluated by measurement of the ouabain-sensitive (Na/K) ATPase activity in the presence and

absence of detergent pretreatment. The possibility exists that this method underestimates the percentage of sealed inside-out vesicles since by the criteria of the ATPase assay, the ATPase activity of inside-out vesicles would be limited by the generation of a Na gradient (Boumendil-Podevin and Podevin, 1983). Furthermore, if some external ATP enters the intravesicular space of right-side-out vesicles, the trapped ATP would be rapidly hydrolyzed, due to the relatively high concentration of ATPase, before binding of ouabain could occur to the outside of right-side-out vesicles. Thus, the homogeneity of the preparation orientation and integrity may be even greater than indicated in these results.

## E. Trypsin activation of membrane-bound renin.

Wilson and coworkers (1976) postulated that membrane-bound renin may represent a higher molecular weight, but less active, precursor of soluble renin. Nishimura et al. (1980) reported that membrane-bound renin can be activated by Triton X-100, phospholipase $A_2$, lysolecithin and melittin such that untreated plasma membrane renin has less than 3% of the activity of the fully activated preparation. Barrett and coworkers (1982) reported that plasma renin activity could be increased 8-fold with the addition of 5 mg trypsin/ml plasma and that a concommitant concentration-dependent decline in renin activity could be observed after addition of soybean trypsin inhibitor (SBTI). Furthermore, Toogood et al. (1983) reported that trypsin digestion by itself does not lead to disruption of the membrane, for the membranes can continue to carry out ATP-stimulated calcium accumulation after treatment with trypsin.

To investigate the nature of a possible plasma membrane translocation of nascent chains of secretory renin, limited proteolysis of PMVs by trypsin was used as a means of dissecting this activity into functional components and to determine if membrane-bound renin is present in an inactive form. Washed isolated PMVs were incubated with trypsin on ice for 30 minutes and the reaction was stopped by the addition of soybean trypsin inhibitor. The rationale was that the translocation activity might be represented by transmembrane protein(s) that should contain cytosol-exposed domain(s) that are readily accessible to proteolytic enzymes (Blobel & Dobberstein, 1975a). On the premise that these domains are globular in nature, conditions were sought that would permit their proteolytic severence in a form

sufficiently intact so that their readdition to the proteolytically depleted and inactivated membranes would restore translocation activity. Other investigators have demonstrated that translocation activity of microsomal membranes can be assayed in cell-free systems (Shields & Blobel, 1978; Blobel & Dobberstein, 1975b; Dobberstein & Blobel, 1977). The results (Figure 18) support the conclusion that membrane-bound renin is present in an inactive, but trypsin activable form. Inactive renin has been defined as the absolute difference in renin activity between trypsin treated samples (representing the total renin present) and the untreated controls (representing endogenous active renin) [Barrett et al., 1982]. According to this definition and in light of these experiments, at least 50% of membrane-bound renin appears to be in an inactive, but trypsin-activable form. Given the proximity of the plasma membrane to the extracellular compartment, and the abundance of cytosolic and membrane proteases similar to trypsin (Gossrau, 1985), it seems reasonable to propose that this high incidence of membrane-bound renin in an inactive form, with a trypsin-sensitive cytosol-exposed domain, represents a readily releasable store of renin. Precedence for this interpretation is found in the in vivo studies of Barrett and associates (1982; 1981). After mild renin stimulation (restraint) in in vivo experiments, active renin increased and inactive renin decreased in a reciprocal manner, but total renin remained unchanged (Barrett et al., 1982).

These data from trypsin activation of isolated PMVs offer evidence for a possible mechanism for Barrett et al's. (1981) in vivo renin activation. After more vigorous stimulation of renin release by ether, Barrett and coworkers (1981) noted a further significant increase of active renin with little or no change of inactive plasma renin concentration. It is well documented that anesthetics influence plasma membrane function (Seeman, 1972; Nishimura et al., 1980b) and since $11.2 \pm 0.6\%$ of the total renin content of the PMVs preparation [i.e. $\Sigma$(EVC, IVC, PMC)] is found in the plasma membrane fraction (Figure 23), a local renin releasing effect on the PM compartment during ether anesthesia seems to be indicated. Furthermore, as propranolol, an adrenergic ß-receptor blocking agent, apparently inhibits the renin release induced by anesthesia (Pettinger et al., 1975), and pertussis toxin, an $\partial_2$-adrenergic blocker and a ß-adrenergic magnifier, potentiates anesthesia-induced renin secretion (Pedraza-Chaverri et al., 1985); a plasma membrane localized renin store is one of the possible sources of the increased plasma renin activity reported by Barrett and coworkers (1982).

In reporting that trypsin digestion per se does not lead to membrane vesicle fragmentation or to large nonspecific holes in the membrane, Toogood et al. (1983) also demonstrated that trypsin treated membranes are highly impermeable to sucrose, suggesting a normal stability of the membranes with regard to their barrier function for small molecules. Furthermore, trypsin digestion of sarcoplasmic reticulum membrane vesicles, preloaded with Ca, stimulates Ca efflux possibly by preferentially exposing Ca channels in the membrane (Toogood et al. (1983). The mechanism of trypsin-stimulated renin release from isolated plasma membrane vesicles may involve a trypsin modification of a Ca ionophore of the channel or pore type in the plasma membrane. Specifically, trypsin hydrolyzes arginine- and lysine-associated peptide and ester bonds (Waymouth, 1982).

**F. Renin secretion from isolated perfused kidneys and renin activity of renal tissue compared to that of isolated renal cortical PMVs of hypophysectomized and sodium depleted rats.**

The pituitary gland is known to influence a wide variety of physiological functions, but the exact mechanism by which it influences arterial blood pressure and the renin-angiotensin system remains to be elucidated. Hypophysectomized (Hx) rats are severely hypotensive (Leathman & Drill, 1944) and despite the fact that low blood pressure is a potent stimulus of renin secretion, Hx rats exhibit an inappropriately low plasma renin activity compared to that usually measured in hypotensive animals (Honeyman et al., 1983). This is even more paradoxical when it is con- sidered that Hx rats have a greatly increased renal renin content but their PRA and basal renin secretion rate in vitro are normal while their response to isoproterenol and Ca omission are signficantly attenuated (Simon et al., 1984). This suggests that the sensitivity of the kidney to release renin may have been suppressed in the absence of the pituitary. Thus, renin release following hypophysectomy might involve indirect biochemical responses to pituitary-deprivation, rather than a direct endocrine response. The plasma membrane compartment may represent a readily releasable pool of renin and may account for the increased plasma renin activity observed in Na-deprived rats. The observation that Hx rat plasma membranes also have a high renin activity comparable to that found in the Na-deprived plasma membranes and yet fail to show an elevated PRA seems to suggest a defect in the Hx rat plasma membrane selectively inhibiting the

release of this membrane pool of renin. The additional observation that a high K medium in the absence of Ca triggers the release of membrane-bound renin, but inhibits renin release in the presence of Ca, lends support to an earlier hypothesis that renin release may involve membrane depolarization and a membrane-localized Ca-mediated response (Fray et al., 1983).

Sedlak (1985) recently reported that one of the long term effects of hypophysectomy in rats is a significant decrease in non-protein sulfhydryl groups (NP-SH), total (T-SH) and protein-bound sulfhydryl groups (PB-SH) in the kidney, thyroid, adrenal, liver and skeletal muscle of Hx rats. In the kidney, the most significant decrease was observed in the amount of PB-SH and the NP-SH, specifically, glutathione-SH (GSH). Sedlak (1985) hypothesized that this decrease in PB-SH and GSH concentrations was caused mainly by a disappearance of the stimulatory effect of tropic hormone on proteosynthesis and biosynthesis of GSH. Numerous studies have reported alterations in proteosynthesis rate, loss of maturation, as well as, decreases in the activity of many enzymes after HX (Greer et al., 1968; Baliga et al., 1973; Trzeciak et al., 1979). Kosower et al. (1972) has suggested that the level of GSH and of oxidized GSH (GSSG) as well as the equilibrium GSH:GSSG play an important role in the regulation of proteosynthesis. High intracellular concentrations of the SH-reducing agent glutathione normally functions to maintain a reducing environment in the cytosolic compartment of the cell, thus preventing the formation of disulfide bonds between neighboring cysteine-SH groups in a polypeptide chain. In the absence of normal concentrations of GSH, zymogens such as renin could conceivably prematurely acquire S-S bridges. This premature folding of the polypeptide chains could block the normal secretory process by trapping the enzyme in some presecretory compartment. This could explain why the increased renal renin content was unaffected by hypophysectomy (Figure 19), while renin release was significantly impaired (see Figure 20 A). Alternatively, if a protein-bound SH group is part of a membrane transport complex, the conversion of L-cysteine to L-cystine residues would be expected to affect the binding of the enzyme to the membrane transport complex.

While the precise influence of a low sodium diet on the renin angiotensin system remains controversial (cf pp. 24 - 33 in thesis introduction), a number of specific effects, of particular relevance to the current project have been reported. At the cellular level, an increased JG cell renin content (Gillies &

Morgan, 1978), an augmented rough endoplasmic reticulum proliferation evidenced by increased D-glucose-6-phosphatase activity and electron microscopy ( Morimoto et al., 1979; Hill et al., 1983 ), more plentiful and more developed Golgi with numerous coated vesicles (Desormeaux et al., 1982; Hill et al., 1983), an increased number of granules per cell (Hill et al., 1983); an increased intracellular K:Na ratio and intracellular Na concentration, and altered Na pump kinetics (Jest et al., 1985; Wilkins et al., 1985) have all been associated with Na depletion. Deep channel-like plasma membrane invaginations were seen by Hill and coworkers (1983), sometimes coming into close proximity to granules in JG cells, but only rarely was there evidence suggesting granule exocytosis. In contrast, exocytotic release of peripolar cell granule material was commonly observed in both immersion fixed tissue and in perfusion fixed tissue after several days of Na depletion (Hill et al., 1983).

At the extracellular and organ levels, Na depletion is believed to increase the number of JG cells (Desormeaux et al., 1983; Lindopp and Lever, 1986), the percent of renin-positive efferent arterioles (Taugner et al., 1981), and the renin activity of deep JGA over superficial JGA. Deep JGA have a decreased ability to respond to acute volume expansion (Kaufman et al., 1980) and would therefore be less sensitive to potential osmotic changes during the isolation procedure.

At the systemic level, Na depleted rats show evidence of an increased responsiveness of the renin-angiotensin release system (Fray, 1978) and subsequently an increased PRA, increased blood pressure, and decreased serum levels of Na (Munoz-Ramirez et al., 1980). They also have increased transmitter stores in adrenergic nerves and decreased transmitter release per nerve impulse as well as attenuated neurohormonal responses to altering stimuli (Nilsson et al., 1985) and an increased TPR index (Omvik and Lund-Johansen, 1986).

Since Na deprivation plays so many important roles in regulating both directly and indirectly the activity of the JGA, and particularly since alterations in the cellular handling of Na have been implicated in essential hypertension (Blaustein, 1984), it was germaine to this project to investigate the effects of a low Na diet at the renal tissue and plasma membrane levels of organization.

In these experiments, renin content, secretion, and PMV storage were significantly altered by changing the amount of Na in the diet. These results are comparable to those reported by others (Fray,

1978; Gillies and Morgan, 1978; Morimoto et al., 1979). Interestingly, plasma membrane renin from the renal cortex of the same Na deprived rats was increased by almost the same proportion as the renal renin content compared to that measured in normal rats; i.e. both tissue renin and plasma membrane renin was 3-fold greater in Na-deprived rats compared to normal rats (Fig. 19). Figure 20 A indicates that isolated perfused kidneys from Na-deprived rats in the absence of any stimulating influence, secrete twofold more renin than kidneys from rats on a normal diet, but plasma membrane renin from Na-deprived rats was fourfold greater than that measured in plasma membranes isolated from rats on a normal diet (Figure 20 B). Hence, while rats on a low sodium diet secrete more renin than rats on a normal diet, their plasma membranes store a substantially greater amount.

Isoproterenol (2.83μM) induced renin release in kidneys from Na-deprived rats as well as in kidneys from rats on a normal diet, but the magnitude of release from Na-deprived rats was greater than that from rats on a normal diet. A similar increased renin secretion rate has been reported when kidneys from Na-deprived rats were exposed to isoproterenol (Fray, 1978) . Correspondingly, plasma membrane renin in membranes isolated from Na-deprived rats was slightly, but not significantly, increased while that measured in plasma membranes from rats on a normal diet was not altered in the presence of isoproterenol (Figure 20B). These experiments demonstrate that although a substantial amount of renin was released from kidneys of both Na-depleted and Na-repleted rats, the renin content of their plasma membranes was maintained at a relatively constant, albeit much higher, level. Renin secretion in absence of Ca was 102-fold greater than in the presence of Ca in kidneys from rats on a normal diet, whereas in kidneys from Na-deprived rats, renin secretion increased 83-fold over that measured in the presence of Ca (Figure 20 A). Plasma membrane renin from Na-deprived rats after 15 minutes perfusion in the absence of medium Ca was slightly, though not signficantly increased over that measured in the presence of medium Ca; whereas membrane-bound renin content did not change in the presence or absence of Ca in plasma membranes isolated from rats on a normal diet.

Taken together, these results suggest that plasma membrane renin from both Na-deprived and normal rats consistently either remained constant or increased slightly in the presence or absence of perfusion media Ca or in the presence or absence of perfusion media isoproterenol. These results provide

evidence for a plasma membrane localized mechanism of renin storage and controlled release. The constancy of this plasma membrane renin argues against a random release of renin from the plasma membrane compartment, as well as the possibility that the observed release may be the result of an artifactual disruption of the membrane structure itself.

In the present study, PMVs isolated from the renal cortex of Na-deprived rats had a significantly greater renin activity per gram of tissue ($p < 0.001$) than PMVs isolated from the renal cortex of normal rats. This data seems to support the earlier hypothesis that Na depletion elevates renal renin content (Ajzen et al., 1965) and suggests that this increase includes the plasma membrane compartment as well as the granular fraction reported earlier (Morimoto et al., 1979).

**G. The study of ionic and calmodulin influences on renin release from isolated JG cell plasma membrane vesicles.**

Ca as well as other ions have been suggested to play a role in renin secretion (Fray, 1977; Wilcox, 1978; Ettienne & Fray, 1979: Fray & Park, 1979) and the present studies may provide clues as to some possible mechanism of action at the level of the plasma membrane. Dietary Cl loading (in the absence of Na loading) has been reported to suppress renin activity in intact Sprague-Dawley rats (Kotchen et al., 1983; Kirchner et al., 1978). In isolated perfused kidneys of such rats, renin release was found to be inversely proportional to Cl reabsorption in the thick ascending limb of the Loop of Henle, but decreasing perfusate Cl, had no effect on renin release from nonfiltering kidneys (Rostand et al., 1985). In the present study, Cl had no effect on renin release from isolated PMVs, though PMVs incubated in medium containing Na released a greater amount of renin than those in Cl-containing medium (Figure 21). Thus the reported inhibitory effect of Cl on renin release (Kotchen et al., 1983) does not directly affect the plasma membrane renin pool of the JG cell, but may exert its influence on renin release indirectly at some other site. According to Rostand and coworkers (1985) this distal site may involve the renal tubular receptor with Cl reabsorption in the TAL and/or at the macula densa being the critical signal for renin release via the macula densa. Whatever the exact mechanism, the JG cell membrane renin pool has failed to respond in a significant manner to the presence of added Cl. A similar

lack of a renin inhibitory response to selective Cl loading was recently reported by Whitescarver et al. (1986) in which Cl loading had no effect on plasma renin activity in either Dahl salt-sensitive or renin-dependent hypertensive rats. This absence of a signficant Cl response may also provide indirect evidence for the low tubular contamination of this JG cell membrane preparation.

One of the early events in the coupling of a stimulus to renin secretion may be the hyperpolarization of the renal JG cell membranes (Fray, 1976; 1978). In support of this hypothesis, Ettienne and Fray (1979) have reported that high concentrations of extracellular Mg activate renin release by a mechanism which involves hyperpolarization of the JG cell membrane and an associated decrease in cytoplasmic Ca. Elevated concentrations of extracellular K, which inhibit renin release (Davis & Freeman, 1976) depolarize the cell membrane (Fishman, 1976). Lowering extracellular K below physiological levels also inhibits renin secretion presumably by inhibiting the Na/K pump and consequently shutting down Na/Ca exchange resulting in an increase in cytosolic Ca (Churchill & Churchill, 1979). By this line of reasoning, lowering the concentration of Na at the cytosolic surface of the plasma membrane (i.e. the surface exposed to the medium in PMV suspensions of mostly inside-out vesicles) would also shut down the Na/K pump and therefore Na/Ca exchange and the permeability of the plasma membrane to Ca. Thus medium Ca would not permeate the plasma membrane (by inference cytosolic Ca would not fall) and renin secretion would be inhibited. This was reflected by the 2-fold increase in media renin specific activity (i.e. the cytosolic compartment). Conversely, increasing media (cytosolic) Na would be expected to stimulate Na/K pump, Na/Ca exchange thus decreasing media Ca, and increasing renin secretion to the intravesicular space or to the membrane compartment. This sequence of events is evidenced by the decreased media (cytosolic) renin specific activity in the presence of high media Na (Figure 21). Furthermore, an accumulation of Na in the cytosolic compartment (i.e. an increase in medium Na in a PMVs suspension of mostly inside-out-vesicles) would depolarize the membrane, and the decrease in membrane potential would allow the opening of voltage-dependent Ca channels and facilitate the movement of Ca down its concentration gradient. In this preparation, PMV were water-washed and resuspended in KRB. Therefore, Ca flux would be reversed in comparison to the whole cell with an established Ca gradient. Ca in this isolated PMVs suspension would move from the EVC to the IVC on opening of

Ca channels. This decreased media Ca would be expected to increase renin secretion to the intravesicular compartment. This renin response is reflected in the decreased renin specific activity of the media (the EVC and by inference the cytosolic space).

To elucidate further the role of Ca in the regulation of renin release, the effects of two Ca related factors were investigated (Figure 22). In the presence of media Ca, plasma membrane renin was significantly less than the control (20 mM Tris-HCl); however, neither media EGTA nor media calmodulin significantly affected membrane renin (though a slight decrease was observed with each factor), but in combination, EGTA with calmodulin caused a substantial release of renin from the PMV significantly lowering the amount of renin in the plasma membrane ($p < 0.01$). This seems to add additional support to the hypothesis that calmodulin may be the intracellular Ca receptor responsible for transducing the Ca signal into the molecular event leading to inhibition of renin secretion (Park et al., 1986). EGTA would be expected to block the formation of the Ca-calmodulin complex (Weiss & Wallace, 1980), a necessary preliminary step in the cascade of processes leading to renin secretion. That EGTA or calmodulin alone were not able to effect renin release from the plasma membrane points to a secondary role for Ca and calmodulin in the renin secretory pathway. Perhaps, as suggested by Park et al. (1986) calmodulin could be a subunit of a Ca-dependent enzyme involved in the inhibition of renin secretion requiring a high concentration of calmodulin antagonists for the inhibition of the enzyme; or, as evidenced here, the aid of an additional Ca chelator coupled with an excess of calmodulin to completely deplete the Ca-calmodulin complex, causing a reversal of an active enzyme form [CaM-Ca-E] for inhibiting renin secretion, to a Ca-depleted, inactive form. In support of this hypothesis, trifluoperazine (Fray et al., 1983; Kawamura & Inagami, 1983), W-7 (Kawamura & Inagami, 1983) and calmidazolium (Park et al., 1986) were all found to be more effective in stimulating renin secretion at low concentrations of extracellular Ca or in the absence of Ca.

Additional experiments designed to determine quantitatively the distribution of renin in the EVC, plasma membrane and IVC confirmed that adding Ca to the media increased EVC renin (and by inference cytosolic renin) and inhibited renin secretion (Figure 23 A). High K in the presence of Ca significantly augmented this response ($p<0.001$) suggesting that depolarizing the plasma membrane with

high K is a significant factor in Ca-mediated inhibition of renin secretion. Combining plasma membrane depolarization in high K media with 0 Ca media (EGTA-supplemented media) caused a highly significant 30% increase in EVC renin ($p < 0.001$) with a concomitant significant decrease in IVC renin (i.e. inhibition of renin secretion), $p < 0.001$ (Figure 23 B). That high K media in the presence or absence of Ca caused such a significant increase in EVC renin strongly suggests that the effect of K on renin release may be mediated by membrane voltage or some other effect of this cation and not by Ca per se.

More direct evidence for a Ca mediated renin response is provided by the divalent cation data presented in Figure 24. Increasing media Ca significantly increased media renin specific activity and by inference decreased renin secretion to the intravesicular space. Increasing media Ba produced a very similar renin response to that evoked by Ca. That Ba can substitute for Ca in sustaining Ca-mediated physiological responses is well documented (Douglas & Rubin, 1964; Hotta & Tsukui, 1968; Uvelius et al., 1974; Hagiwara & Byerly, 1981; Uvelius & Sigurdsson, 1981; Tsien, 1983). In addition, Somlo and Hasson-Voloch (1987) very recently presented kinetic data showing the Ba inhibits membrane-bound Na/K-ATPase in isolated PMV of the electrocyte of Electrophorus electricus. Ba is also known to permeate the Ca channel in several excitable cells (Hagiwara and Byerly, 1981; Tsien, 1983) and was recently demonstrated to permeate the chromaffin cell membrane at least 10 times better than do Ca ions (Artalejo et al., 1987). Interestingly, the hydrated radius of Ba is reported to be smaller (0.415nm) than that of Ca (0.659 nm). This is nearly the same as the radius of hydrated K (0.413 nm) (Hansen et al., 1984). The small size of hydrated Ba can explain how the ion has easy access to the interior of the cell through channels gated too small to admit Ca and could explain its10-fold greater permeability.

In contractile responses, Ebeigbe & Aloamaka (1985) reported that the relaxation rates following high-K contractions were similar for Ca, Sr, and Ba suggesting a common mechanism for sequestration and extrusion. Both Ba and Ca have been reported to bind to calmodulin (Wolff et al., 1972) and both exhibit similar membrane stabilizing effects in smooth muscle cells of the rat tail artery (Ebeigbe & Aloamaka, 1985). In the present divalent cation dose-response study, both Ca and Ba significantly increased the amount of renin released into the media (and by inference, the cytosolic space) thus

reflecting a similar inhibition of renin secretion. Raising Ca concentration is known to inhibit renin secretion ( Kisch et al., 1976; Watkins et al., 1976; Kotchen et al., 1977) presumably via membrane depolarization and Ca influx. These experiments lend support to this hypothesis by demonstrating the direct correlation between increased cytosolic concentrations of Ca and Ba and increased cytosolic renin (and by inference a decreased renin secretion).

The renin secretory mechanism appears to be able to selectively distinguish among divalent cations since neither Mn nor Mg elicited the same response observed with Ca and Ba (Figure 24), though the Mg response was similar to that of Mn and was not signficantly different from that observed with Cl in Figure 21. The cation in the chloride experiments was Co and Smith and coworkers (1985) reported that Mg, Mn and Co can completely substitute for each other in studies of metal binding sites in Escherichia coli $F_1$-ATPase. In their study, fully reconstituted enzymes were active as ATPases and were able to rebind to $F_1$-depleted membranes and catalyze ATP-driven proton pumping across membranes. Both Mg and Mn have been reported to stimulate renin secretion (Churchill & Lyons, 1976; Fray, 1977; Wilcox, 1978; Ettienne and Fray, 1979; Logan and Chatzilias, 1980) and both cations are believed to be Ca antagonists in excitation-contraction coupling and stimulus-secretion coupling in hormone release (Hermansen and Iversen, 1978). In the present study, adding Mg or Mn to the incubation media reversed the effects of Ca and Ba on renin release to the extravesicular space. It seems reasonable to postulate that the decreased renin specific activity of the media may reflect an increased renin secretion to the intravesicular space, thus supporting the stimulatory role of Mg and Mn in renin secretion.

The reported reciprocal relationship between K and renin secretion (Churchill & Churchill, 1980) is supported by the data in Figure 25. Depolarizing concentrations of K (i.e. >140mM K) increased the renin specific activity of the EVC (and by inference inhibited renin release), whereas concentrations < 140mM K decreased the renin specific activity of the EVC and apparently stimulated renin release. That Ca is involved in this reciprocal relationship between K and renin secretion is evidenced by Figure 26 where increasing the concentration of K in the presence of media Ca shifted the

linear dose-response curve to the left and in the absence of media Ca (EGTA) the curve shifted to the right. Fishman (1976) has demonstrated that high concentrations of K depolarize the JG cell and others have shown that such depolarizing concentrations of K inhibit renin secretion in isolated perfused kidneys (Fray, 1980a), renal cortical slices (Naftilan and Oparil, 1982; Churchill, 1985; Park et al., 1986), isolated glomeruli (Baumbach and Leyssac, 1977), and isolated JG cells (Fray and Laurens, 1981; Kurtz et al., 1984). This K-linked inhibition of renin secretion is believed to mimic the depolarizing effects of high perfusion pressure and to involve an influx of Ca through voltage sensitive Ca channels. Evidence for this hypothesis is provided by the observation that raising perfusion pressure causes constriction in renal afferent arterioles (Edwards, 1983), presumably via depolarization-induced Ca influx. That voltage sensitive Ca channels are involved is evidenced by the fact that inhibition of renin secretion by high perfusion pressure is blocked by verapamil (Fray, 1980a). Furthermore, the calcium influx mechanism may be regulated by calmodulin since calmodulin has been shown to be a component of the Ca channel (Johnson, 1984) and high perfusion pressure inhibition of renin release is reversed by calmodulin inhibitors such as trifluoperazine and calmidazolium (Park et al., 1986; Fray and Park, 1986). The data in Figure 26 supports these hypotheses since the effectiveness of depolarizing concentrations of K in inhibiting renin release was augmented in the presence of media Ca (an experimental condition mimicking Ca influx) and attenuated in the absence of media Ca (an experimental condition mimicking the effect of blocking the Ca channels and resultant Ca influx).

## H. The ubiquitous distribution and postulated role of plasma membrane localized extrarenal renin.

Recent advances in molecular biologic techniques have enabled several groups of investigators to document that renin and angiotensinogen genes are expressed in many extrarenal and extrahepatic sites (Ganten et al., 1976; Hirose et al., 1980; Re et al., 1981, 1982; Naruse et al., 1981, 1983; Doi et al., 1984; Naruse et al., 1985; Pandey & Inagami, 1986; Dzau & Re, 1987). Furthermore, angiotensin as characterized by high performance liquid chromatography, radioimmunoassay, and quantitative autoradiography can also be detected in many tissues (Hermann et al., 1982; Phillips and Stenstrom, 1985;

Gehlert et al., 1986). Although once attributed to the nonspecific proteolytic activity of cathepsin D (Reid, 1977; Hackenthal et al., 1978), recent biochemical and immunological studies using specific antirenin antibody distinguished renin from other proteolytic enzymes in various tissues (Inagami et al., 1978; Guyene et al., 1980; Hackenthal et al., 1980; Hirose et al., 1980; Naruse et al., 1981). In reviewing the literature as early as 1976, Ganten et al. reported that angiotensinogenase activity, i.e. enzymes capable of forming angiotensin I during incubation with renin substrate, had been found in no less than 37 different tissues of humans, dogs, cattle, rabbits, mice and rats. Since this early review, numerous studies (as cited above) have demonstrated renin or reninlike enzymes (angiotensinogenases, isorenin) in an ever growing variety of extrarenal tissues such as uterus (Hackenthal et al., 1980) , pineal gland (Hirose et al., 1980), adrenal (Naruse et al., 1983), blood vessel walls (Swales et al., 1983), testes (Pandey et al., 1984), pituitary (Mizuno et al., 1985a), brain (Dzau et al., 1986), heart, (Dzau & Re, 1987). The presence of renin in extrarenal tissues raises an intriguing hypothesis that there exists a tissue renin-angiotensin system which can locally regulate tissue functions.

Naruse et al. (1985) has demonstrated that renin is not a contaminant of tissue preparations due to entrapped plasma, but is, indeed, endogenous to each tissue. The human brain renins from pineal and pituitary glands have been reported to share some biochemical features with well-known kidney renin, such as molecular weight (37,000-45,000 d.), optimum pH (6.0-7.0), the presence of trypsin-activable inactive renin, and a glycoprotein nature (Mizuno et al., 1985b). However, the electrofocusing pattern of renin from pituitary tissue (pI 4.43, and pI 5.77) differed from that of plasma and kidney enzymes previously reported, a discrepancy which the authors interpreted as evidence for the endogenous synthesis of renin in the brain tissue (Mizuno et al., 1985b). Iwao et al. (1982), investigating the distribution of exogenously administered $^{125}$I-labeled renin in mice, found no significant uptake of renin in tissues other than the kidney, further indicating endogenous synthesis of renin in these extrarenal tissues.

However, since the granules containing renin in most extrarenal cells are probably lysosomes (Barajas & Salido, 1986), it is tempting to speculate that perhaps renin was never "intended" to circulate, but only to act as a local agent in the control of vascular tone. Control of renin release is probably multifactorial, but may be mediated by the sympathetic nervous system and locally controlled by ions such

as intracellular calcium concentration (Barajas & Salido, 1986; Churchill, 1985). Because the lysosomal system functions both in the uptake (heterophagocytosis) and discharge (secretion) of materials, it is conceivable that renin is avidly taken up and stored throughout the body for use in control of local vascular phenomena. The absence of evidence of uptake in mice as reported by Iwao et al. (1982) does not necessarily mean uptake does not occur. These negative results could reflect species differences, metabolic state at the time of assay or perhaps the frequency or amount of uptake was beyond the limits of the method employed and therefore went undetected.

Alternatively, since the JG cells have the genetic machinery to synthesize renin (Levens et al., 1981), there may be a population of cells distributed throughout the body, in analogy to the amine precursor uptake and decarboxylation system, which is capable of synthesizing renin in situ for a very localized release. If renin is being synthesized locally and released in a manner similar to that postulated for JG cells, or if renin is taken up by the extrarenal organs reported to contain renin, one would expect to find renin in the plasma membrane of these organs as is true of the JG cell membrane. Membrane-bound renin has been reported for the submaxillary gland of the Swiss Webster mouse (Wilson et al., 1976). In the present study, a significant amount of renin was found in the plasma membrane fraction of homogenized brain, heart, spleen, liver, lung, and skeletal muscle of Sprague-Dawley rats (Figure 27). The heart plasma membrane fraction had the highest amount of renin specific activity of all extrarenal tissues examined (i.e. 80% of that found in the kidney plasma membrane fraction). This result confirms the recent report of the existence of renin in isolated cardiac myocytes (Dzau & Re, 1987) and extends this finding by describing a measurable amount of cardiac renin in the plasma membrane fraction of homogenized cardiac tissue. This finding, coupled with the earlier reports of myocardial angiotensin II binding sites (Wright et al., 1984), the demonstration that angiotensin and its analogs appear to have a positive inotropic effect on the myocardium (Ahmed et al., 1975), and that nifedipine, which is known to have negative inotropic properties, lowered the intracellular renin activity of isolated cardiac myocytes in vitro (Dzau & Re, 1987), indicates that this system is subject to regulation and may be involved with the autocrine or paracrine control of cardiac contractility.

Plasma membrane involvement in the postulated cardiac renin-angiotensin system, as well as

other extrarenal renin-angiotensin systems, is suggested by the presence of specific angiotensin II receptors on the surface of cardiac cell membranes (Wright et al., 1984), brain membranes (Bennet & Snyder, 1976), adrenal cortex membranes (Naruse et al., 1985), and the growing evidence of internalization of these receptors (Robertson and Khairallah, 1971). Angiotensin II may thus have important effects on the cellular metabolism of the myocardium and other renin-positive tissue cells since angiotensin II receptors have also been reported on intracellular organelles such as mitochondria and nuclei (Robertson and Khairallah, 1971). Specific nuclear binding of angiotensin II by rat liver and spleen nuclei has also been reported (Re et al., 1981). Although the physiologic relevance of hormone internalization for cellular events other than receptor down-regulation is unclear, these observations are intriguing as a broad distribution of renin in diverse extrarenal tissue plasma membranes was demonstrated in this study. It is tempting to suggest that plasma membrane renin may serve as a readily releasable pool of renin for local renin-angiotensin system functions or it may be internalized in the phagosomal system of the extrarenal cell, and may be activated intracellularly by fusion with a primary lysosome. Since Triton X-100 solubilization was necessary to measure renin activity of extrarenal plasma membranes, it seems likely that most extrarenal plasma membrane renin is present in an inactive form as was determined for renal plasma membranes.

## I. Summary and conclusions.

Methods for the preparation of a renal cortical cell suspension enriched in JG cells of greater than 90% purity and the isolation of a JG cell membrane vesicle population consisting of 87% sealed inside-out-vesicles were devised. Both of these methods gave consistent and high yields of renin-containing cells and plasma membrane-bound renin which could only be removed from the membrane by harsh detergent solubilization or sonication. The JG cell enriched suspension was shown to be physiologically responsive by the demonstration of normal secretory responses to forskolin, cAMP and ionomycin. The plasma membrane vesicle preparation was biochemically characterized with appropriate marker enzyme analyses and their integrity and orientation were established by an ATPase assay and

concanavalin A affinity chromatography.

The subcellular mechanism for the control of renin secretion was investigated using isolated renal cortical plasma membrane vesicles from normal, hypophysectomized and Na-depleted Sprague-Dawley rats with particular emphasis on the presence of inactive, trypsin-activable, renin in the plasma membrane compartment and the effect of medium ions, especially Ca and Ca-related factors on plasma membrane renin.

As a result of these studies, it is proposed that the plasma membrane compartment may represent a readily releasable pool of renin and may account for the increased plasma renin activity observed in Na-deprived rats. Normal Sprague-Dawley rats consistently sequester $11.2 \pm 0.6\%$ of the total renin specific activity of isolated PMV within the plasma membrane compartment. The observation that Hx rat plasma membranes have a high renin activity comparable to that found in the Na-deprived plasma membranes (2-3-fold > plasma membranes from normal rats) and yet fail to show an elevated PRA seems to suggest a defect in the Hx rat plasma membrane selectively inhibiting the release of this membrane pool of renin. The additional observation that a high K medium in the absence of Ca triggers the release of membrane-bound renin but inhibits renin release in the presence of Ca, lends support to an earlier hypothesis that control of renin release may involve membrane depolarization and a membrane-localized Ca-mediated response (Fray et al., 1983).

In conclusion, the JG cell enriched renal cortical cell suspension and the purified preparation of rat renal cortical plasma membranes described herein are rapid, reproducible in vitro models that are physiologically responsive at the whole cell level to known renin secretogogues, and responsive at the plasma membrane level to trypsin activation, hypophysectomy, sodium dietary deprivation, media ionic concentrations as well as other renin secretory stimuli not reported here. These model systems offer useful methods for elucidating the regulatory roles of specific cellular and membrane-associated phenomena in the process of renin secretion.

# APPENDIX I

## RENIN ASSAY PROGRAM FOR MACINTOSH PLUS COMPUTER

Program written by David J. Lush, Ph.D.

```
picogram values of standards
pg(1)=:  really zero, but log 1=0
pg(2)=5
pg(3)=12.5
pg(4)=25
pg(5)=50
pg(6)=125
pg(7)=250

standards indicates number of standards
standards=7

repeat indicates replicate counts
repeat=2

DIM st(standards, repeat)

FOR n=1 TO standards
lgpg(n)=LOG(pg(n))/LOG(10)
NEXT n

FOR n=1 TO repeat
INPUT "Total counts";t(n)
tav=tav+t(n)
NEXT n
PRINT: tav=tav/repeat

FOR n=1 TO repeat
INPUT "Nonspecific";ns(n)
nsav=nsav+ns(n)
NEXT n
PRINT:nsav=nsav/repeat

FOR nn=1 TO standards
FOR n=1 TO repeat
PRINT "Standard";nn;:INPUT st(nn,n)
stav(nn)=stav(nn)+st(nn,n)
NEXT n
PRINT:srav(nn)=stav(nn)/repeat
NEXT nn

FOR n=1 TO standards

p(n)=(stav(n)-nsav)/tav*100
NEXT n
```

```
INPUT "Number of UNKNOWNS, including substrate";samples
DIM un(samples, repeat), unav(samples),pun(samples)

s$="Unknown"
FOR nn=1 TO samples
IF nn=samples THEN s$="unknown (substrate)"
PRINT: PRINT s$;nn
FOR n=1 TO repeat
PRINT TAB(15):INPUT un(nn,n)
unav(nn)=unav(nn)+un(nn,n)
NEXT n
NEXT nn
CLS

correct:
INPUT "All Unknown Counts Correct (y or n)";d$
IF d$="y" THEN ok
IF d$="n" THEN gosub fixit
GOTO correct

ok:
FOR n=1 TO samples
unav(n)=unav(n)/repeat
pun(n)=(unav(n)-nsav)/tav*100
NEXT n
PRINT:INPUT "DILUTION FACTOR";df
PRINT:INPUT "GENERATION TIME (hours)";h

standard curve:
CLS
printit:
q$=DATE$
CALL TEXTFACE(1)
PRINT TAB(20);"Radioimmunoassay of...";q$
CALL TEXTFACE(0)
pa$="% Bound"
FOR n=1 TO 7
CALL MOVETO(30,50+15*n)
PRINT MID$(p$,n,1)
NEXT n

FOR n=25 TO 225 STEP 40
CALL MOVETO(60,n)
PRINT INT(112.5-n/2)

NEXT n

CALL MOVETO(95,240):PRINT"0"
CALL MOVETO(165,240):PRINT"1"
CALL MOVETO(235,240):PRINT"2"
CALL MOVETO(305,240):PRINT"3"
CALL MOVETO(145,265):PRINT"Log pg AI"
```

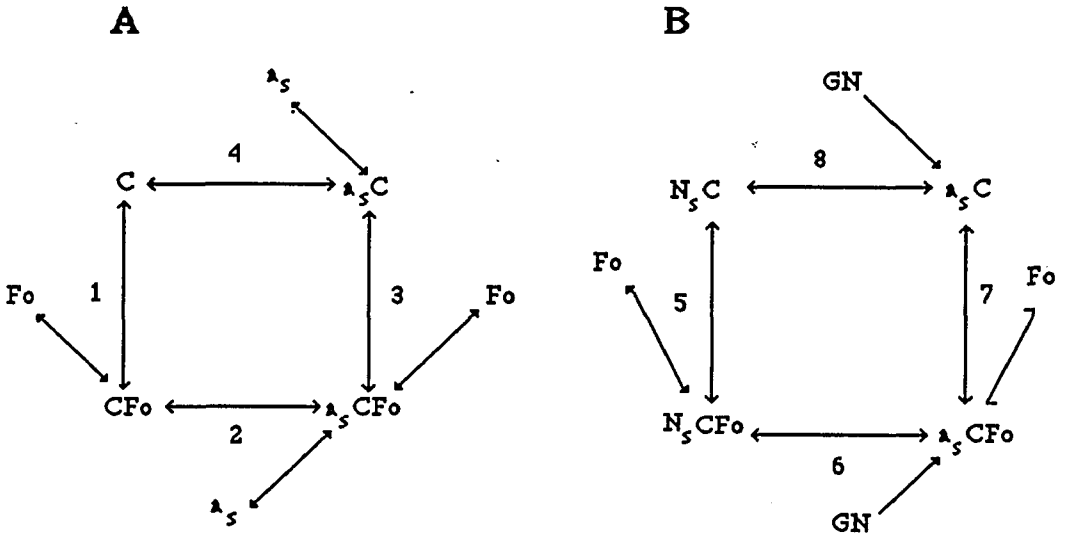
```
LINE (100,20)-(100,220)
LINE -(310,220)
FOR n=20 TO 220 STEP 20
LINE (95,n)-(100,n)
NEXT n

FOR n=100 TO 310 STEP 70
LINE (n, 225)-(n,220)
NEXT n

x=100+(210*1gpg(1)/3)
y=220-(200*p(1)/100)
CIRCLE(x,y),2
LINE (x,y)-(x,y)
    FOR n=2 TO standards
        x=100+(210*1gpg(n)/3)
        y=220-(200*p(n)/100)
        LINE - (x,y)
        CIRCLE(x,y),2
    NEXT n
LOCATE 4,41:PRINT"No. pg    % Bound"
    FOR n=1 TO standards
        LOCATE n+4,40:PRINT n
        LOCATE n+4,44:PRINT pg(n)
        LOCATE n+4,51:PRINT (CINT(p(n)*10))/10
    NEXT n

IF ff THEN ff=0: RETURN

BUTTON 1,1"Proceed", (370,220)-(425,240)
BUTTON 2,1,"Alter",(370,245)-(425,265)
WHILE DIALOG(0)<>1:WEND
but=DIALOG(1)
BUTTON CLOSE 1
BUTTON CLOSE 2
IF but=2 THEN GOTO alter

FOR n=2 TO standards

m(n)=(p(n-1)-p(n))/1gpg(n-1)-1gpg(n))
c(n)=p(n)-(m(n)*1gpg(n))
NEXT n

GOTO wrapup

fixit:
CLS
INPUT"Change UNKNOWN #",n
INPUT"Count #",c
PRINT"From...";un(n,c);
INPUT"To...",un(n,c)
RETURN
```

```
alter:
CLS
INPUT"Change STANDARD #",n
INPUT"Count #",c
PRINT"From...";st(n,c);
INPUT"To...",st(n,c)
stav(n)=0
FOR q= 1 TO repeat
stav(n)=stav(n)+st(n,q)
NEXT q
stav(n)=stav(n)/repeat
p(n)=(stav(n)-nsav)/tav*100
GOTO standard curve

calc:
FOR q=2 TO standards
IF pun(n)>p(q) THEN pg=EXP(2.30258*(pun(n)-c(q))/m(q)):RETURN
NEXT q
pg=EXP(2.30258*(pun(n)-c(standards))/m(standards)):RETURN

wrapup:
ff=1
OPEN "LPT 1:" FOR OUTPUT AS # 1
WINDOW OUTPUT # 1
GOSUB printit:PRINT:PRINT:PRINT:PRINT:PRINT:PRINT:PRINT
n=samples:GOSUB calc
IF pg<1 THEN pg=1
pg=(INT(pg*1000))/1000
PRINT:PRINT "Substrate = ";pg;"pg":sb=pg
PRINT"Dilution Factor = ";df
PRINT"Generation Time = ";h;"hours"
PRINT:PRINT"Sample No.   CPM    pg/tube    ng/h    Corrected ng/h"
PRINT

FOR n=1 TO samples-1
    GOSUB calc:IF pg<1 THEN pg=1
    ng=pg*df/h
    ngsb=(pg-sb)*df/h:IF ngsb<0 THEN ngsb=0

    PRINT USING "###";n;
    PRINT TAB(10);:PRINT USING "#####";un(n1);
    PRINT TAB(20);:PRINT USING"#####";pg;
    PRINT TAB(30);:PRINT USING"#####";ng;
    PRINT TAB(40);:PRINT USING"#####";ngsb
        FOR q=2 TO repeat
        PRINT TAB(10);:PRINT USING"#####";un(n,q)
        NEXT q
    PRINT
NEXT n
CLOSE #1
END
```

# APPENDIX II

## A Working Model for Stimulation of Adenylate Cyclase by Forskolin

Adapted from Shi et al. (1986).




**In Figure A (activation of free C),** forskolin is assumed to act at a site on the catalytic component of adenylate cyclase (C). Forskolin ($F_0$) binding to the catalytic component (low affinity) results in the formation of $CF_0$ (reaction 1) resulting in activation of the basal state. Secondly, it is proposed that the hormone and or Gpp(NH)p-activated enzyme further potentiates the forskolin-activated enzyme through an indirect action of $a_S$ (reaction 2). The forskolin binding affinity of each component is different with low affinity to C and with high affinity to $a_SC$ (suggested by calculation, not shown by binding data) and binding is fully reversible in both equations. The amount of $a_SC$ and C are different in the sample, even in maximally activated conditions. $a_SC$ only contributes 10-20% of the total enzyme. The true meaning of this limit remains to be studied. Thus, it is believed that $a_SC$ has high affinity but low capacity, and C has low affinity but high capacity for forskolin. This model also explains the observed GDP-sensitive and GDP-insensitive forms of forskolin activated cyclase. Depicted in

reactions 2 and 4 is the reversibility of the $a_sC$ and $a_sCF_0$ activated forms. These activated forms can be deactivated by GDP and GDPßS to form C or $CF_0$; reaction 4 represents the GDP-sensitive forskolin activated form.

**Figure B (activation of $N_sC$ complex)** shows parallel action of forskolin in the case of $N_sC$ complex. Information regarding this complex and its regulation are limited at this time, except for the assumptions that reactions 5,6,7 and 8 are similr to reactions 1,2,3, and 4 of Figure A, and reactions 6 and 8 are different from reactions 2 and 4.

The model recognizes that forskolin and Gpp(NH)p each produce characteristic activation of adenylate cyclase and that there is a further activation which is produced by the two agents together. This is represented as reactions 2 and 4, and 6 and 8 showing two levels of $a_s$ activation of the cyclase.

# APPENDIX III

## DISSERTATION ABBREVIATIONS

AC: adenylate cyclase

ACE: angiotensin converting enzyme

AMM: alphamethyl mannoside

ANG I, II, III: angiotensins

ANP: atrial natriuretic peptide

ANRL: antihypertensive neutral renomedullary lipid

APRL: antihypertensive polar renomedullary lipid

CaM: calmodulin

CDE: cell damaging enzyme

CRB: Concanavalin A running buffer

CSB: Concanavalin A starting buffer

DG: diacylglycerol

EVC: extravesicular compartment

GC: guanylate cyclase

GFR: glomerular filtration rate

GHR: Goldblatt hypertensive rat

Gi: inhibitory G protein

Gs: stimulatory G protein

GSH: glutathione sulfhydryls

GSSG: oxidized glutanthione

Hx: hypophysectomized

Hypox: hypophysectomized

IOV: inside-out vesicle

IP3: inositol triphosphate

IVC: intravesicular compartment

JG: juxtaglomerular

JGA: juxtaglomerular apparatus

JGC: juxtaglomerular cell

KHB: Krebs Henseleit bicarbonate buffer

KRB: Krebs Ringer buffer

LDH: lactate dehydrogenase

LV: leaky vesicle

NP-SH: non-protein sulfhydryl groups

PB-SH: protein-bound sulfhydryl groups

PDE: phosphodiesterase

PDP-4: peptidyl dipeptidase-4

PG: prostaglandin

PIP2: phosphoinositol diphosphate

PKC: protein kinase C

PM: plasma membrane

PMC: plasma membrane compartment

PMVs: plasma membrane vesicles

PRA: plasma renin activity

R.S.A.: relative specific activity

RAS: renin angiotensin system

RBF: renal blood flow

Ri: inhibitory receptor

RIC: renomedullary interstitial cells

ROV: rightside-out vesicle

Rs: stimulatory receptor

S.A.: specific activity

SAB: sodium acetate buffer

ROV: rightside-out vesicle

Rs: stimulatory receptor

S.A.: specific activity

SAB: sodium acetate buffer

SBTI: soybean trypsin inhibitor

SDS: sodium dodecyl sulfate

SHR: spontaneously hypertensive rat

SNS: sympathetic nervous system

T-SH: total sulfhydryl groups

T.A.: Total activity

TFP: trifluoperazine

TMB-8: 8(N,N-diethylamino) octyl 3,4,5-trimethoxy-benzoate

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