GLUCOCORTICOID REGULATION OF RAT RENAL SODIUM POTASSIUM ADENOSINE TRIPHOSPHATASE

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KLEIN

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
F. EDWARD HÉBERT SCHOOL OF MEDICINE
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

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Name of Candidate: Laura E. Klein
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Thesis and Abstract Approved:

James Ferris Committee Chairperson	29 March 90 Date
Howard Bryant Committee Member	29MAR90 Date
David Clough Committee Member	29 March 90 Date
Lochhead Committee Member	29 March 1990 Date
Doris Corcoran Committee Member	29 March 1990 Date

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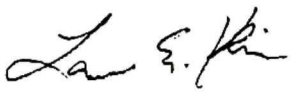

Laura E. Klein
Department of Physiology
Uniformed Services University
of the Health Sciences

ABSTRACT

Title of Dissertation: Glucocorticoid Regulation of Rat Renal Sodium Potassium Adenosine Triphosphatase

Laura Elizabeth Klein, Doctor of Philosophy, 1990

Dissertation directed by: Chu-Shek Lo, Ph.D.
Associate Professor
Department of Physiology

Sodium potassium adenosine triphosphatase (NaK-ATPase) is the integral plasma membrane protein which maintains the transmembrane electrochemical Na^+ and K^+ gradients required for many vital physiological functions. Glucocorticoids have been shown to enhance NaK-ATPase activity in various tissues including the kidney. An objective of this work was to investigate two possible mechanisms for glucocorticoid regulation of rat renal NaK-ATPase activity: 1) through an increase in filtered sodium load and 2) by increasing the number of NaK-ATPase units through an enhanced mRNA content. The experiments involved three groups of animals: adrenalectomized rats, adrenalectomized corticosterone-treated rats, and sham operated (control) rats. The temporal responses in GFR, filtered Na^+-load, and NaK-ATPase activity were compared in the three groups. In adrenalectomized rats, renal cortical NaK-ATPase activity was increased by 31% ($p \leq 0.05$) 6 h after corticosterone administration

and reached a maximal increase of 41% ($p \leq 0.05$) by 24 h. In the medulla, the activity increased to a maximum above control of 65% ($p \leq 0.05$) within 6 h. GFR and filtered Na^+ load did not change during the 24 h after corticosterone administration.

After adrenalectomy, α and β subunit levels decreased 48% and 52% ($p \leq 0.05$), respectively, below those found in control animals. Two hours after corticosterone administration in adrenalectomized rats, the α and β subunit content had risen to values that were not significantly different from those in control animals. Radioactively labeled complementary DNAs were used, in hybridization studies, to determine if this increase in subunit levels was associated with a rise in the mRNA content following corticosterone administration. After adrenalectomy, α and β subunit mRNA levels were reduced 61 and 64% ($p \leq 0.05$), respectively, below control (sham operated). Within 1 h of corticosterone administration, messenger RNA levels returned to control values.

It is concluded that corticosterone restores renal NaK-ATPase activity in adrenalectomized rats by acting at the level of renal tubular epithelial cells, prior to any enhanced sodium delivery, to increase the levels of the NaK-ATPase subunits, at least in part, through increased mRNA content.

GLUCOCORTICOID REGULATION OF

RAT RENAL SODIUM POTASSIUM ADENOSINE TRIPHOSPHATASE

by

Laura Elizabeth Klein

Dissertation submitted to the Faculty of the Department of Physiology
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADH -	antidiuretic hormone
cDNA -	complementary sequence of deoxyribonucleic acid
c-erbA -	the cellular gene derived from the v-erbA gene of avian erythroblastosis virus
C-terminal -	carboxy terminal
DOC -	deoxycholic acid
EBr -	ethidium bromide
EcoRI, HindIII,- PstI	endonucleases which cut at specific locations within characteristic DNA sequences; restriction enzymes
FITC -	fluorescein isothiocyanate
GFR -	glomerular filtration rate
HRP -	Horseradish Peroxidase
IgG -	immunoglobulin G
kDa -	kilodalton
Mg-ATPase -	Magnesium Adenosine Triphosphatase
MOPS-	3-[N-Morpholino]propanesulfonic acid
MOPS buffer -	0.02 M MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0
mRNA -	messenger ribonucleic acid
NaK-ATPase -	Sodium Potassium Adenosine Triphosphatase
N-terminal -	amino terminal
pA1N -	recombinant pSV2neo plasmid containing a full length NaK-ATPase α1-cDNA
PAH -	Para-aminohippuric acid
P_i -	inorganic phosphate

poly A^+ RNA -	polyadenylated RNA; describes 5'poly A tails, characteristic of most eukaryotic messenger RNAs
prb19G -	recombinant pBR322 plasmid (622 bp deleted) containing an insert that consists of a full length NaK-ATPase β-cDNA with some 5'and 3' untranslated regions
RPF -	renal plasma flow
SDS -	sodium dodecyl sulfate
SDS-PAGE -	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSC -	20x SSC = 3 M NaCl, 0.3 M sodium citrate, pH 7.0.
TPBS -	phosphate buffered saline containing 0.05% (v/v) Tween 20
TCA -	trichloroacetic acid
TSM-	20 mM Tris-HCl, pH 7.4, 0.9g% NaCl, 10 g% nonfat dry milk, 1 mM sodium azide, 100 U/ml penicillin and 100 μg/ml streptomycin
v-erbA -	avian erythroblastosis virus gene

RATIONALE

NaK-ATPase activity is critical to the maintenance of cellular osmotic equilibrium and to a large array of tissue specific functions required for homeostasis. Despite extensive investigation, a comprehensive understanding of the factors regulating this enzyme's activity has remained elusive. One purpose of this work was to evaluate the hypothesis that rat renal NaK-ATPase activity is enhanced by glucocorticoid actions at the epithelial cell level. Initial studies were designed to establish whether corticosterone administration restores renal cortical and outer medullary NaK-ATPase activity in adrenalectomized rats to the increased level observed in sham operated rats. Previous work had demonstrated that glucocorticoids produce increases in glomerular filtration and Na^+-reabsorption (Raisz et al., 1957; Yunis et al., 1964). Renal function studies were conducted, in this project, to determine whether the observed changes in NaK-ATPase activity occurred after, and possibly as the result of, changes in substrate (Na^+) delivery to renal tubular epithelial cells.

A second hypothesis tested in this work states that the production of an enhanced level of enzyme units mediates the glucocorticoid induced increase in NaK-ATPase activity. Polyclonal antibody and complementary DNA probes were used to assess the enzyme subunit and messenger RNA levels, respectively, in kidneys from adrenalectomized and sham operated rats with or without corticosterone administration. These studies are the first to demonstrate glucocorticoid modulation of NaK-ATPase subunit precursor levels and their contribution to both an increased number of enzyme units and a resulting enhanced NaK-ATPase activity.

Characterization of NaK-ATPase

Enzyme Structure

Sodium Potassium Adenosine Triphosphatase contains two well characterized protein subunits (α, M_r ~ 112,000; β, M_r ~ 55,000). Reconstitution of these two subunits into phospholipid vesicles in the presence of ATP is sufficient to generate Na^+/K^+ transport across the vesicles (Goldin, 1977). Binding of Na^+ and ATP occurs on the α subunit region extending from the inner surface of the plasma membrane, while K^+ and the specific inhibitor, ouabain, bind to α subunit sites on the outer membrane surface (Glynn, 1968; Ruoho & Kyte, 1974). Sequencing and hydrophobicity studies suggest that this subunit contains up to 8 long hydrophobic regions which may permit the protein to repeatedly span the plasma membrane's lipid bilayer and form a protein pore to allow for transmembrane ion passage (Ovchinnikov et al., 1987). The β subunit is also thought to span the plasma membrane (Girardet et al., 1981). Although the specific role of the β subunit is not understood, its presence is required for maintenance of enzymatic activity (Sweadner and Goldin, 1980). *In vivo* incorporation studies with labeled carbohydrate precursors in rat kidneys (Lo et al., 1984) support earlier findings, from brine shrimp (Peterson et al., 1978), eel electroplax (Churchill et al., 1979) and rabbit renal medulla (Peters et al., 1981), that both subunits are sialoglycoproteins.

Recent studies have demonstrated that there are at least three isoforms of the NaK-ATPase α subunit which are encoded by a multigene family (Herrera et al., 1987). The three α subunit genes are all located on different chromosomes and possess unique patterns of tissue and developmental stage-specific expression (Emanuel et al., 1987; Shyjan and Levenson, 1989). These isoforms may form the basis for NaK-ATPase functional and developmental diversity and may explain variations in the ouabain sensitivity of NaK-ATPase (Herrera et al., 1987; Kent et al., 1987; Orlowski & Lingrel, 1988).

The gene coding for a β subunit of NaK-ATPase has been mapped to a chromosomal location that is not tightly linked to any of the α subunit genes. It also exhibits a distinct tissue-specific pattern of mRNA expression (Emanuel et al., 1987; Kent et al., 1987). Despite these findings, many studies indicate that the synthesis of the α and β subunits is coordinated so as to result in approximately equal levels of expression (Emanuel et al., 1986; Geering et al., 1982; Mercer et al., 1986; Tamkun & Fambrough, 1986). These findings suggest that the control mechanism responsible for coordinating subunit expression is complex and may not function at the transcriptional level (Emanuel et al., 1987). More recently, a second β subunit mRNA has been identified with a different tissue specific pattern of expression (Martin-Vasallo et al., 1989). The identification of one or more isoforms of the NaK-ATPase β subunit opens up the possibility that multiple β isoforms may be expressed and combined with multiple α isoforms in a tissue specific manner. These combinations may play a role in mediating the various levels of enzyme activity and the functional roles of NaK-ATPase in

different tissues.

Localization

The functional units of NaK-ATPase are embedded in all animal cell plasma membranes. With the exception of specialized salt concentrating tissues (e.g., duck salt gland, shark rectal gland) the highest specific activities are found in cell membranes of excitable tissues and of actively absorbing and secreting epithelial cells (Hokin et al., 1973; Hopkins et al., 1976). The enzyme is localized at the basolateral surface of these cells and, in the kidney, the highest NaK-ATPase specific activity is found in the thick ascending limb of the loop of Henle and the distal convoluted tubule (Garg et al., 1985; Kyte, 1976). Selective detergent extraction of plasma membranes from the outer medulla can yield a highly purified form of NaK-ATPase (Jorgensen, 1975).

Proposed enzyme mechanism

Sodium and potassium ions are believed to traverse the plasma membrane in response to conformational changes induced in NaK-ATPase by the hydrolysis of ATP. Evidence for different NaK-ATPase conformations derives from two studies. Glynn and Karlish (1975) noted that phosphorylation of the enzyme by [γ-^{32}P]ATP occurs in the presence of sodium and magnesium ions and that the addition of potassium results in dephosphorylation. When NaK-ATPase was subjected to trypsin digestion in the presence of varying Na^+, K^+, $Mg^{2+}$and ATP concentrations, electrophoresis of the resulting peptides demonstrated that the sites of protease attack had changed (Jorgensen, 1975). How the proposed conformational changes

affect Na^+/K^+ membrane translocation is not understood. The basic enzymatic mechanism originally proposed by Post & Sen (1965) and Albers et al. (1974), and modified by Karlish, et al. (1978), involves two major conformations for NaK-ATPase, termed E_1 and E_2. In the E_1 form, the enzyme has a high affinity site for ATP and three high affinity binding sites for sodium ions. Upon phosphorylation of NaK-ATPase, following the binding of the sodium ions and the release of ADP, a conformational change to the E_2 form occurs. This is accompanied by a decreased affinity for sodium ions and their release into the extracellular fluid. The conformational change is also accompanied by the formation of high affinity sites for two potassium ions on the extracellular surface of the phosphorylated enzyme. Binding of the potassium ions and dephosphorylation are followed by ATP binding to a low affinity site on the enzyme. This induces the conformational change back to the E_1 form and release of the potassium ions into the cytoplasm, beginning the cycle once more. The actual number and nature of the conformational changes required for NaK-ATPase activity remains controversial.

Physiological significance of NaK-ATPase

NaK-ATPase is required to maintain constant low Na^+ and high K^+ intracellular concentrations relative to those concentrations in the extracellular fluid. This low intracellular Na^+ concentration protects cells from the osmotic pressure caused by impermeant cytoplasmic ions. The sodium concentration gradient also provides the driving force for a large array of physiological functions. These include the repolarization of excitable tissue required for nerve impulse transmission and

muscle contraction (Skou, 1957), the functioning of hepatic amino acid and bile acid symport uptake systems (Miner et al., 1980), secretion of water and electrolytes from salivary glands (Schwartz & Moore, 1968), glucose and amino acid absorption in the small intestine (Hafkenscheid, 1973), and renal tubular reabsorption of these nutrients (Jorgensen, 1980). NaK-ATPase activity varies greatly with respect to tissue type. Since the renal outer medulla has the highest specific NaK-ATPase activity of all mammalian tissues investigated, the kidney is a tissue of choice for studying mammalian NaK-ATPase. The activity of this enzyme also varies with respect to growth and development (Schmidt & Horster, 1977; Rane & Aperia, 1985), suggesting that hormones may be involved in the regulation of NaK-ATPase activity and gene expression.

Hormonal modulation of NaK-ATPase

The effects of insulin, catecholamines, thyroid hormones and adrenal steroids on NaK-ATPase activity have been investigated most thoroughly and will be described here. It should be noted, however, that many other hormones and growth factors have been thought to regulate NaK-ATPase activity.

Insulin

Insulin has been demonstrated to enhance NaK-ATPase activity in frog skeletal muscles (Gavryck et al., 1975; Kanbe & Kitasato, 1986), cultured myocytes (Rosic et al., 1985), rat adipocytes (Resh et al., 1980), lymphocyte cell membranes (Hadden et al., 1972), hepatoma cells (Gelehrter et al., 1984) and kidney (DeFronzo, 1981; Rasch & Andreassen,

1986). These studies suggest that the response is not mediated by de novo protein synthesis but is secondary to enhanced sodium influx.

The osmotic diuresis seen in uncontrolled diabetes indirectly activates renal NaK-ATPase by enhancing GFR and sodium filtration. This activation of NaK-ATPase is proposed to be an important mechanism for countering extensive electrolyte loss (Wald & Popovtze, 1984). Any direct increase in NaK-ATPase activity resulting from insulin administration would be masked by an accompanying decrease in Na^+ delivery to the cell. To offset this obstacle, insulin was administered to an isolated, perfused kidney, and directly into the left renal artery of a dog with plasma glucose levels clamped to maintain a high GFR and RPF (DeFronzo, 1981; Rostand et al., 1980). An insulin stimulated antinatriuretic effect occurred in distal portions of the nephron. This supports a direct insulin mediated increase in renal NaK-ATPase activity. The administration of anti-insulin serum caused a brisk natriuresis in control rat kidneys while having no significant effects on GFR or urine flow (Rostand et al., 1980). The rapid response suggests that insulin/receptor binding increases NaK-ATPase activity in the renal tubular epithelium by activating already formed enzyme units. One mechanism proposed for activation is via enhanced Na^+/H^+ exchange which increases intracellular sodium concentration (Resh et al., 1980). Administration of the aldose reductase inhibitor, sorbinil, or a diet supplemented with myoinositol prevented the decreased glomerular NaK-ATPase activity seen in acute Streptozotocin diabetes. This suggests that insulin deficiency causes the activation of aldose

reductase which depletes myoinositol and endogenous inositol-containing membrane phospholipids (Cohen et al., 1985). According to this scenario, insulin indirectly affects NaK-ATPase activity via the maintenance of plasma membrane structure.

Catecholamines

Norepinephrine and dopamine enhance NaK-ATPase activity in brain (Svoboda et al., 1986), brown adipose tissue (Horwitz & Eaton, 1975; Svoboda et al., 1986), skeletal muscle (Cheng et al., 1977; Clausen & Flatman, 1977) and crude synaptosome preparations (Powis, 1981) but have no such effect in liver and kidney. The action is rapid and is not specific to NaK-ATPase since Mg-ATPase activity is also enhanced. The stimulation is not prevented by catecholamine antagonists in concentrations sufficient to antagonize many pharmacologic effects. cAMP does not seem to act as an intermediate in the stimulation of these enzymes (Senft et al., 1968) and the mechanism is poorly understood. Explanations most often suggested are that: a. catechols and catecholamines may act as chelating agents and suppress the inhibitory effect of cytoplasmic factor(s), b. they, like insulin, may activate the Na^+/H^+ transporter which enhances sodium influx and leads to a compensatory increase in NaK-ATPase activity and c. they induce changes in phospholipids or other membrane components, unmasking additional enzyme sites through conformational changes.

Thyroid hormones

The most thoroughly investigated regulation of NaK-ATPase activity involves the actions of thyroid hormones. A thyroid hormone dependent increase in enzyme activity has been demonstrated in cultured hepatocytes (Ismail-Beigi et al., 1979) small intestine (Liberman et al., 1979), cardiac and skeletal muscle (Asano et al., 1976; Philipson & Edelman, 1977), submandibular and parotid glands (Lo et al., 1981), thymus and spleen (Klein & Lo, 1985) and kidney (Lo et al., 1976). This increase in NaK-ATPase activity results in a lowered intracellular sodium concentration and an enhanced intracellular potassium concentration in liver slices from euthyroid rats (Ismail-Beigi & Edelman,1973). It accounts for one third to one half of the triiodothyronine induced increase in oxygen consumption and heat production in whole kidney homogenates (Ismail-Beigi & Edelman, 1971). The results of ouabain binding and phosphorylated intermediate studies suggest that thyroid hormone augments this specific activity through an accumulation of NaK-ATPase units (Lo et al., 1976, Lo & Lo, 1981, Lo et al., 1981). This accumulation was found, in labeled methionine incorporation studies with the kidney cortex, to be the result of enhanced NaK-ATPase synthesis (Lo & Edelman, 1976; Lo & Lo, 1980). Recent studies demonstrate that this increase in enzyme synthesis correlates with an increase in NaK-ATPase subunit mRNAs (Chaudhury et al., 1987). These findings agree with the currently held view that thyroid hormone receptors, like those of steroid hormones, belong to a superfamily of ligand-responsive transcription factors (Evans, 1988; Green & Chambon, 1986). Such receptors

bind to specific DNA regions and either stimulate or repress transcription initiation by RNA polymerase II at specific promoters. Data from nuclear run-off assays suggest that thyroid hormone may also affect the expression of NaK-ATPase via post-transcriptional regulatory processes such as the processing of nuclear RNA precursors and the rate of mRNA delivery to the cytoplasm, both of which are capable of altering the rate of mRNA degradation (Gick et al., 1988).

Adrenal steroids

In 1966, Chignell and Titus demonstrated that adrenalectomy reduced rat renal NaK-ATPase activity by 40-50%. Although aldosterone administration returned Na^+ retention to normal within 3 h, only glucocorticoids restored NaK-ATPase activity. Many other workers have provided data supporting these findings in the kidney (Hendler et al., 1972; Charney et al., 1974; Fisher et al., 1975; Rodriguez et al., 1981; Sinha et al., 1981; Klein et al., 1984b). Glucocorticoid administration to adrenalectomized rats has also been shown to restore NaK-ATPase activity in the intestine (Charney et al., 1975), cardiac muscle (Klein et al., 1984) and submandibular gland (Bartolomei et al., 1983). Studies with selected nephron segments (enriched by sedimentation or microdissection) have demonstrated that glucocorticoids regulate NaK-ATPase activity throughout the nephron (Doucet et al., 1986; Garg et al., 1981; Garg et al., 1985; Rayson & Edelman, 1982; Rayson & Lowther, 1984) while mineralocorticoid modulation of NaK-ATPase activity occurs in the collecting ducts. Such results are in agreement with the pattern of ^3H-aldosterone binding, suggesting the collecting tubule as the

target site of mineralocorticoid action (Doucet & Katz, 1981; Farman & Bonvalet, 1983). Mineralocorticoids also regulate NaK-ATPase expression in toad bladder epithelial cells (Geering et al., 1982). Work with isolated, superfused tubule segments (Rayson & Edelman, 1982) supports the *in vivo* findings (Fisher et al., 1975) that glucocorticoids directly stimulate NaK-ATPase activity and that this effect is independent of changes in the glomerular filtration rate. Kinetic data suggest that this increase in NaK-ATPase activity is due to an increased number of functional enzyme units (Sinha et al., 1981). This may be caused by activation of preexisting enzyme units, stimulation of NaK-ATPase synthesis or inhibition of its degradation. An examination of the currently accepted mechanism of steroid hormone action and the role of glucocorticoid action in renal function would suggest the preferred approach for further investigation.

Mechanism and physiological role of glucocorticoid action

Mechanism of glucocorticoid action

The hydrophobic structure of the glucocorticoid molecule facilitates its entry into target cells by passive diffusion. Although this is thought to be the primary route across the plasma membrane, evidence for facilitated transport has been presented (Harrison et al., 1974; Rao et al., 1976; Rao et al., 1977).

Binding studies with tritiated glucocorticoids revealed that they attach to a high affinity, low capacity cytoplasmic protein (Toth & Aranyi, 1983). This receptor is extremely labile but hormone binding

protects it against heat inactivation. The serum transport protein does not bind to synthetic hormones such as triamcinolone acetonide and dexamethasone. Use of these compounds permitted quantitation of glucocorticoid/cytoplasmic receptor binding (Beato & Feigelson, 1972; Giannopoulos, 1973). Whether glucocorticoid receptors are localized in the nucleus and bound to DNA in the absence of the hormone is still controversial (Becker et al., 1986).

Extensive investigation has revealed the complex structure of the glucocorticoid receptor. Limited proteolysis of glucocorticoid receptor preparations suggested that this binding protein contains at least three functional domains (Wrange & Gustafsson, 1978; Gustafsson et al., 1987). These were described as the N-terminal immunodominant region, the central DNA binding domain and the C-terminal steroid binding domain. Further characterization became possible when glucocorticoid receptor complementary DNAs were isolated. These were added to *in vitro* transcription and translation systems to synthesize large quantities of the functional receptor. They were also engineered to contain base insertions in selected locations (insertional mutants). The proteins synthesized *in vitro* from insertional mutants were observed for alterations in hormone and DNA binding (Giguere et al., 1986; Hollenberg et al., 1985). The glucocorticoid receptor has a C-terminal, steroid-binding domain containing a single binding site for glucocorticoids (Wrange et al., 1986). When this region of the glucocorticoid receptor was compared with the corresponding region of other steroid receptors, varying though significant amino acid homologies were observed. For example, the carboxy-terminal region of the mineralocorticoid receptor

has a 57 percent amino acid identity with the corresponding glucocorticoid receptor region (Arriza et al., 1987). Between the steroid and DNA binding domains is a shorter region which is not significantly conserved among the steroid receptors. The region is highly hydrophilic and has a predicted secondary structure which is rich in turn and coil. Such characteristics suggest that this region is located on an exposed surface of the globular receptor protein and is flexible (Krust et al., 1986). This predicted "molecular hinge" could permit direct interaction between the steroid and DNA binding regions of the receptor. The observed increase in receptor affinity for DNA, following glucocorticoid binding, might result from this association (Giguere et al., 1986). The centrally located DNA-binding domain of the glucocorticoid receptor contains significant homology with the receptors for other steroid hormones (Arriza et al., 1987; Conneely et al., 1986; Danielsen et al., 1986; Gronemeyer et al., 1987; Hollenberg et al., 1985; Jeltsch et al., 1986; Kumar et al., 1987; Loosfelt et al., 1986; Weinberger et al., 1985), thyroid hormones (Sap et al., 1986; Weinberger et al., 1986), retinoic acid (Petkovich et al., 1987), vitamin D_3 (McDonnell et al., 1987) and with the v-erbA oncogene product of avian erythroblastosis virus (Green et al., 1986; Krust et al., 1986). Work in two laboratories has shown that the product of the cellular gene c-erbA functions as a high affinity thyroid hormone receptor (Sap et al., 1986; Weinberger et al., 1986). These receptors are now believed to comprise a superfamily of transcriptional regulatory proteins which stem from a common ancestral gene (Evans, 1988; Green & Chambon, 1986). A 94% identity exists between the DNA-binding region of the human

mineralocorticoid and glucocorticoid receptors (Arriza et al., 1987). This domain of both the rat and human glucocorticoid receptors was found to contain repeated units rich in the basic amino acids cysteine, arginine and lysine (Hollenberg et al., 1985; Miesfeld et al., 1986). Such units were first noted in the transcription factor IIIA from *Xenopus* oocytes and were later found in other nucleic acid-binding proteins (Berg, 1986; Miller et al., 1985). Miller and colleagues proposed a model to describe the binding of these proteins to DNA. The repeated basic unit of the DNA binding protein is predicted to fold around a zinc ion to form a finger-like structure capable of binding to a half turn of DNA. Complementary DNAs for the glucocorticoid receptor gene were used to construct insertional mutants. These constructs contained additional bases which produced amino acid insertions in the receptor protein. The insertions were specifically designed to disrupt the "zinc fingers". Such mutant receptors bound to the glucocorticoid normally, ruling out a role for this region in hormone binding. They did not translocate to the nucleus or bind DNA *in vitro* and were unable to modulate transcriptional activation (Giguere et al., 1986). The secondary structure of the central region is, therefore, critical to DNA binding and to glucocorticoid induction of transcription. The N-terminal region varies greatly in length and sequence among the steroid/thyroid superfamily. It is clearly immunodominant since most antibodies raised in various laboratories are directed against this domain (Gametchu & Harrison, 1984; Giannopoulos, 1973; Gustafsson et al., 1987; Okret et al., 1981; Westphal et al., 1982). The first 106 amino acids of the glucocorticoid receptor are not essential for transcriptional activity but help to

modulate the magnitude of the transcriptional response (Danielsen et al., 1986; Greene et al., 1986; Hollenberg et al., 1987; Miesfeld et al., 1987).

In cell-free studies neither steroid nor receptor alone bound to DNA. However, the hormone-receptor complex bound to DNA at a very slow rate at 0°C (Higgins et al., 1973; Kalimi et al., 1975) and this binding was accelerated at 25°C and in the presence of physiological levels of Ca^{2+} or isotonic saline. Based on kinetic, thermodynamic and sedimentation studies, the process of hormone-receptor complex activation resembles protein denaturation reactions, suggesting that a conformational change in the receptor is responsible for the enhanced affinity (Atger & Milgrom, 1976; Giannopoulos, 1975; Kalimi et al., 1975). Molybdate was shown to stabilize the glucocorticoid-receptor complex and inhibit its activation (Leach et al., 1979; Lee et al., 1981). This finding permitted the structures of activated and nonactivated complexes to be compared using chromatography, electrophoresis and sucrose density gradient centrifugation (Catelli et al., 1985; Holbrook et al., 1983; Mendel et al., 1986; Wrange et al., 1984). The nonactivated complex has a molecular weight of 300-kDa and a sedimentation coefficient of 9.2 S. The activated complex has a molecular weight of 94-kDa and a sedimentation coefficient of 4.8 S (Higgins et al., 1973; Holbrook et al., 1983). A 90-kDa protein released from the complex upon activation was shown to be a cytoplasmic heat shock protein. This protein may function in the processing and/or transport of newly synthesized receptors or in maintaining the inactive state of the receptor (Catelli et al., 1985; Sanchez et al., 1985). Both the 90 and 94-kDa proteins were later shown

to be phosphorylated in the nonactivated complexes, suggesting a possible role for dephosphorylation in the activation process (Miesfeld et al., 1986).

The DNA region which binds the activated glucocorticoid-receptor complex has been identified (Geisse et al., 1982; Govindan et al., 1982; Payvar et al., 1981; Pfahl, 1982; Scheidereit et al., 1983). Genes which exhibit increased transcription initiation in response to glucocorticoids (growth hormone, metallothionine, mouse mammary tumor virus) were cloned and reacted with the activated complex. Binding regions were identified with exonuclease III protection studies and DNAase I footprinting studies (von der Ahe et al., 1986; Cordingley et al., 1987; Webster et al., 1988). Gene transfer experiments next demonstrated that binding of the activated complex to this receptor binding DNA region induced transcriptional initiation. The region was placed upstream from a promoter and from a gene not normally induced by glucocorticoids. The addition of this DNA sequence caused glucocorticoids to induce transcription from the normally unresponsive gene (Chandler et al., 1983; Hynes et al., 1983; Karin et al., 1984; Lee et al., 1981; Ponta et al., 1985; Slater et al., 1985). The receptor binding DNA region could be ligated in either orientation and at great distances from the promoter without diminishing the glucocorticoid induction. These are characteristics of an inducible enhancer, this one called the glucocorticoid response element (Maniatis et al., 1987).

How hormone-receptor complex binding to the response element enhances transcriptional initiation is not known. Sequences within the promoter region of the mouse mammary tumor virus become hypersensitive

to degradation by DNAase I following activated receptor binding (Hager, 1983; Peterson et al., 1985; Zaret and Yamamoto, 1984). This suggests a reorganization of nucleoprotein structure in the promoter region, and possibly larger chromatin regions. Such a structural change may permit the binding of other activating proteins to nearby promoters and increase accessibility of RNA polymerase II (Cordingley et al., 1987).

Physiological effects of glucocorticoids

Glucocorticoids influence development, cell replication, metabolism and the expression of a wide array of genes in virtually all cells of the body (Funder, 1987). The primary glucocorticoid effects include what has been called a "translocation" of protein from muscle stores to visceral organs, such as the liver. Enhanced muscle catabolism and diminished amino acid uptake into the muscles provide precursors for the synthesis of specific proteins in the liver. The mobilization of protein from muscle, and fatty acids from peripheral adipose tissues also provides the liver with substrates for gluconeogenesis. Glucocorticoids promote glucose storage in the liver by enhancing glycogen formation and inhibiting its utilization. These actions have profound effects on many major organ systems including potentiation of the peripheral arteriolar response to vasoconstrictors, antagonism of vitamin D action in the gastrointestinal system, generalized catabolic effects in muscles, bone, skin and lymphoid system, and stimulatory effects on the central nervous system (Baxter & Forsham, 1972).

Glucocorticoid actions increase renal plasma flow, glomerular filtration rate and free water clearance (Kleeman et al., 1975; Raisz et

al., 1957). Patients suffering from adrenal cortical insufficiency (Addison's disease) are unable to excrete a water load and have a reduced urine concentrating capacity. These symptoms can be ameliorated with glucocorticoid therapy but not with aldosterone (Schwartz & Kokko, 1980; Yunis et al., 1964). Two hypotheses have been proposed to explain the glucocorticoid mediated control of water regulation. One is based on Schwartz and Kokko's finding that glucocorticoids augment the hydroosmotic response to ADH in the collecting ducts. Collecting ducts, in adrenalectomized rabbits, respond to ADH with a diminished rate of intracellular cAMP accumulation. Their findings also support earlier studies showing that corticosteroids diminish phosphodiesterase activity (Senft et al., 1968; Stoff et al., 1973). Glucocorticoids are thought to increase cAMP accumulation in response to ADH by inhibiting it's degradation by phosphodiesterase. This hypothesis states that restoration of urine concentrating capacity by glucocorticoids results from permissive effects on ADH action. A second hypothesis is that glucocorticoids enhance sodium reabsorption at the thick ascending limb of Henle's loop by increasing NaK-ATPase activity. By transporting greater amounts of sodium from the filtrate into the medullary interstitium, this increased NaK-ATPase activity would enhance the osmotic pressure in the interstitium. This would enable the kidneys to produce a more concentrated urine in the presence of equally high ADH levels. This theory can, therefore, account for the observed significant ADH independent modulation of water reabsorption by glucocorticoids (Yunis et al., 1964). It also suggests a mechanism by which glucocorticoids permit excretion of a free water load. An increased NaK-ATPase activity in

this region would result in a more dilute urine being delivered to the collecting ducts and excreted in the absence of ADH release. Elucidation of the role of glucocorticoids in modulating renal NaK-ATPase activity may contribute to an understanding of an important physiological mechanism for the maintenance of water and electrolyte balance.

METHODS

Regulation of hormonal environment

Surgically adrenalectomized male, Sprague Dawley rats weighing between 240 and 340 g were supplemented with glucocorticoids during studies to determine the roles of these hormones in the modulation of NaK-ATPase activity. Surgical adrenalectomy followed anesthesia with chloral hydrate (36 mg/100 g body weight, i. p.). In pilot studies, adrenalectomized rats showed an increase in urinary Na^+ and a decrease in urinary K^+, providing evidence for the success of the surgical technique. After surgery, the rats were allowed to recover from adrenalectomy for 5 to 7 days prior to further manipulation. They were maintained on Rat Chow (Agway 1257) *ad libitum* and 0.9 % saline. In all glucocorticoid replacement studies, the animals were given subcutaneous doses of corticosterone in a 0.5% ethanol vehicle or vehicle alone.

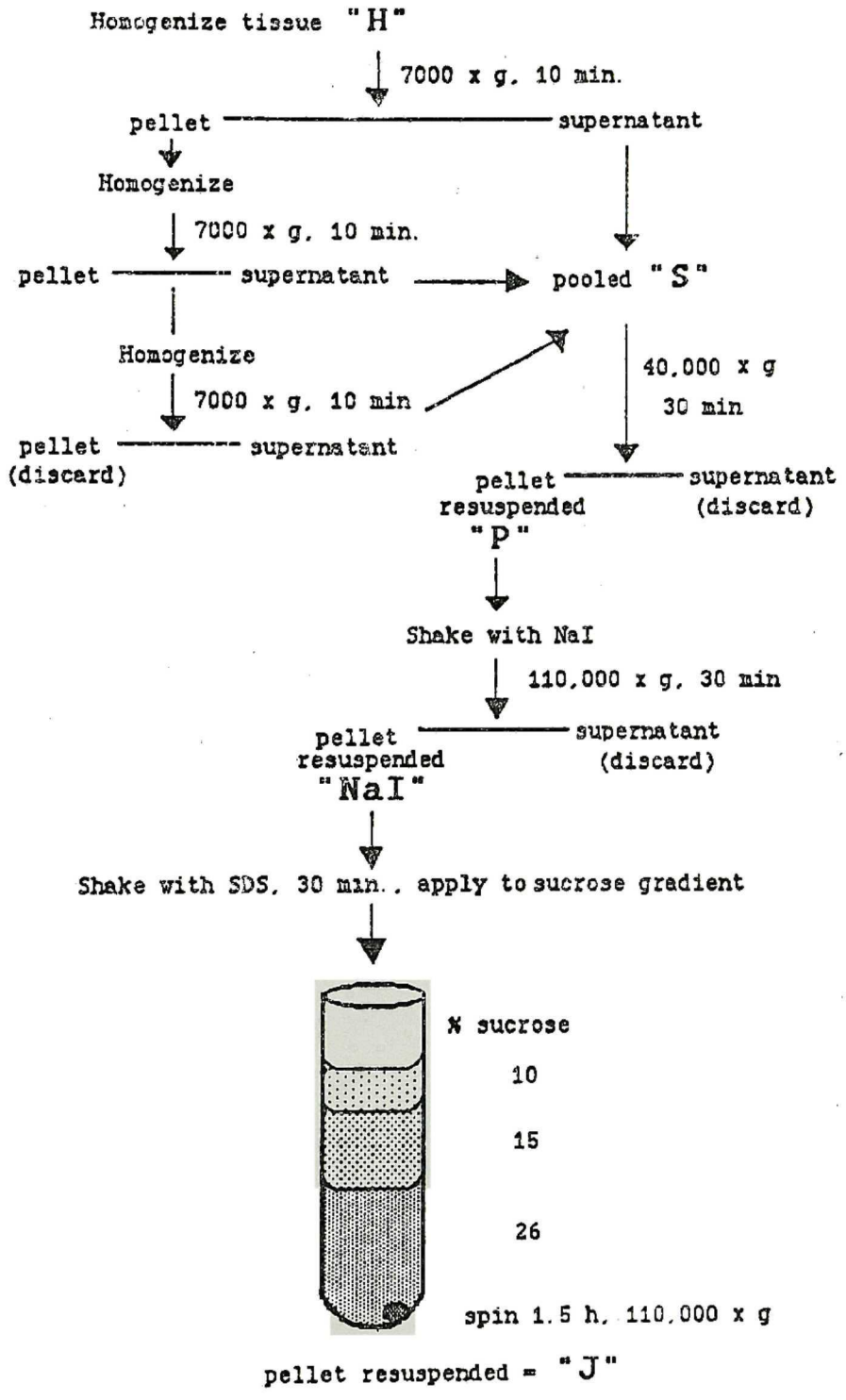
NaK-ATPase enrichment

At the appropriate time point, following hormone replacement, the rats were decapitated with a single guillotine stroke and both kidneys

were perfused via the abdominal aorta with 15 ml of ice-cold 0.155 M NaCl. After the kidneys were isolated and decapsulated, the cortex, outer medulla and papilla were collected by sharp dissection. Respective zones from both kidneys were pooled, weighed and suspended in ice cold Solution A (10 ml/g wet weight) (1 mM EDTA, 0.03 M DL-Histidine, 0.25 M sucrose, pH 7.2) containing 0.1% deoxycholic acid (DOC). Following the method outlined in Figure 1, I applied ten strokes from a teflon glass motor driven Potter-Elvehjem homogenizer to disrupt the tissue and then centrifuged the homogenate for 15 minutes at 7000 x g, at 4°C in a Sorval SS-34 rotor. The resulting supernatant (S) fraction was placed on ice. Following resuspension of the pellet in 12 ml of solution A + DOC and homogenization with 5 strokes, the centrifugation was repeated. S fractions were combined and the pellet again resuspended and homogenized (5 strokes), this time in 6 ml of solution A + DOC. Following the third centrifugation, all three S fractions were pooled and centrifuged for 30 minutes at 40,000 x g in the SS-34 rotor, at 4°C. The resulting supernatant was discarded and the microsomal pellets (P) resuspended in a total of 1.2 ml of solution A. An equal volume of solution C (50 mM Imidazole, 2 mM EDTA, pH 7.5) containing 30 g% sodium iodide was added, dropwise, into the P fraction while vortexing. The mixture was shaken on ice for 10 minutes and then centrifuged for 45 minutes at 110,000 x g in a DuPont-Sorvall A-841 fixed angle ultracentrifuge rotor. Each pellet was resuspended with sonication in 1 ml of solution B (25 mM Imidazole, 0.25 M sucrose, 1 mM EDTA, pH 7.5).

Figure 1. Enrichment protocol for NaK-ATPase.

Rat kidneys were homogenized in the presence of 0.1 g% deoxycholic acid, the homogenates subjected to differential centrifugation and the pellets homogenized. The cycle of homogenization and centrifugation was repeated twice and the combined supernatants were centrifuged to yield pellets containing the microsomal fraction (P). Upon resuspension, this fraction was reacted with sodium iodide to solublize peripheral membrane proteins and yield an enriched microsomal fraction (NaI). This was further solublized with sodium dodecyl sulfate and subjected to sucrose density gradient ultracentrifugation, resulting in the formation of small pellets which, upon resuspension, displayed enhanced NaK-ATPase specific activity (J).


Homogenize tissue "H"
7000 x g, 10 min.
pellet
supernatant
Homogenize
7000 x g, 10 min.
pellet
supernatant
pooled "S"
Homogenize
7000 x g, 10 min
40,000 x g
30 min
pellet
(discard)
supernatant
pellet
resuspended
"P"
supernatant
(discard)
Shake with NaI
110,000 x g, 30 min
pellet
resuspended
"NaI"
supernatant
(discard)
Shake with SDS, 30 min., apply to sucrose gradient
% sucrose
10
15
26
spin 1.5 h, 110,000 x g
pellet resuspended = "J"

The NaI fraction and ATP were diluted in solution C to give a final concentration of 111 mg of protein and 149.6 mg of ATP in a total volume of 80 ml. I added 10 ml of 4.65 mg/ml SDS in solution C, dropwise, while vortexing the mixture at room temperature and swirled the mixture for 30 minutes at room temperature. Immediately after this, the mixture was applied to the top of a sucrose density gradient consisting of three layers with 10, 15 and 26 g% sucrose in solution C, in volumes of 4.8, 6 and 12 ml, respectively. The gradient was centrifuged for 1.5 hours at 110,000 x g, at 4°C in the DuPont-Sorval A-841 fixed angle ultracentrifuge rotor. After decanting the supernatant and drying the upper half of the polycarbonate tube with a swab, I scraped the small, translucent pellet from the surface of the tube and resuspended it with sonication in 100 μl of solution B. This J fraction and aliquots of all fractions obtained from the earlier purification steps were stored at -80°C and NaK-ATPase specific activity, total activity, and fold purification were determined for each.

Enzyme, protein and RNA assays

NaK-ATPase activity was calculated as the difference between the inorganic phosphate released in the presence and absence of 1 mM ouabain. In the presence of this specific inhibitor of NaK-ATPase, the inorganic phosphate released reflects Mg^{2+}-dependent ATPase activity. The samples (homogenate, "S", "P", "NaI", "J") were incubated for 15 minutes at 37°C in enzyme assay solution A (100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 100 mM Tris, 1 mM EDTA, pH 7.4) or B (enzyme assay solution A + 1 mM ouabain), following initiation of the reaction by the addition of ATP

(3 mM final concentration). Addition of 0.2 ml ice-cold 30% TCA was used to terminate the reaction and the inorganic phosphate content was determined by the method of Fiske and Subbarow (1925). Inorganic phosphate production is linear with time for 20 minutes under these conditions (Lo et al., 1976). Specific activity is expressed as μmoles of inorganic phosphate produced per hour per mg protein and per mg DNA. Protein content was determined by the method of Lowry (Lowry et al., 1951). In other studies, total RNA concentrations were determined by assaying ribose with the orcinol method of Kerr and Seraidarian (1945) and by determining the optical density at 260 nm. Mg-ATPase activity was calculated to determine whether hormonal effects on NaK-ATPase activity are selective or the result of a generalized membrane hypertrophy.

One hypothesis proposed to explain glucocorticoid enhancement of renal NaK-ATPase is that it occurs in response to the accelerated delivery of sodium to the epithelial cells, which results from increased renal blood flow and glomerular filtration rate (Jorgensen, 1980; Katz & Epstein, 1967). While both whole animal (Fisher et al., 1975; Sinha et al., 1981) and *in vitro* studies (Doucet et al., 1986; Rayson & Edelman, 1982) provide data supporting a direct glucocorticoid effect at the cellular level, there are conflicting findings (Westenfelder et al., 1977). Determining the sequence of glucocorticoid effects on renal function and NaK-ATPase activity is critical to understanding the mechanism of this hormone's modulation of NaK-ATPase. A rapid increase in renal function followed by a rise in NaK-ATPase activity would support a mechanism relying on enhanced Na^+ delivery. In contrast, if NaK-ATPase activity increased more rapidly than renal function, it would suggest a direct

glucocorticoid effect on renal epithelial cell NaK-ATPase that is not secondary to enhanced sodium delivery.

Glucocorticoid effects on renal function

The time course of the glucocorticoid mediated increase in renal blood flow and glomerular filtration rate was determined in three groups of adrenalectomized rats representing time-points in the experiment, i.e., 0, 12 and 24 h after corticosterone treatment (1 dose, 1 mg/100 g body wt.). A group of sham operated animals was also studied. Following induction of anesthesia, as described above, a catheter (PE-10) filled with heparinized saline was inserted into the left carotid artery and another into the right external jugular vein. A third catheter (PE-50) was placed in the bladder. After 30 minutes of urine output and 1 ml of arterial blood were collected, a priming 0.5 ml bolus of inulin (3 mg/ml) and para-aminohippuric acid (PAH) (1 mg/ml) in heparinized saline was administered via the jugular vein. This was followed by a sustaining infusion of the same solution with a constant infusion pump (Harvard Apparatus) at a rate of 0.018 ml/min for 2 h to reach a constant level of inulin and PAH. Urine was collected throughout the infusion and the final volume recorded. During the final 5 minutes of the infusion a 1 ml blood sample was obtained. Hematocrits were determined for both blood samples. After the erythrocytes were removed with centrifugation, protein-free plasma was obtained by addition of $CdSO_4$ (0.9 %) and 0.1 N NaOH followed by removal of the precipitates with an additional round of centrifugation. Sodium and potassium concentrations were ascertained in plasma and urine with a Beckman Klina atomic

absorption flame photometer and expressed as meq/liter. The urine and plasma samples were assayed spectrophotometrically for both inulin and PAH using the anthrone method (Davidson and Sackner, 1963) and the method of Smith (Smith, 1956), respectively.

Antisera were prepared against the NaK-ATPase α and β subunits to selectively observe the effect of glucocorticoids on the levels of these proteins. Isolation of these subunits was, therefore the next step required.

Antigen preparation

To prepare polyclonal antibodies specifically directed against the subunits of NaK-ATPase, both antigens had to be isolated from other plasma membrane proteins. The intact enzyme fraction having the highest specific activity (J fraction), was electrophoresed through an SDS-polyacrylamide gel to separate the proteins according to their molecular weights. In preparation for SDS-polyacrylamide gel electrophoresis, 200 μl of a solubilizing solution containing 30 g% SDS and 30 vol% glycerol, 2 M β-mercaptoethanol and 0.003 g% pyronin Y (Sigma) were added to 400 μl of the J fraction. The mixture was incubated at 37°C for 30 minutes, diluted with four times it's volume of sample diluting buffer (0.0625 M Tris, pH 6.8) and applied to a preparative slab gel trough. This 3 mm thick gel had a 3 g% acrylamide stacking gel, pH 6.8, and a 9 g% acrylamide separating gel, pH 8.8, both containing 0.1 g% SDS. The electrophoresis buffer consisted of 200 mM glycine, 2 mM Tris, 0.1 g% SDS, pH 8.3. The electrophoresis ran at 10°C at a constant power of 3 Watts until the dye line reached the bottom of the slab. Using an

LKB vertical electrophoresis unit with two gels, this required approximately 3.5 hours.

After discarding the stacking gel and cutting two vertical strips from different regions of the separating gel, each strip was placed on a quartz plate and scanned at 280 nm in a Beckman DU-8 spectrophotometer with a gel scanning module. Locations of the α and β subunits were marked on the gel strips with dots of ink and the strips were placed in their original locations on the slab gel. Strips were cut across the slab gel in the locations of the α and β subunits and each subunit preparation was stored separately at 4°C.

After two such runs were completed (4 slab gels), the gel strips with a given subunit were pooled and cut into small pieces. The proteins were electroeluted from the gel at 4°C for three hours with an Electrophoretic Sample Concentrator (Instrumentation Specialties Company). The same buffer used for electrophoresis was placed in the large buffer tanks and 5 ml of a ten fold dilution of this buffer added to the sample cups. The buffer containing the eluted proteins was withdrawn from the small well in each cup and stored at -80°C in 1.5 ml Eppendorf microtubes.

When 15 ml of buffer containing a particular subunit had been collected, the samples were pooled and concentrated to approximately 1 ml in an Amicon MMC Multimicro Ultrafiltration apparatus containing a 25 mm YM10 Diaflo ultrafiltration membrane (10-kDa MW cut-off). The protein concentration was determined and a small sample was electrophoresed on a horizontal analytical slab gel, 1.5 mm in thickness, by the same procedure as that described for the preparative gel

electrophoresis. This gel was fixed overnight in a solution containing methanol and acetic acid (50 ml% and 10 ml%,respectively) and the proteins stained for 2 h in the same solution containing 0.1 g% Coomassie brilliant blue. The background was destained in 7% acetic acid with 2.5% glycerol. If only one protein band, representing either the α or β subunit of NaK-ATPase, was seen per sample well, the concentrated sample was aliquoted into microfuge tubes and stored at -80°C. If more than one band could be seen, preparative SDS-PAGE, scanning, and electroelution were repeated to improve the purification.

The rat antigens were used to inoculate rabbits and elicited production of a mixture of antibodies directed against various sites on the NaK-ATPase subunits.

Antigen inoculations and monitoring of antibody titres

Preimmune serum was obtained by bleeding adult New Zealand rabbits from the external ear vein, prior to antigen inoculation. Each rabbit was inoculated with 200 μg of the purified α or β subunit of NaK-ATPase. This antigen was first mixed with Freund's complete adjuvant to give a final volume of 1 ml and then injected into 10-15 subcutaneous sites on the animal's back. Approximately 5 ml of blood was drawn weekly from each rabbit and the serum monitored for antibody titre with Laurell rocket immunoelectrophoresis (Laurell, 1966). Antiserum was mixed with melted agarose to give a final agarose concentration of 1 g% agarose in barbital buffer (20 mM diethylbarbituric acid, 70 mM Tris, 0.35 mM calcium lactate, 2.0 mM sodium azide, pH 8.6) and 0.1 ml of either anti-α or anti-β antiserum. The agarose/antiserum mixture (3.5 ml) was poured

between two plates to form 0.5 mm thick slabs. Wells were punched in the congealed agarose, antigen added to the wells and electrophoresis carried out in barbital buffer, overnight, at a constant current of 1 mA. The agarose slabs were then blotted and air dried to thin films and rinsed in 500 ml 0.9 g% saline and deionized water. After being stained in a solution of methanol and acetic acid (50 ml% and 10 ml%, respectively) with 0.1 g% Coomassie brilliant blue for 10 minutes they were destained in the same solution minus the dye. The area under the rocket shaped precipitates was measured (by weighing a paper tracing of the "rocket"), as it is inversely related to the antibody titre in the antiserum sample. The antibody titre was initially boosted every thirty days with inoculations containing 50 μg of antigen in Freund's incomplete adjuvant. When the titre increased significantly, as evidenced by significantly decreased area under the rockets, 50 ml of serum was taken from each rabbit every 14 days and the titre boosted only when the rocket size increased.

The crude antiserum contains numerous plasma protein fractions, including five categories of immunoglobulins. The next goal was to prepare a solution enriched in the desired immunoglobulin fraction.

Antibody collection and enrichment

Blood was collected from the inoculated rabbits via their external ear veins and the serum prepared by pelleting red cells with a clinical centrifuge at 400 x g. Serum samples were aliquoted and stored at -80°C. A saturated ammonium sulfate solution (0.55% of the final volume, pH 8.1-8.2 with concentrated ammonium hydroxide) was rapidly

added to the serum samples. After thoroughly mixing the solution at room temperature, I centrifuged it at 16,000 x g for 30 minutes at 4°C and decanted the supernatant. The precipitate was dissolved in 1 serum volume of 0.15 M NaCl containing 0.001 M sodium azide. A 6 ml sample was ultrafiltrated against dialysis buffer (0.02 M Tris-HCl, pH 8.0, 0.028 M NaCl, 0.02 g% sodium azide) for one hour in an Amicon MMC Multi-micro Ultrafiltration apparatus and applied to a 45 ml column of DEAE Affi-Gel Blue. This column matrix had previously been washed, using a Buchner funnel with five volumes of prewash buffer (0.10 M acetic acid, pH 3.0, 1.4 M NaCl, 40% v/v isopropanol), followed by 10 bed volumes of dialysis buffer. The sample was eluted from the column with dialysis buffer and collected in 6 ml aliquots with an LKB fraction collector while being monitored with the LKB Uvicord I. The 6 to 8 aliquots containing the unbound protein were pooled, concentrated to 6 ml in the Amicon MMC apparatus, aliquoted and stored at -80°C.

The ability to form insoluble antigen-antibody complexes demonstrates antibody binding to immobilized antigens. Inhibition of enzyme activity through antibody interactions with NaK-ATPase subunits was investigated in an effort to characterize the IgG fraction. These steps were critical in assuring that studies with these antisera provided information about the actual NaK-ATPase α and β subunits.

Antibody characterization

Laurell rocket immunoelectrophoresis was the primary method used to monitor polyclonal antibody titre. This method is not ideal for demonstrating antiserum specificity since an optimal ratio of antibody

to antigen is required for the formation of insoluble complexes. A lack of precipitate formation between the antisera and control antigens could therefore be due to a less than optimal ratio of the two, rather than to antigen-antibody specificity. An additional set of techniques was employed to demonstrate binding. In these experiments, a crude membrane preparation (P fraction) was subjected to analytical sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, Western blotting was used to electrophoretically transfer the separated proteins onto a sheet of nitrocellulose paper. One sheet of nitrocellulose and eighteen sheets of filter paper cut to the same dimensions as the separating gel, were soaked briefly in transfer buffer (200 mM glycine, 2 mM Tris base, pH 8.3, 1 g% SDS), and used to "sandwich" the gel between the two electrodes of an LKB electrophoretic transfer apparatus. For 90 minutes at room temperature, a constant current of 0.8 mA per cm^2 of gel transferred the separated proteins from the gel to the nitrocellulose. The nitrocellulose was air dried and the strip containing molecular weight standards was cut out, stained with amido black and destained with 7% acetic acid. Antiserum specificity was demonstrated by the binding of the antibodies to the immobilized α and β subunits and attachment of fluorescent, colored or radioactively labeled ligands to the antibodies. The strips of nitrocellulose paper were incubated in phosphate buffered saline containing 0.05% (v/v) Tween 20 and 0.3 g% bovine serum albumin (TPBS + BSA). After 30 minutes I added the primary antiserum, either α (1:500 dilution), β (1:100 dilution) or preimmune serum (1:100 dilution) and proceeded with the incubation for another hour at room temperature. The nitrocellulose

strip was rinsed repeatedly in TPBS and reacted with a goat anti-rabbit antibody bound covelantly to a biotin molecule. Since many avidin molecules can bind selectively and tightly to each biotin, reacting the strip with an avidin:chromophore complex results in signal amplification and localization of the specific antigen for the primary antibody. The two complexes used were avidin:fluorescein isothiocyanate (FITC) and avidin:biotin:Horseradish Peroxidase (HRP). FITC fluoresces upon exposure to U.V. radiation and HRP turns purple when reacted with its chemical substrate, 4-chloro-1-naphthol, in the presence of hydrogen peroxide. Antiserum specificity is demonstrated by the appearance of single bands in the location of the α or β molecular weight and no bands when incubated with the preimmune serum.

Still greater sensitivity, in detecting binding, was obtained by attachment of ^{125}I-labeled protein A to the bound antibody on a Western blot. The blot was incubated in TSM (20 mM Tris-HCl, pH 7.4, 0.9 g% NaCl, 5 g% nonfat dry milk, 1 mM sodium azide, 100 U/ml penicillin and 100 μg/ml streptomycin) for 6 h at room temperature (Kaufmann et al., 1987). An appropriate volume of preimmune serum or antiserum was added and after a 12 h incubation at 4°C, the blot was rinsed repeatedly in 20 mM Tris-HCl, pH 7.4, 0.9 g% NaCl, 0.05 % (v/v) Nonidet P-40, and 2 M urea, followed by a rinse with the same solution minus the detergent and urea (TS buffer). A final incubation in TSM containing 5-10 μCi radio-labeled iodine (^{125}I) covalently bound to protein-A resulted in the attachment of this radioactive complex to the Fc regions of the anti-bodies and the labeling of the nitrocellulose in the location of the antigen. After rinsing and air drying the nitrocellulose, I exposed it

to x-ray film (Kodak X-OMAT) for an appropriate length of time at room temperature and developed the autoradiograph. The location of the darkened region corresponds to that of the α or β subunit and the density of this region is related to the quantity of the antigen.

The effect of antigen-antibody binding on NaK-ATPase activity was assessed by preincubating the enriched membrane fraction (J fraction) with varying amounts of anti-α ,anti-β or preimmune serum at 4°C for 1 hour prior to assaying enzyme activity. Inhibition of NaK-ATPase activity by one or both of the antisera, but not by equal concentrations of the preimmune serum, provides functional evidence that the antibodies bind specifically to the NaK-ATPase subunits. The antibodies can then be used to identify and quantitate the subunits in crude membrane fractions from rat kidneys, providing information about the effect of adrenalectomy and glucocorticoid administration on these protein levels.

Quantitation of NaK-ATPase subunits

Variation in transfer efficiency makes Western blotting a less than ideal method for quantitation. Slot blots were prepared from duplicate, serial diluted P fractions which were applied to nitrocellulose strips held in a vacuum manifold. These blots were radiolabeled by the same procedure used for Western blots. Autoradiography permitted the locations of the slots to be visualized and quantitated by counting the γ radiation.

An increase in the number of NaK-ATPase subunits could be attributed to increased synthesis or decreased degradation of the subunits in response to the glucocorticoid treatment. Since control of

transcription initiation is recognized as a major mechanism of steroid action, the demonstration of an accompanying increase in the levels of messenger RNAs coding for the α- and β-subunits would suggest that glucocorticoids stimulate NaK-ATPase synthesis.

Preparation of total RNA from kidneys

Rat kidneys were rapidly dissected and frozen in liquid nitrogen. Two grams of this tissue were added to 13 ml of the homogenization buffer (5 M guanidinium isothiocyanate, 10 mM EDTA, 50 mM Tris pH 7.6, 8 vol% β-mercaptoethanol) in a 50 ml polypropylene tube and disrupted with a Brinkman polytron on setting 9. Addition of 15 ml of IAC (chloroform:isoamyl alcohol, 24:1) was followed by brief mixing with the polytron. The mixture was centrifuged for fifteen minutes at 4300 x g in a Sorvall SS-34 rotor at 15°C. The upper, aqueous phase containing the RNA was collected and combined with five volumes of 4 M lithium chloride, mixed and stored for 12 hours at -20°C. After spinning the resulting precipitate at 9800 x g in the SS-34 rotor for 90 minutes at 4°C, I resuspended it with the polytron in three volumes of 3 M LiCl. This mixture was centrifuged for one hour at 9800 x g, 4°C and the pellet was dispersed in 21 ml of resuspension buffer (10 mM Tris, pH 7.5, 1 mM EDTA, pH 8.0, 0.1 g% SDS) and an equal volume of IAC. After mixing and a 20 minute centrifugation at 4300 x g, 4°C, the upper aqueous layer was collected, mixed with 0.05 vol 6 M ammonium acetate and 2.5 vol 100% ethanol and stored for 24 hours at -20°C to precipitate the RNA. This RNA was pelleted at 9800 x g for 30 minutes at 4°C, washed gently three times with 80% ethanol and air dried. The pellet

was dissolved in 800 μl of sterile, diethylpyrocarbonate treated, distilled water.

Although this analysis of glucocorticoid effects on NaK-ATPase subunit mRNA levels used the total RNA, preliminary Northern blotting and hybridizations were carried out with a more highly purified fraction, the polyadenylated RNA. Since this fraction comprises a small percentage of the total RNA, its use in preliminary studies provided enhanced sensitivity and aided in establishing optimal experimental conditions.

Preparation of polyadenylated RNA

Total RNA in 400 μl of elution buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) was heated to 65°C for five minutes, and an equal volume of binding buffer II (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 M NaCl) was added. Following quick cooling in an ethanol-ice slurry, it was applied to an oligo(dT)-cellulose column (0.1 g swollen to 0.5 ml). The eluate was reheated, quick cooled and reapplied to the column, which was then rinsed with 15 ml binding buffer I (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 M NaCl). The remaining, bound RNA was eluted in three 0.4 ml aliquots of elution buffer. The sample was stored at -20°C as a precipitate in ammonium acetate and ethanol and analyzed for NaK-ATPase subunit mRNA levels.

In the next procedures, the RNA was separated by size with denaturing agarose gel electrophoresis and transferred to a nylon membrane. Hybridization with radiolabeled cDNAs permitted analysis of

specific polyadenylated RNA sizes, the amount of RNA degradation and preliminary quantitation.

Denaturing agarose gel RNA electrophoresis and Northern blotting

The RNA (0.05-5.0 μg) was denatured with incubation at 55°C for 15 minutes in a mixture containing 1x MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0), 6% formaldehyde and 50% formamide. After mixing this with 0.2 volumes of formaldehyde loading buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 50 ml% glycerol, 0.4 g% bromophenol blue, 0.4 g% xylene cyanol), the sample was applied to a well in a slab gel containing 1.2 g% agarose in 1x MOPS, 1 ml% formaldehyde, and 0.3 μg/ml ethidium bromide (to permit visualization of the RNA). The gel was submerged in 1x MOPS and run at a constant voltage of 5 volts/cm gel until the leading dye front had moved ~80% of the gel length.

Upon completion of the electrophoresis the RNA bands were photographed on an ultraviolet transilluminator (LKB) and the gel was placed in contact with a prewetted strip of nylon on a vacuum manifold (Phamacia LKB VacuGene XL). The vacuum was applied and the gel was covered for 10 minutes with each of three solutions: distilled DEPC treated water, Denaturation solution (10 mM NaCl, 50 mM NaOH) and Neutralizing solution (1.0 M Tris,pH 7.4). The Transfer solution, 20 x SSC (3.0 M NaCl, 0.3 M sodium citrate, pH 7.0) was next used to fill the apparatus and cover the gel for 2 hours. The transferred RNA was UV cross-linked to the nylon sheet by exposure for 60 seconds.

Hybridization of a single strand of DNA to its complementary RNA was used to selectively identify and quantitate the mRNAs coding for the

NaK-ATPase subunits. Dr. Robert Levenson generously supplied *E-coli* transfected with specific plasmids. These plasmids contain the NaK-ATPase α and β subunit full length cDNAs. The next procedures included culturing large quantities of these bacteria, isolation and restriction of the plasmids, and separation and labeling of the cDNA fragments.

Isolation and labeling of complementary DNAs

The bacteria of interest are transfected with one of two plasmids, pA1N or prb19G. Each of these plasmids contains the entire coding region for one of the two NaK-ATPase subunits, α and β, respectively. A single colony from each of these plates was grown in Luria-Bertani (LB) broth until there was one liter of each in the log phase of growth, as evidenced by an $O.D._{600}$ of 0.6. Chloramphenicol was next added to give a concentration of 170 μg/ml and incubation of the bacterial suspensions continued for 12 h at 37°C with constant swirling. This arrested bacterial growth and genomic DNA reproduction while permitting replication of plasmid DNA. The bacteria were harvested, lysed and the total DNA (genomic and plasmid) precipitated. Following resuspension in TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA), addition of cesium chloride to a concentration of 1 g/ml and ethidium bromide (EBr) to 1 mg/ml, the DNA were centrifuged in a Beckman VTi-80 vertical rotor for 20 h at 219,000 x g at 18°C. EBr was added to permit visualization of DNA bands with a UV light source. A 20 gauge needle attached to a 3 ml syringe was used to remove the lower band containing the plasmid DNA. I extracted the EBr from the plasmid DNA with a solution of butanol saturated with CsCl

and water and precipitated the DNA with sodium acetate and ethanol as described previously.

The cDNAs were restricted from the remainder of the plasmid DNA with the appropriate enzymes and separated on the basis of size with agarose gel electrophoresis. The plasmid pA1N was constructed with the α coding region bounded by base sequences that are recognized by the enzyme HindIII. The plasmid prb19G contained the β coding region and was similarly engineered to contain sequences recognized and cut by the enzyme EcoRI. Enzyme reactions were run to result in complete restriction of known amounts of plasmid DNA. The restricted DNA was then loaded on a preparative slab gel containing 1.2 g% agarose in 1X TGA (40 mM Tris, pH 8.1, 20 mM sodium acetate, 1 mM EDTA) and run at a constant voltage of 5 V/cm. The gel also contained 0.1 μg/ml EBr which permitted localization and removal of the portion of gel containing the cDNA insert. This slice of gel was placed in dialysis tubing which was filled with 1x TGA buffer and returned to the electrophoresis tank. After a 3 hour run at 5 V/cm, the polarity was reversed for 2 minutes, the buffer (now containing the insert) was removed and the membrane rinsed with additional buffer. The DNA inserts were next isolated from any agarose and concentrated by running the sample through an Elutip-d ion exchange column (Schleicher & Schuell). The resulting small volume permitted precipitation of the DNA with 0.05 volumes of 6 M ammonium acetate, 2 volumes of ethanol and 20 μg of glycogen (Boehringer Mannheim). The cDNA was spun down in an Eppendorf microfuge and resuspended in TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA). A random primer DNA labeling kit (Boehringer Mannheim) was used to incorporate

[α-^{32}P]dCTP into the cDNA. This method uses random hexanucleotides as primers which bind to the denatured cDNA. The large subunit of DNA polymerase I (Klenow fragment) then polymerizes a mixture of unlabeled dATP, dGTP and dTTP along with the labeled dCTP, using the purified cDNA as the template. The reaction was run for 6 h at 37°C and terminated by removal of the unincorporated nucleotides from the labeled cDNA with a passage over the Elutip-d column. The dCT^{32}P-labeled cDNA fragments were then ready to incubate with the Northern blotted or directly applied RNA (slot blots) to selectively quantitate mRNAs coding for NaK-ATPase subunits.

Prehybridization, DNA/RNA hybridization and autoradiography

The Northern blots were placed in a sealable plastic pouch and reacted with 10 ml prehybridization buffer (1% bovine serum albumin, 7% SDS, 0.5 M phosphate, pH 7.0 0.001 M EDTA) (Church & Gilbert, 1984), containing 1 mg% heat denatured salmon sperm DNA, for 1 h at 65°C. The hybridization was performed for 12 h at the same temperature in the same solution containing a radiolabeled and heat denatured, full length NaK-ATPase α1 or β1 complementary DNA (cDNA). The blots were rinsed two times, for 15 minutes each in 150 ml of 1X SSC + 0.1 g% SDS at room temperature, followed by two additional rinses in the same volume of 0.25X SSC + 1 g% SDS at 60°C, for 10 minutes each. They were exposed at -70°C to X-OMAT AR film with two intensifying screens (DuPont Cronex). The Northern blots were also probed with a nick-translated plasmid p5B which contains a sequence corresponding to mouse 18S rRNA (Bowman

et al., 1981). This served as an internal control for the analysis of α and β subunit mRNAs.

One objection to the use of Northern blots quantitatively is that variation might result from differences in transfer of RNA from different regions of the gel. Preparation of serial dilutions of the RNA samples and application directly to the nylon (slot blots) eliminates this variability. It also permits identification of a range of RNA concentrations which produces linearly increasing signals.

RNA slot blots

The total RNA samples were diluted to 200 μg of RNA in 50 μl. These were added to sterile 1.5 ml tubes containing 30 μl 20 x SSC (1 x SSC contains 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and 20 μl 37% formaldehyde. Each mixture was then incubated at 65°C for 15 minutes and 15 x SSC was used to prepare three serial dilutions. A 100 μl volume from each dilution was applied with suction to a prewetted nylon sheet, employing a vacuum manifold. The RNA, UV cross-linked to the nylon, was prehybridized and hybridized with radiolabeled cDNAs for NaK-ATPase α, and β subunits as described for the Northern blots. Autoradiography was followed by scanning of the X-ray film with a Beckman DU-8 spectrophotometer.

RESULTS

NaK-ATPase enrichment

The initial enzyme enrichment procedure described in Figure 1 produced NaK-ATPase in membrane vesicles, maintaining the enzyme's higher order structure and permitting the specific activity to be used to assess purification (Table 1). Differential centrifugation of the 7000 x g supernatant (S fraction) from the crude kidney homogenate produced a microsomal pellet (P fraction) showing a 19% increase in NaK-ATPase specific activity. Further solubilization of membrane proteins with sodium iodide produced a NaI fraction with a 107% enhancement of specific activity, compared with the P fraction. Incubation of the NaI fraction with SDS and sucrose density gradient ultracentrifugation resulted in a 293% increase in specific activity relative to the NaI treated microsomes and an 864% increase in NaK-ATPase specific activity relative to the S fraction. Figure 2 shows an SDS-polyacrylamide gel stained with Coomassie brilliant blue. An equal amount of protein (50 μg) from each step in the purification procedure was electrophoresed and a step-wise enrichment of the proposed α and β subunits can be seen to accompany increases in specific activity.

NaK-ATPase activity

The NaK-ATPase activities of S fractions from the renal cortex and medulla decreased 28 and 51%, respectively, following adrenalectomy (Figure 3). NaK-ATPase activity responded to multiple injections of

Table 1. Enzyme and protein assay data for NaK-ATPase enrichment from rat kidney homogenates.

The S fraction is the combined supernatant following three rounds of homogenization and centrifugation at 7000 x g. The P fraction is the microsomal pellet. NaI is the microsomal pellet after treatment with sodium iodide. J is the pellet formed after SDS extraction and sucrose gradient ultracentrifugation. Purification values represent specific activity normalized to that of the S fraction.

	S	P	NaI	J
volume (ml)	57.0	1.7	1.3	0.1
total protein (mg)	214.1	45.7	12.6	1.0
total activity (μmol P_i/h)	26434.0	5944.0	4387.0	1079.0
yield (%)	100.0	22.4	16.6	4.0
specific activity (μmol P_i/mg Pr/h)	109.6	130.2	269.3	1058.0
purification (specific activity normalized to that of S fraction)	1.0	1.2	2.5	9.6

Figure 2. SDS-PAGE of enriched NaK-ATPase fractions.

An equal amount of protein (50 μg) from each of the preparations assayed in Table 1 was subjected to electrophoresis in a 3 g% polyacrylamide stacking gel and a 9 g% separating gel under reducing conditions. The proteins were fixed and stained with Coomassie brilliant blue to show a progressive enrichment of the NaK-ATPase α and β subunits.

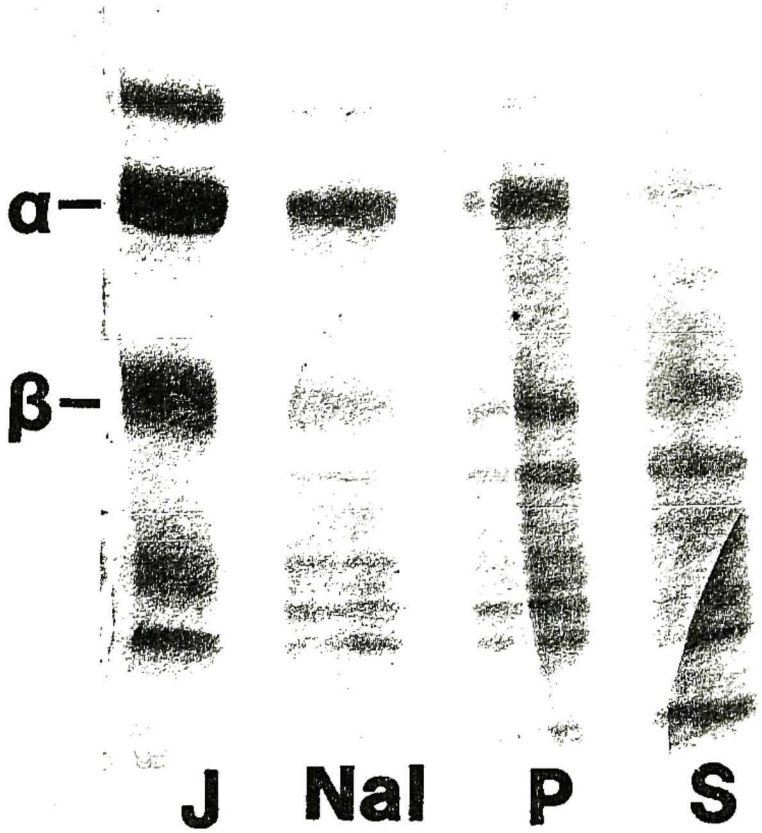
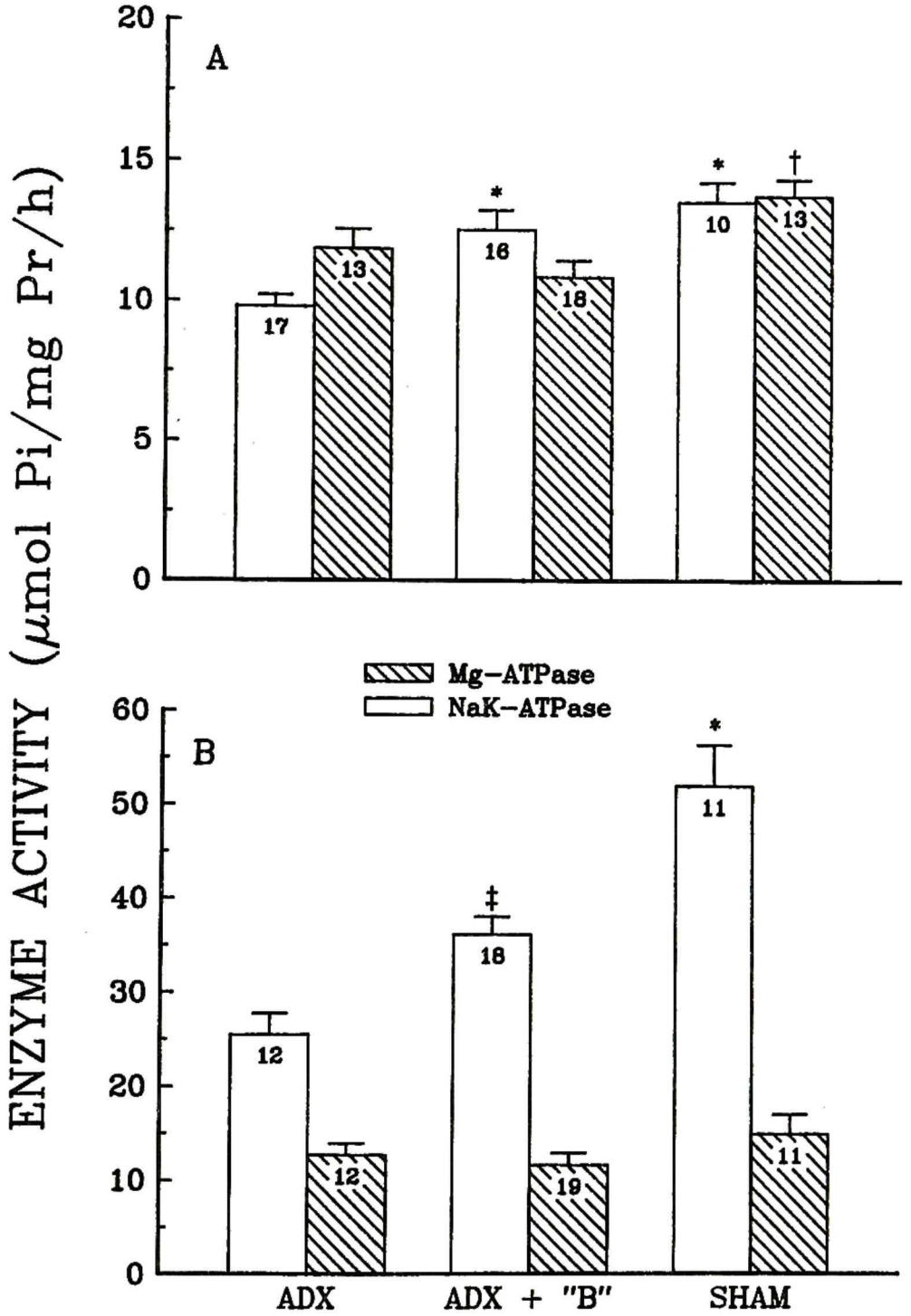

α–
β–
J
NaI
P
S

Figure 3. Effect of multiple doses of corticosterone on renal cortical and outer medullary NaK-ATPase and Mg-ATPase specific activities. Adrenalectomized rats were injected with either dilutent (ADX) or corticosterone (250 μg/100 g BW) twice daily for 3 successive days (ADX + B). Sham operated rats were administered diluent according to the same schedule. The rats were killed and renal cortices (A) and outer medullas (B) were dissected for enzyme enrichment. Enzyme activities of the S fractions are expressed as μmol P_i released per mg protein per h. The number of observations is in printed in the bars. Values are the mean ± SEM. *P* values are determined with an analysis of variance followed by Duncans multiple range test.

* $P \leq 0.05$ when compared with the adrenalectomy + diluent group.

† $P \leq 0.05$ when compared with the adrenalectomy + "B" (corticosterone) group.

‡ $P \leq 0.05$ when compared to both adrenalectomy + diluent and sham.


ENZYME ACTIVITY (μmol Pi/mg Pr/h)
A
B
Mg–ATPase
NaK–ATPase
20
15
10
5
0
60
50
40
30
20
10
0
17
13
16
18
10
13
12
12
18
19
11
11
*
†
‡
ADX
ADX + "B"
SHAM

corticosterone (250 μg/100 g BW, twice daily, 3 days), reaching 93% of sham operated values in the cortex and 69% in the medulla. The lack of Mg-ATPase response supports a selective enrichment of NaK-ATPase rather than a generalized plasma membrane hypertrophy.

NaK-ATPase activity in both cortical and medullary tissue from the adrenalectomized rats was significantly increased 6 h following a single injection of corticosterone (1 mg/100 g BW) (Figure 4). Cortical NaK-ATPase activity increased by 31% at this time, rising to a peak of 41% by 24 h before decreasing almost to the diluent level (zero time point) by 48 h. At the 6 h time point the medullary NaK-ATPase activity was 65% greater than that seen in the diluent treated animal. The activity in this tissue remained elevated thru 48 h, at which point it was 25% greater than the adrenalectomized + diluent value and significantly less than the 24 h value. Mg-ATPase activity was unaffected at all time points in the cortex and was only transiently enhanced (at the 12 h time point) in the medulla.

The dose dependence of NaK-ATPase activity in the cortex and outer medulla was observed 24 h after a single injection of corticosterone (Figure 5). The increase in renal cortical NaK-ATPase activity reached 35, 48 and 55% following administration of 100, 250 and 500 μg corticosterone/100 g BW, respectively. In the outer medulla, the same doses enhanced NaK-ATPase activity by 0.3, 22 and 40%, respectively. None of the above doses of corticosterone produced a change in Mg-ATPase activity in either tissue.

Figure 4. Temporal changes in renal cortical and outer medullary NaK-ATPase and Mg-ATPase specific activities in the S fraction after corticosterone.

Adrenalectomized rats were administered a single dose of corticosterone (1 mg/100 g BW). Six, 12, 24 and 48 h, following the injection, the rats were killed and the renal cortices (A) and outer medullas (B) were removed for enzyme preparation. Activities are expressed as μmol P_i released per mg protein per h. The number of observations is in parenthesis. Values are the mean ± SEM. *P* values are determined with analysis of variance followed by Duncan's multiple range test.

* $P \leq 0.05$ when compared to the diluent treated (zero time point) group.

† $P \leq 0.05$ when compared to the 24 h corticosterone treated group.

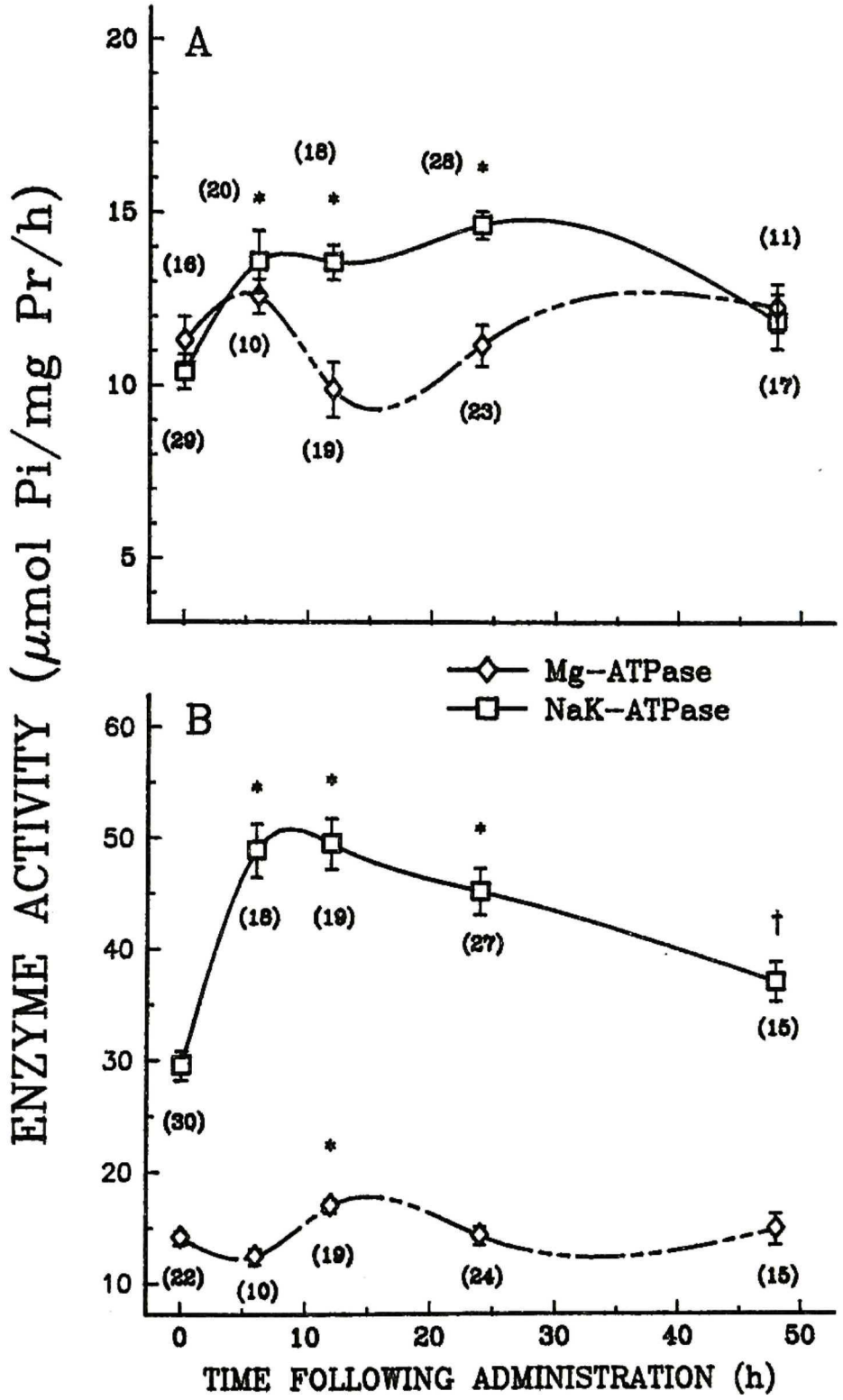
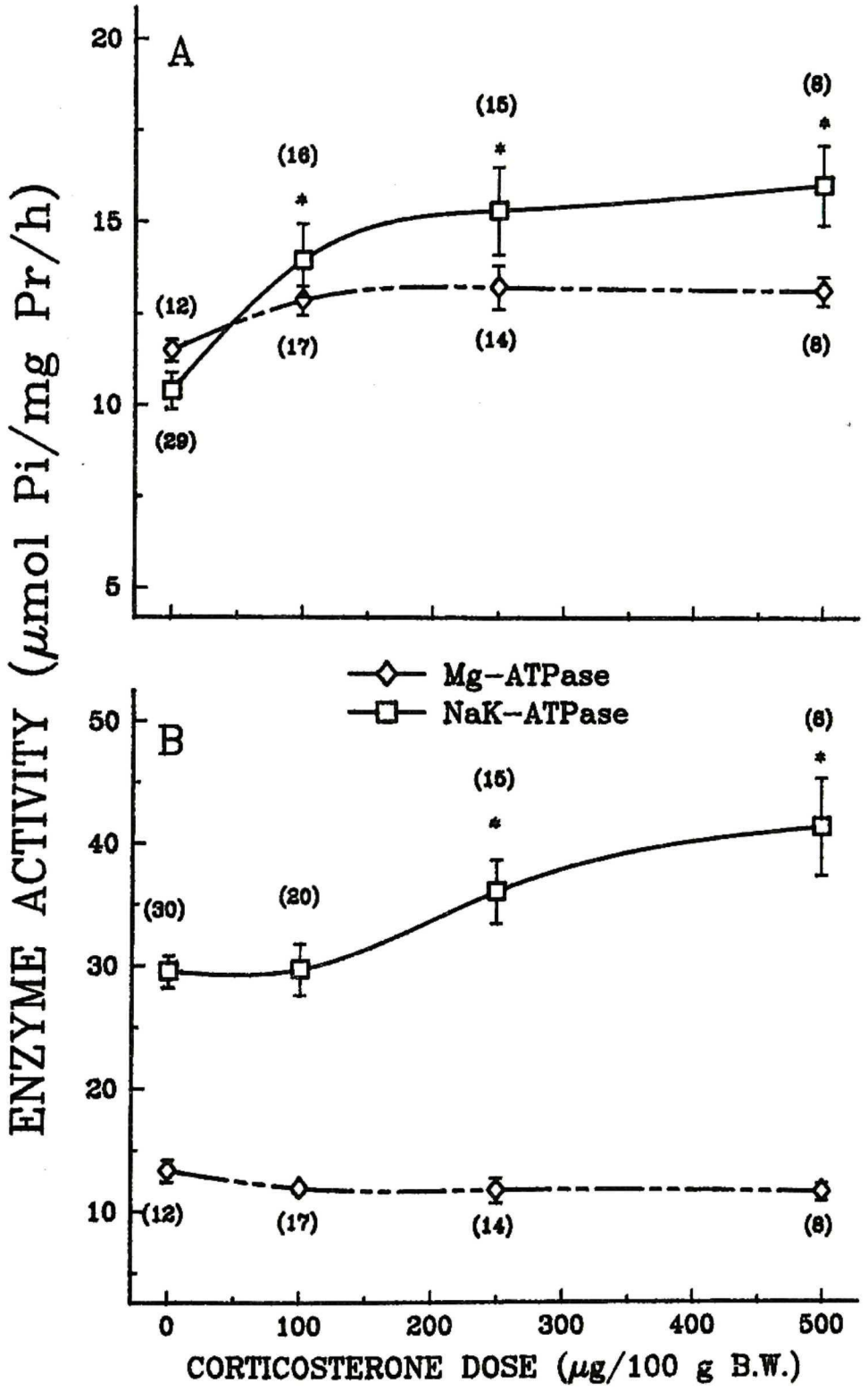

A
B
20
15
10
5
60
50
40
30
20
10
0
10
20
30
40
50
(20)
(18)
(28)
(16)
(10)
(11)
(29)
(19)
(23)
(17)
(30)
(18)
(19)
(27)
(15)
(22)
(10)
(19)
(24)
(15)
Mg–ATPase
NaK–ATPase
ENZYME ACTIVITY (μmol Pi/mg Pr/h)
TIME FOLLOWING ADMINISTRATION (h)

Figure 5. Dose response of renal cortical and outer medullary NaK-ATPase and Mg-ATPase specific activities in the S fraction after corticosterone.

Adrenalectomized rats were injected with a single dose of corticosterone 24 h prior to decapitation and dissection of cortices (A) and medullas (B) for enzyme preparation. Enzyme activities are expressed as μmol P_i released per mg protein per h. The number of observations is in parenthesis. Values are the mean ± SEM. *P* values are determined with an analysis of variance followed by Duncans multiple range test.

* $P \leq 0.05$ when compared to the diluent treated (zero dose group).


A
B
Mg–ATPase
NaK–ATPase
ENZYME ACTIVITY (μmol Pi/mg Pr/h)
CORTICOSTERONE DOSE (μg/100 g B.W.)
(29)
(12)
(16)
(17)
(15)
(14)
(8)
(30)
(20)
0
100
200
300
400
500
5
10
15
20
30
40
50

Renal function studies

The plasma and urine inulin and PAH concentrations produced by the sustaining infusion are presented in Table 2. Within the 7 day recovery period, adrenalectomy decreased values for all the renal function parameters observed (Figure 6). Renal plasma flow decreased by 37%, accompanied by a 52% drop in the glomerular filtration rate. Sodium filtration and reabsorption were both diminished by 63-64%. Within 12 h following administration of a single dose (1 mg/100 g body wt.) of corticosterone, RPF was essentially restored with a 33% increase and maintained at the 24 hour time point. In contrast, values for GFR, Na^+ filtration and Na^+ reabsorption were not changed at 12 and 24 h following administration of corticosterone. Hematocrits, plasma sodium and potassium concentrations were unchanged throughout these studies.

Antigen preparation

The enrichment of putative α and β protein bands upon electrophoresis of the J fraction was sufficient to allow the gel to be cut in these locations. Following electroelution and ultrafiltration, the newly purified proteins were again electrophoresed and stained (Figure 7). If obvious contamination remained, another round of preparative SDS-PAGE and electroelution was performed. The resulting proteins were mixed with adjuvant and used to inoculate rabbits for production of anti-α and anti-β polyclonal antibodies.

Table 2. Concentrations of inulin and PAH in the plasma and urine following a 2 h sustaining infusion.

Adrenalectomized rats were given a single, subcutaneous injection of the vehicle (0 h) or corticosterone (1 mg/100 g body weight) either 12 or 24 h prior to the surgery. Sham operated animals received the vehicle. Anesthetized rats had cannulas placed in the left carotid artery, right external jugular vein and the bladder. Following a thirty minute baseline collection and a priming bolus, the rats were infused for 2h with inulin and PAH in heparinized saline. Urine was collected, throughout the infusion, and plasma samples were obtained, during the last 5-10 minutes of the infusion. Colorimetric assays were used to determine inulin and PAH concentrations in the plasma and urine samples. Concentrations are given in μg/ml and values are expressed as the mean $\pm$ SEM. *P* values were determined with analysis of variance followed by Duncan's multiple range test. Number of observations is in parenthesis.

* $P \leq 0.05$ when compared to the sham operated group.

	Sham	Adrenalectomized vehicle (0 h)	Corticosterone (12 h)	Corticosterone (24 h)
Inulin (μg/ml)				
Plasma	13.68 ±4.29	23.03 ±1.45	21.67 ±2.55	18.50 ±2.61
Urine	2276 ±405	2941 ±309	2358 ±312	3131 ±466
PAH (μg/ml)				
Plasma	2.87 ±0.22	3.44 ±0.30	4.47 ±0.43	5.08* ±0.80
Urine	920 ±126	1127 ±185	1021 ±168	1543 ±272
N	(10)	(17)	(13)	(11)

Figure 6. Temporal changes in four measures of renal function following adrenalectomy and supplementation with corticosterone. Anesthetized rats had cannulas placed in the left carotid artery, right external jugular vein and the bladder. Following a thirty minute baseline collection and a priming bolus, the rats were infused for 2h with inulin and PAH in heparinized saline. Urine was collected, throughout the infusion, and plasma samples were obtained, during the last 5 minutes of the infusion. The treatment groups included sham operated rats (control), adrenalectomized rats given diluent (zero time) and adrenalectomized rats given corticosterone (1 dose, 1 mg/100 g BW) either 12 or 24 h prior to the collection. Inulin and PAH concentrations were determined spectrophotometrically. Renal plasma flow and glomerular filtration rates are expressed as ml per minute per g kidney weight. Sodium filtration and reabsorption are expressed as μeq per minute per g kidney weight. The number of observations is in parenthesis. Values are expressed as the mean ± SEM. *P* values are determined with analysis of variance followed by Duncan's multiple range test.

† $P \leq 0.05$ when compared to the sham operated group.

* $P \leq 0.05$ when compared to the diluent treated group.

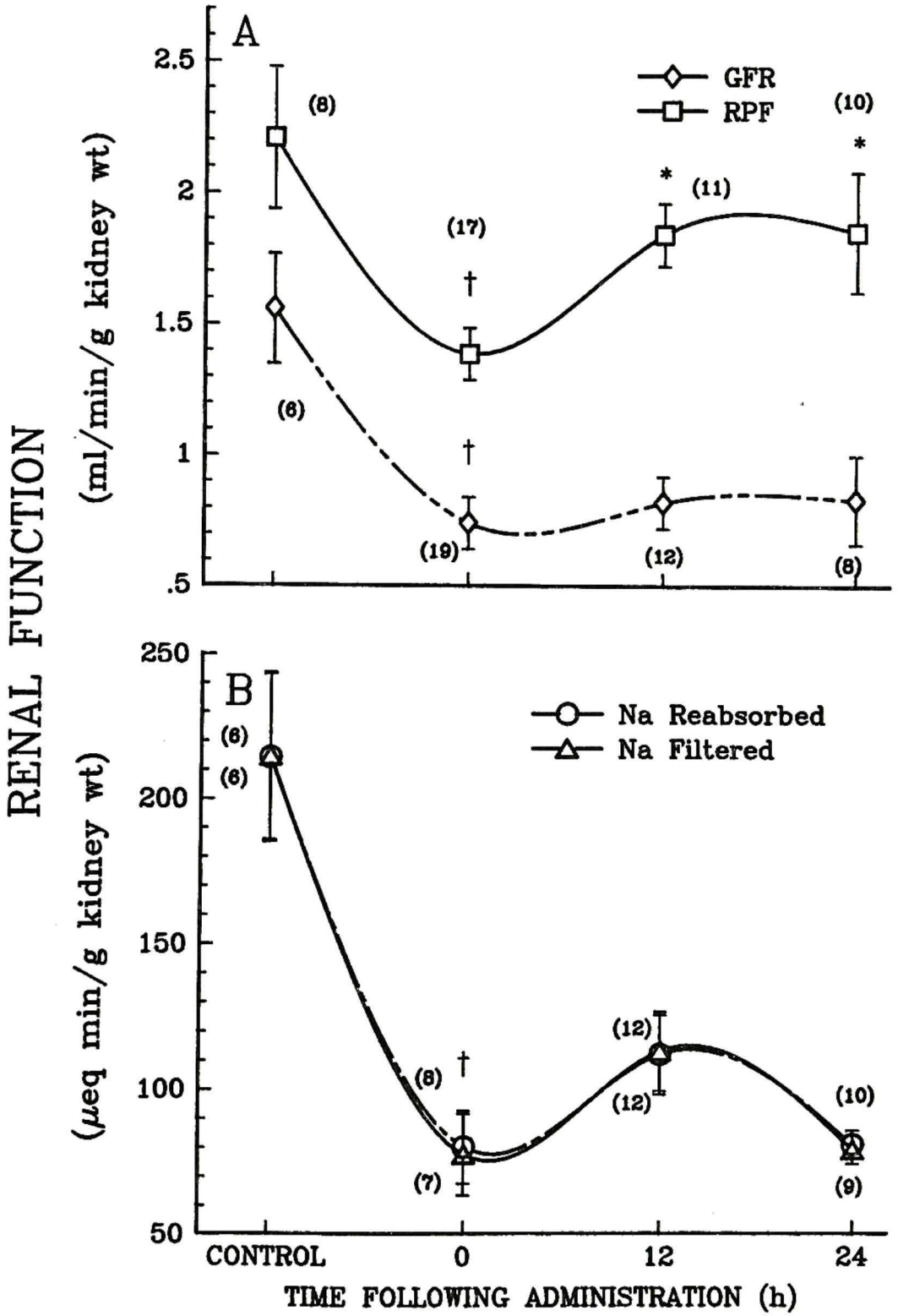
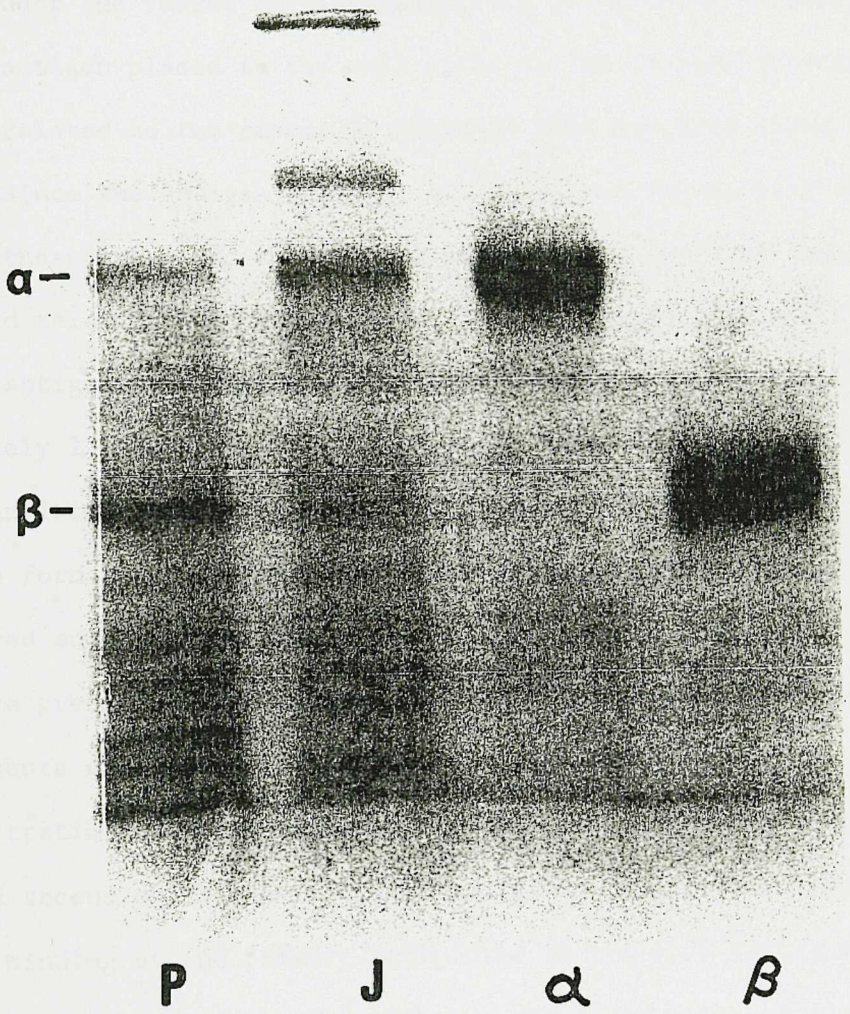

RENAL FUNCTION
A
(ml/min/g kidney wt)
2.5
2
1.5
1
.5
GFR
RPF
(8)
(6)
(17)
(19)
(11)
(12)
(10)
(8)
*
†
B
(µeq min/g kidney wt)
250
200
150
100
50
Na Reabsorbed
Na Filtered
(6)
(6)
(8)
(7)
(12)
(12)
(10)
(9)
CONTROL
0
12
24
TIME FOLLOWING ADMINISTRATION (h)

Figure 7. A Coomassie stained SDS-polyacrylamide gel showing progressive enrichment and isolation of the NaK-ATPase subunits. Equal amounts (50 μg) of protein from three steps in the purification procedure were solublized in SDS, subjected to SDS-PAGE and stained with Coomassie brilliant blue. The microsomal fraction, P, was prepared with differential centrifugation of a kidney homogenate. The J fraction resulted from further solubilization with NaI and SDS followed by sucrose density gradient ultracentrifugation. The isolated α and β subunits were produced by preparative SDS-PAGE of the J fraction, localization of the desired bands by scanning with UV densitometry and electroelution of the proteins.


α
β
P
J
α
β

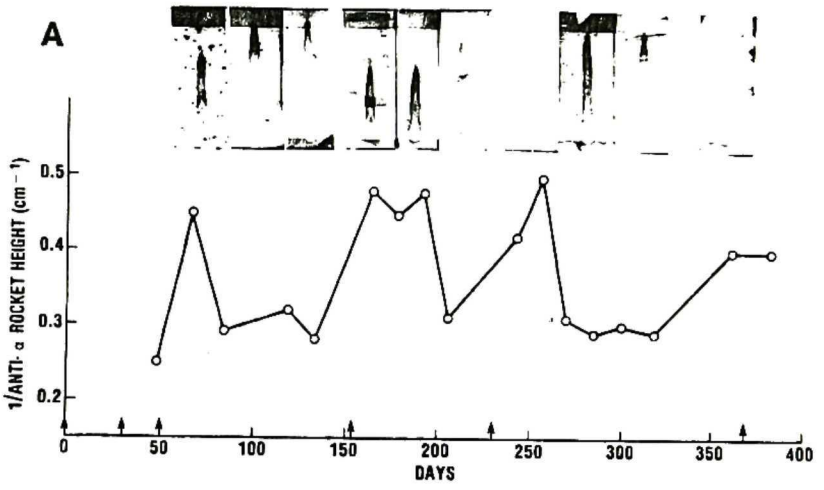
Polyclonal antibody preparation and characterization

Screening with Laurell rocket immunoelectrophoresis demonstrated the existence of polyclonal antibodies which formed complexes with the putative NaK-ATPase α and β subunits (Figure 8A and B, respectively). The area under the rocket shaped precipitate is directly related to the amount of antigen placed in the well prior to immunoelectrophoresis and inversely related to the amount of antibody that has been mixed with the agarose. Since the antigen quantity and antiserum volume were kept constant, these areas and titration curves constructed from these areas represented relative antibody titres. This indicated when additional boosts of antigen were required to maintain antibody production for approximately 1 year without inducing tolerance. The method also provided an initial means of characterizing the antibodies, since the ability to form insoluble complexes with the appropriate antigen demonstrated antibody presence.

Since precipitation of antibody-antigen complexes requires that the components exist in an optimum ratio, this method is poorly suited for demonstration of specificity. By localizing the antigens with HRP-conjugated second antibodies (Figure 9), it was possible to demonstrate selective binding of the primary antibodies to proteins with the relative molecular masses of the NaK-ATPase α and β-subunits. Figure 9 shows five nitrocellulose strips onto which electrophoresed proteins from a kidney microsomal preparation (P fraction) had been Western blotted. When the primary antiserum was directed against the α-subunit (A and B upper band), the appearance of a single band suggests that these

Figure 8. Laurell rocket immunoelectrophoretic assessment of relative serum anti-α (A) and anti-β (B) titre.

Following three initial inoculations with purified NaK-ATPase subunit (200 μg) in Freund's complete adjuvant, serum samples were collected weekly and assayed for relative anti-α (A) and anti-β (B) titres with Laurell rocket immunoelectrophoresis. Additional inoculations with antigen (50 μg) in incomplete adjuvant were administered (at the arrows) in response to diminishing antibody titre. This drop was indicated by the increased area under the "rocket" shaped precipitates (in panel above graph).


A
1/ANTI- α ROCKET HEIGHT (cm⁻¹)
0.5
0.4
0.3
0.2
0
50
100
150
200
250
300
350
400
DAYS

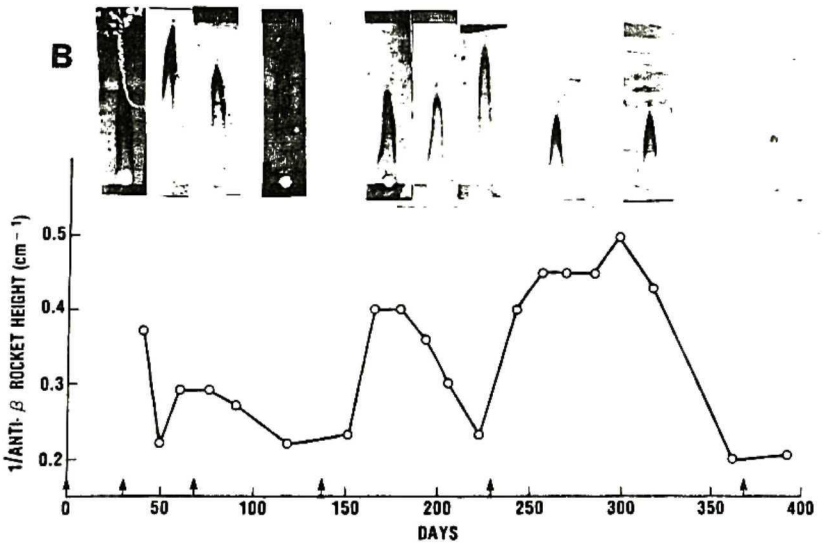
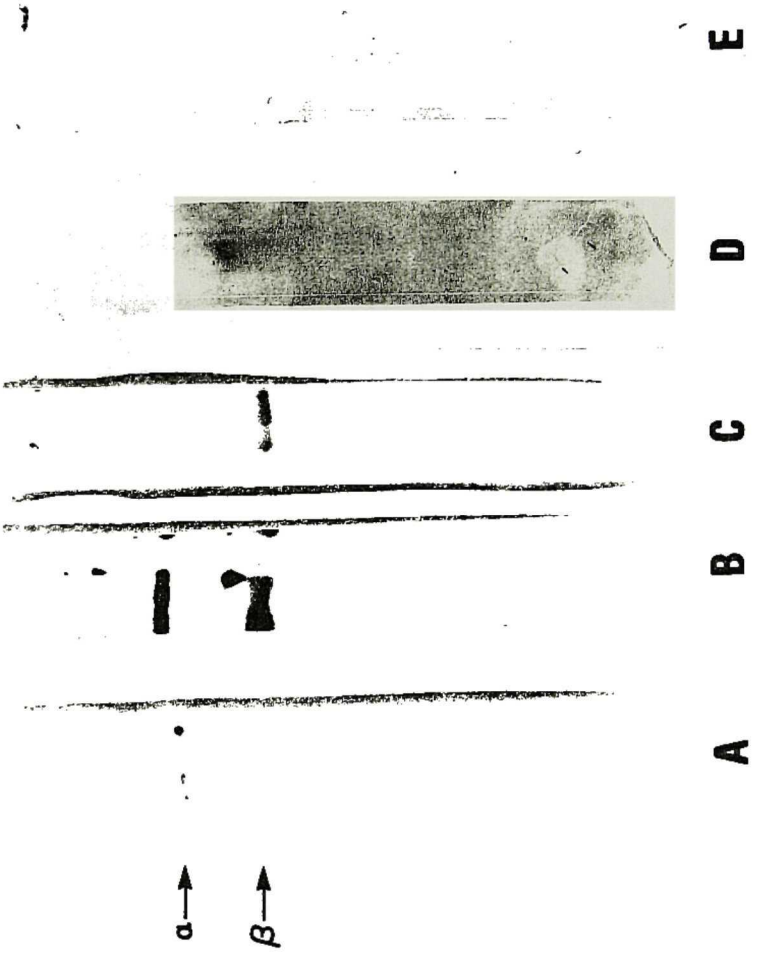

B
1/ANTI- β ROCKET HEIGHT (cm⁻¹)
0.5
0.4
0.3
0.2
0
50
100
150
200
250
300
350
400
DAYS

Figure 9. Horseradish Peroxidase labeling of Western blots. Following SDS-PAGE of enriched membrane fraction (J, 50 μg protein) and Western blotting, the nitrocellulose strips were incubated in the presence of (A) anti-α serum, (B) both anti-α and anti-β sera, (C) anti-β serum, (D) no serum, or (E) preimmune serum. They were next reacted with the biotinylated second antibody followed by avidin: biotin: HRP complex and the HRP substrate with hydrogen peroxide.


E
D
C
B
A
α
β

polyclonal antibodies are directed against the various antigenic sites on a protein with the molecular weight of the α subunit. The band seen in lane C and the lower band in B is in the molecular weight range of the β-subunit. Incubation with either no primary antiserum (D) or pre-immune serum (E), even with longer exposure to the enzyme substrate (note the darker background) resulted in an absence of detectible bands.

The Western blotting procedure was repeated with P fractions (8 μg of total protein each) from adrenalectomized rats, and sham operated rats. These blots were incubated with the antibodies against NaK-ATPase α or β subunits and labeled with ^{125}I-labeled protein A. Autoradiographs from blots probed with the putative anti-α and anti-β antibodies show single bands for proteins having apparent molecular weights of ~100 kd and ~50 kd (Figure 10).

Any change in NaK-ATPase specific activity which results from the presence of these antibodies would support binding of these polyclonal antibodies to the subunits of NaK-ATPase. When aliquots of an enriched plasma membrane fraction (J fraction) were preincubated with increasing concentrations of preimmune, anti-α or anti-β serum and then assayed for NaK-ATPase specific activity, the data in Figure 11 resulted. Preincubation of the membranes in anti-α serum produced increasing inhibition of NaK-ATPase specific activity (normalized to the control) with decreasing antiserum dilution in the range investigated (1:50 to 1:2). Preincubation with preimmune serum had no effect on NaK-ATPase activity as did interaction with anti-β serum, except at the highest concentration, which actually enhanced the specific activity.

Figure 10. Western blots of kidney microsomal fractions (P) labeled with ^{125}I-Protein A.

Samples were prepared from rats which were: a. adrenalectomized and given a single dose of the vehicle 12 h before, b. adrenalectomized and given a single dose of corticosterone (1 mg/100 g BW) 12 h before, or c. sham operated. The blots were incubated with (A) anti-α serum or (B) anti-β serum followed by incubation with ^{125}I-labeled protein A and autoradiography.

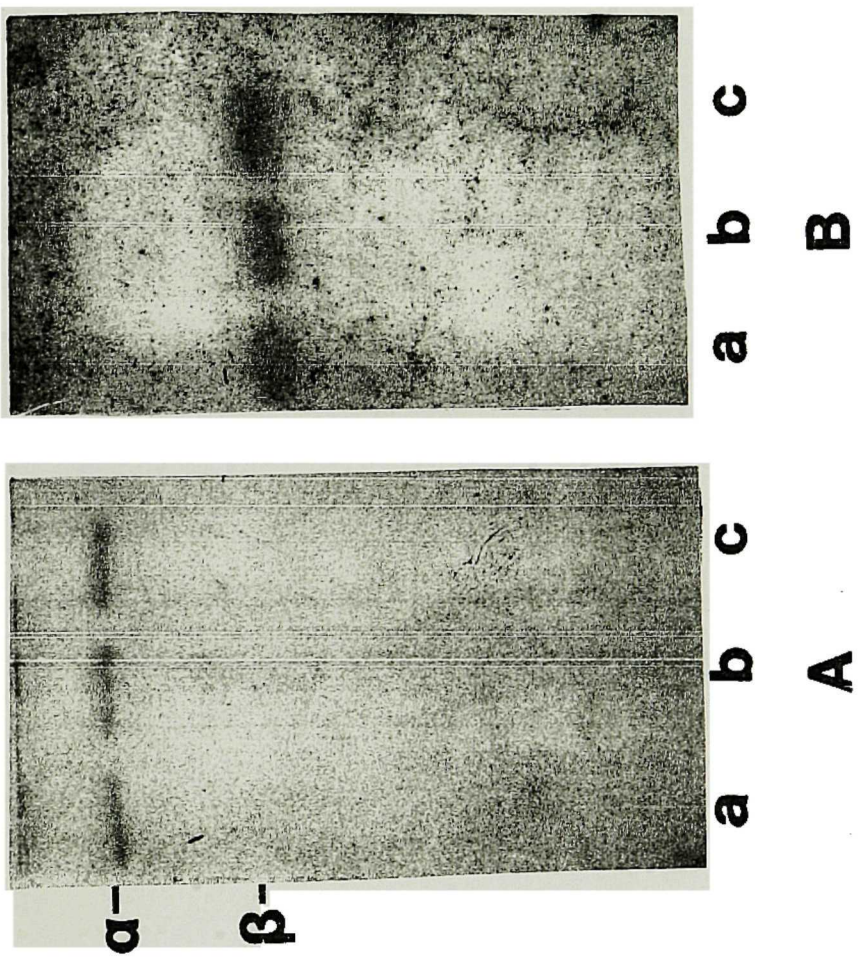
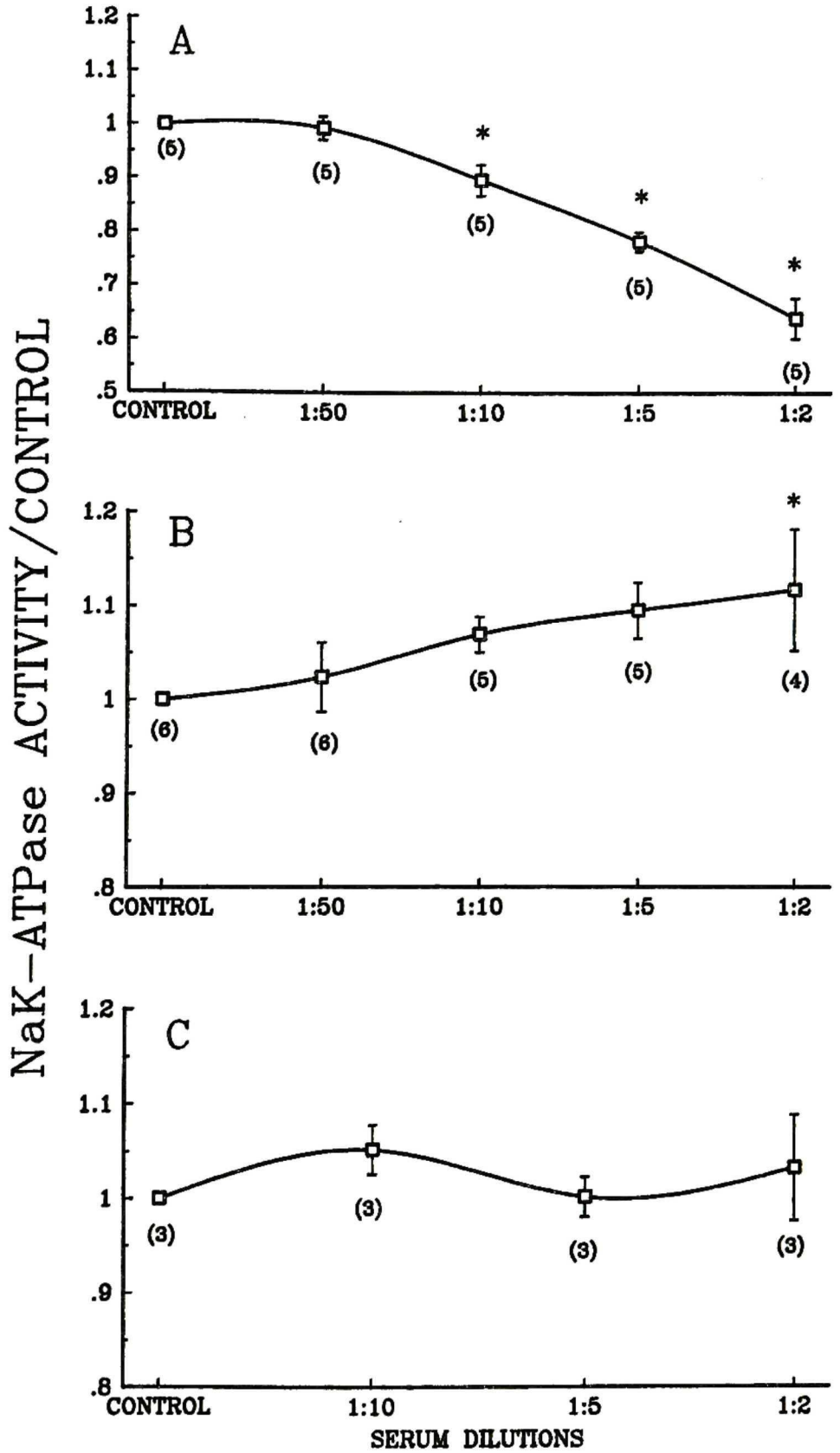

α-
β-
a b c
A
a b c
B

Figure 11. NaK-ATPase activity inhibition by preincubation with anti-sera.

J fraction samples (100 μg protein in each) were preincubated with varying dilutions of (A) anti-α serum, (B) anti-β serum or (C) preimmune serum, in a final volume of 1 ml at 4°C for 60 minutes prior to assaying enzyme activity. NaK-ATPase activities are normalized to the control (no serum) values and expressed as mean ± SEM. *P* values are determined by analysis of variance followed by Duncan's multiple range text.

* $P \leq 0.05$ when compared to the control.


A
B
C
NaK-ATPase ACTIVITY/CONTROL
1.2
1.1
1
.9
.8
.7
.6
.5
(5)
(5)
(5)
(5)
(5)
*
*
*
CONTROL
1:50
1:10
1:5
1:2
(6)
(6)
(5)
(5)
(4)
*
(3)
(3)
(3)
(3)
SERUM DILUTIONS

Quantitation of NaK-ATPase levels with slot blots

Quantitation of subunit levels was repeated with direct application of serially diluted P fractions to nitrocellulose (slot blots). This method demonstrated (Figure 12) that α and β subunit levels decreased by 48 and 52% following adrenalectomy, respectively. Within 2 h of a single corticosterone administration to adrenalectomized rats, the α and β subunit levels were restored to sham operated levels with 74 and 67% increases, respectively.

Denaturing agarose electrophoresis of total RNA and Northern blotting

To separate RNAs on the basis of molecular weight, it is necessary to prevent intrachain hybridization that may result from folding of these single stranded molecules. This denaturation of the RNA samples was assured by heating to 55°C in the presence of formaldehyde and maintained by electrophoresis through a formaldehyde containing agarose gel. Figure 13 is a photograph of such a gel containing 1 μg/ml EBr and 5 μg total RNA per well. The upper and lower discrete bands in each well are the 28 S and the 18 S ribosomal RNA bands, respectively, and the smear of lighter staining material is caused by the heterogeneous mRNAs in each sample. The total RNA was quantitated by reading the $O.D._{260/280}$. Ribosomal RNA bands provided preliminary evidence that each well contained a comparable amount of RNA. The photograph in Figure 14 shows a Northern blot prepared by transferring the RNA from the gel in Figure 13. While some of the RNA, particularly in the higher molecular weight range, was seen to remain in the gel, the overall transfer appeared to have been quite uniform.

Figure 12. Quantitation of NaK-ATPase subunit levels with antibody probed slot blots.

Microsomes (P fraction) from rats which had been adrenalectomized (ADX), adrenalectomized and given a single dose of corticosterone (1 mg/100 g BW) 2 h before (ADX + "B"), or sham operated (SHAM) were serially diluted and applied to nitrocellulose using a vacuum manifold. Following incubation with either anti-α serum (open bars) or anti-β serum (hatched bars) and ^{125}I-labeled protein A, radioautography was used to localize the slots. These nitrocellulose slots were cut and the gamma radiation counted. The subunit quantities are expressed as cpm per 100 ng protein. *P* values are determined with analysis of variance followed by Duncan's multiple range test. The number of observations is shown inside the bars.

*,† $P \leq 0.05$ when compared to the ADX group.

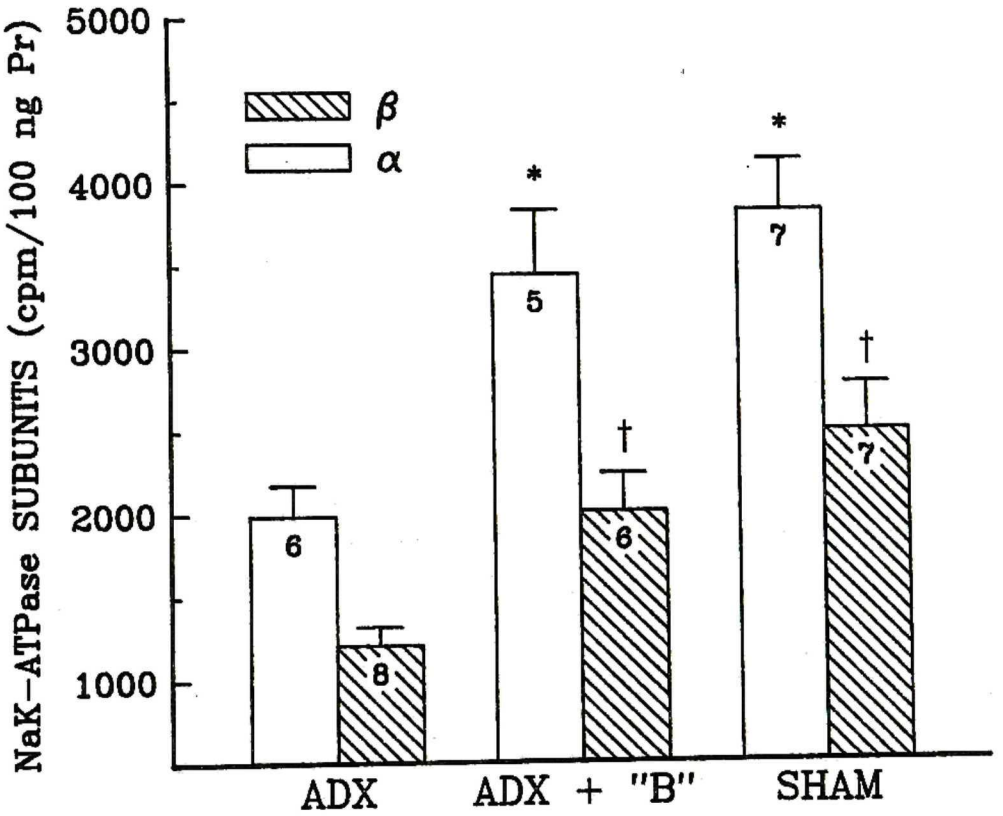

NaK-ATPase SUBUNITS (cpm/100 ng Pr)
5000
4000
3000
2000
1000
β
α
*
†
6
8
5
6
7
7
ADX
ADX + "B"
SHAM

Figure 13. Denaturing agarose gel electrophoresis of total RNA from rat kidneys.

An equal amount of heat-denatured total RNA was placed in each of six wells in a formaldehyde-agarose gel containing 1μg/ml ethidium bromide and electrophoresed in 1 X MOPS buffer at 5 V/cm of gel for approximately 1.5 h.

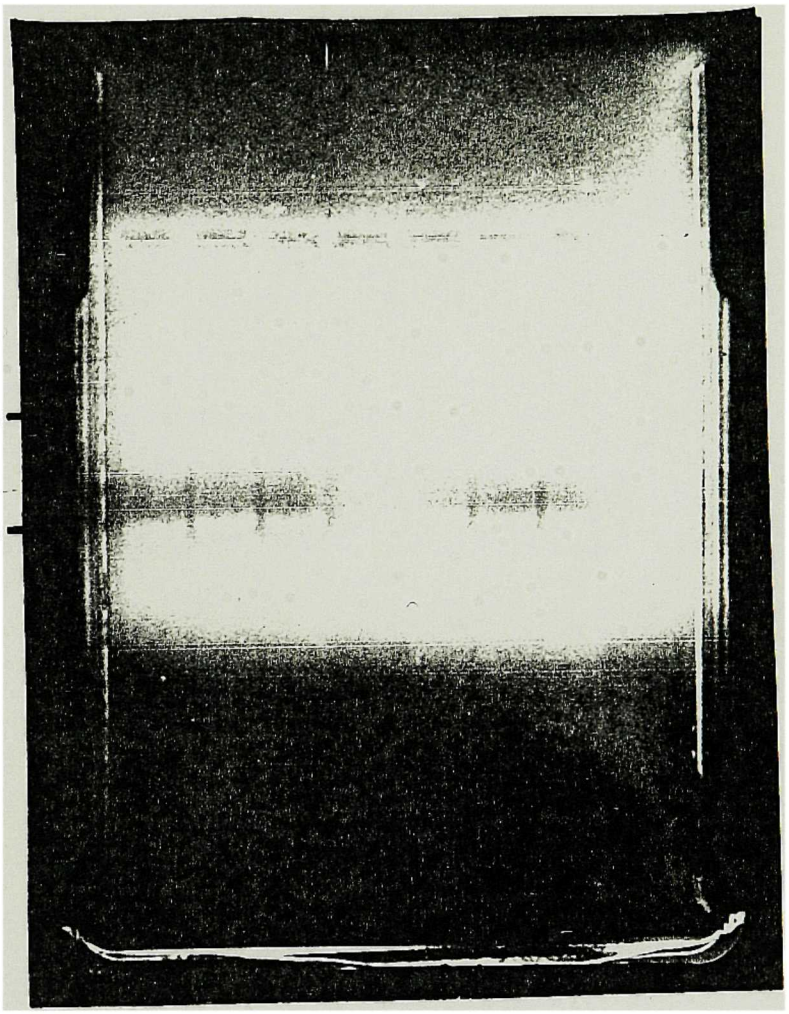
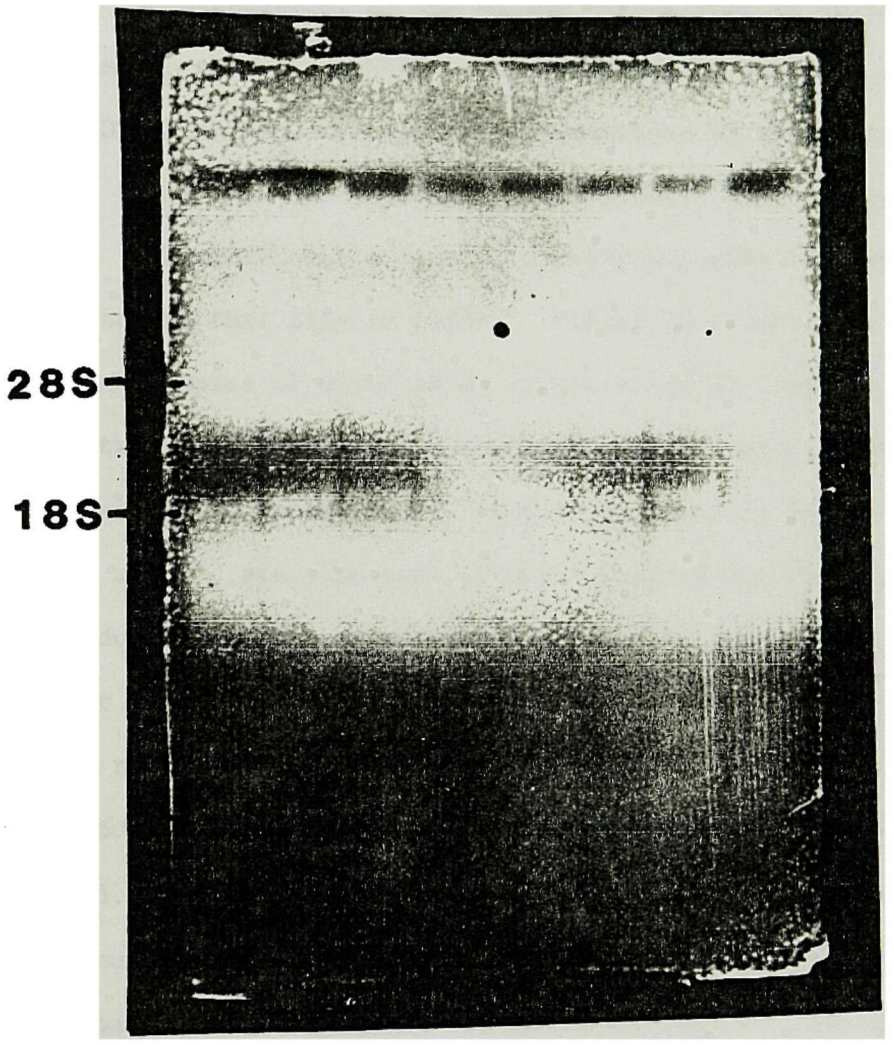

28S
18S

Figure 14. Northern blot showing ethidium bromide stained total RNA. The RNA shown in Figure 13. was transferred to this sheet of nylon for 1.5 h in 20 X SSC with a vacuum blotting apparatus.


28S
18S

Preparation of cDNAs for α, β and 18 S ribosomal RNA

Following growth of a single bacterial colony from each plate of transfected *E-coli* and the separation of plasmid DNA from bacterial genomic DNA, the plasmids were subjected to restriction and the insert DNA (cDNA) separated from the plasmid DNA with agarose electrophoresis. Figure 15 shows the arrangement of the α- and β-cDNA inserts in their respective plasmids, pA1N and prb19G. The plasmid pA1N contains a 3.2 kb rat α1-cDNA linkered with sequences recognized by the restriction enzyme HindIII. This cDNA was inserted at the HindIII site of the vector pSV2neo. The prb19G has a 1.25 kb rat brain β-cDNA linkered with EcoRI and inserted at that site in pBR322. Figure 16 shows a gel containing duplicate samples of uncut pA1N, (lanes A and C), and the result of restricting these two samples with HindIII (lanes B and D). Duplicate unrestricted prb19G preparations, containing the full length β cDNA insert (lanes F and H), are also seen in this figure along with EcoRI restriction products from the same samples (G and I). Lane E. contains molecular weight standards (HindIII cut lambda). The unrestricted 8.9 kb pA1N DNA runs faster than its true molecular weight, appearing here at ~7 kb, due to supercoiling. Once cut by HindIII, the 5.7 kb vector DNA and 3.2 kb insert (the α-cDNA) run true to size in lane B. Lane C shows a number of higher MW contaminants which explains the many unidentifiable bands appearing upon restriction in lane D. The uncut prb19G also supercoils and runs as a ~3 kb sized piece of DNA rather than at the rate for a 4.98 kb strand. Upon restriction, two resulting bands run at the expected rates for 3.7 kb and 1.25 kb (the β-cDNA) molecules.

Figure 15. Diagram of plasmid constructs containing cDNAs for NaK-ATPase α and β subunits.

pA1N consists of the vector pSV2neo with a 3.2 kb α1 cDNA insert between sequences recognized by the restriction enzyme HindIII. prb19G was constructed by deleting 622 bp from pBR322 and ligating a 1.25 kb β cDNA into the EcoR1 restriction site.

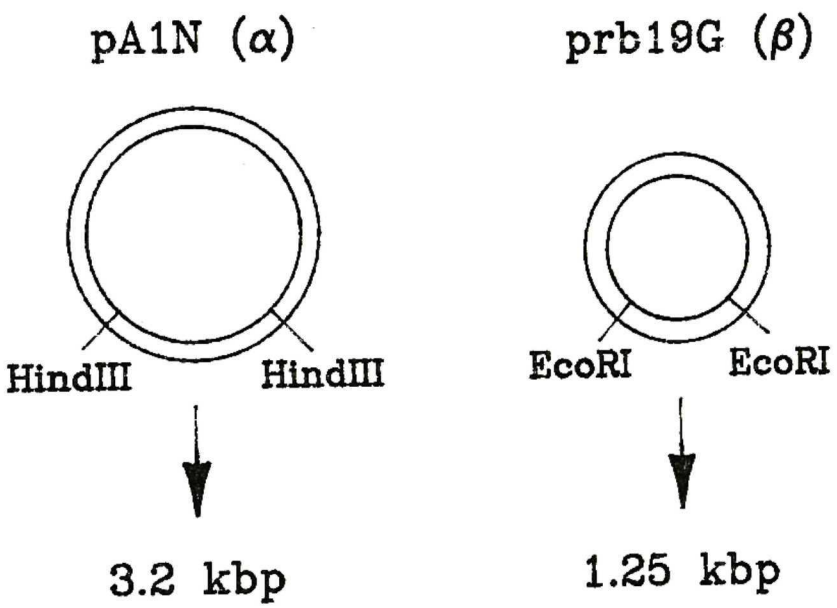
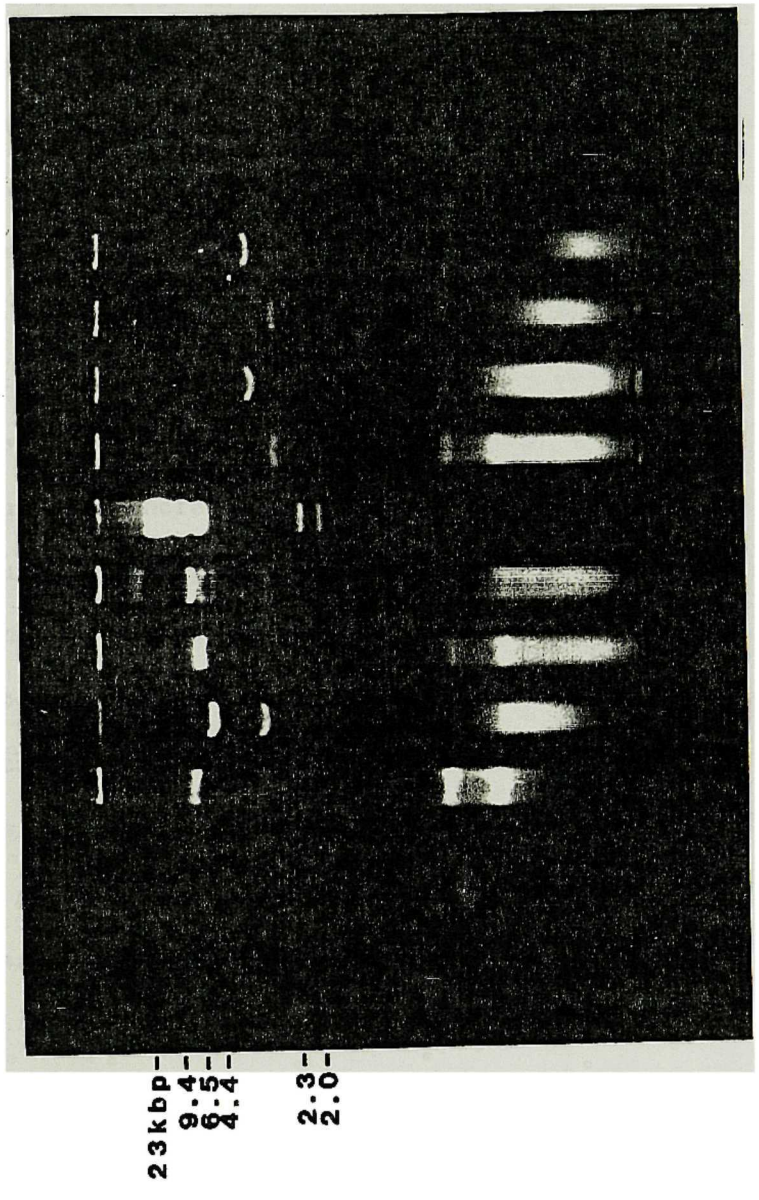

pA1N (α)
HindIII
HindIII
3.2 kbp
prb19G (β)
EcoRI
EcoRI
1.25 kbp

Figure 16. Analytical agarose gel electrophoresis of plasmid DNA. Lanes A and C contain the intact plasmid pAlN DNA. Lanes B and D have the same amount (0.5 μg) of pAlN which has been restricted with HindIII. Lane E contains 1 μg of the molecular weight standards (HindIII restricted lambda phage DNA). Lanes F and H each contain intact prb19G. The same quantity (0.5 μg) of prb19G restricted with EcoRI is in G and I.


23kbp
9.4
6.5
4.4
2.3
2.0
A B C D E F G H I

Northern blots

The purpose of the preliminary hybridization of a Northern blot was to establish that methods used for preparing, labeling and reacting the α-cDNA would result in an autoradiograph with a visible signal that reflected the quantity of RNA in each sample. The autoradiograph in Figure 17 was produced by preparing a sample enriched in polyadenylated (poly A^+) RNA and electrophoresing serial dilutions of this sample in five different wells. The quantity in the wells is: A. 0.5 μg, B. 1 μg, C. 1.5 μg, D. 2.5 μg and E. 5.0 μg. When the Northern blot was hybridized in the presence of 50 ng of α-cDNA (4×10^6 cpm) the autoradiograph produced had a single discrete signal in each well. The signal and background had increasing intensity throughout the range of RNA quantities used. This provided assurance that 5 μg samples of total RNA could be compared in future studies and that there would be sufficient probe to produce signals reflecting the relative α-mRNA quantity in each sample and that a visible signal would likely increase linearly.

As a preliminary comparative study, poly A^+-RNA was isolated from the kidneys of normal and adrenalectomized rats and 0.5 μg of each was electrophoresed, blotted and hybridized. Figure 18 shows a significant decrease in the signal produced upon adrenalectomy. (Lane B) and indicates the need for: 1. studies using total RNA, 2. demonstration that glucocorticoid replacement would restore the signal, 3. investigation into glucocorticoid effects on the level of β-mRNA and 4. an internal standard to demonstrate that the response is not due to errors in total RNA quantitation or handling.

Figure 17. Autoradiograph of increasing quantities of polyadenylated RNA from rat kidney.

RNA was heat denatured and electrophoresed through a denaturing formaldehyde-agarose (1.2%) gel. Amounts (μg) of RNA per lane are as follows: 0.5 (A), 1.0 (B), 1.5 (C), 2.5 (D) and 5.0 (E). The blot was incubated at 65°C with $dCT^{32}P$ labeled α cDNA (1.3×10^6 cpm/ml) and exposed to X-OMAT film for 48 h at -80°C with intensifying screens.

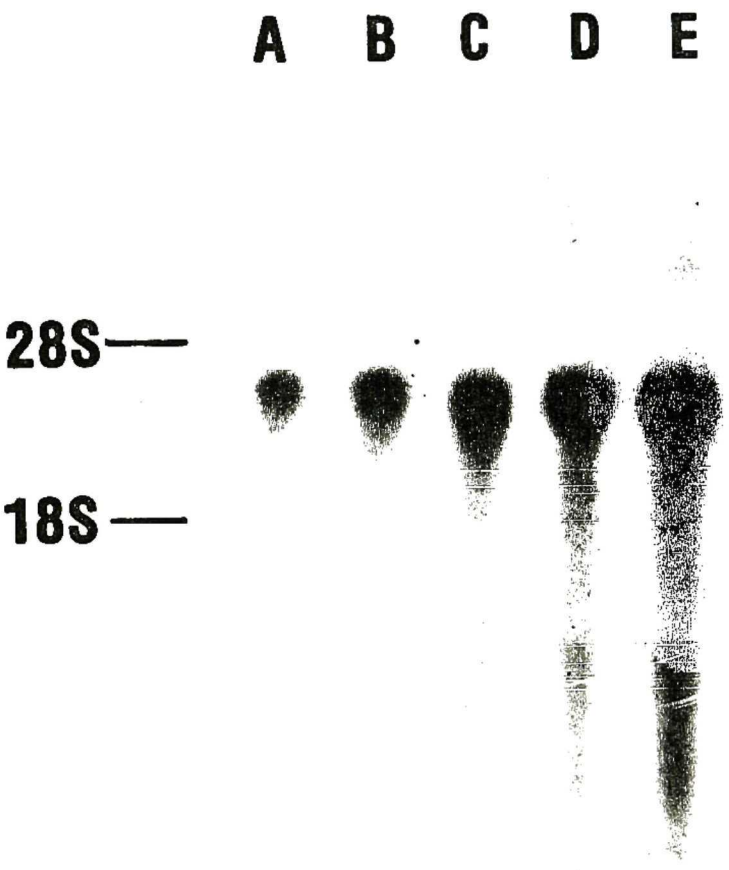
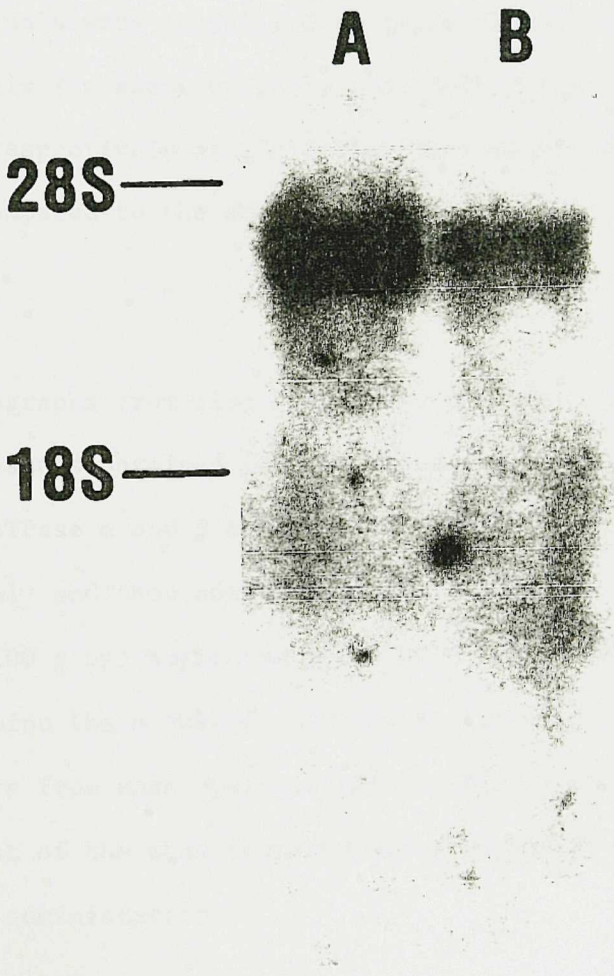

A
B
C
D
E
28S
18S

Figure 18. Autoradiograph with polyadenylated RNA from sham operated and adrenalectomized rats.

Polyadenylated RNA (0.5 μg) from the kidney of an untreated rat (A) and an adrenalectomized rat (B) were electrophoresed in a denaturing formaldehyde-agarose gel and Northern blotted. The blot was incubated at 65°C with $dCT^{32}P$ labeled α cDNA (1.3×10^6 cpm/ml) and exposed to X-OMAT film for 48 h at -80°C with intensifying screens.


A
B
28S
18S

Autoradiographs were produced from Northern blotted total kidney RNA probed with α and β subunit cDNAs and an 18 S ribosomal RNA probe (Figure 19). The blot probed with α cDNA produced an autoradiogram with bands demonstrating a 5.0 kb long mRNA. When that blot was stripped and reprobed with β cDNA the film showed three bands. The most prominent signal appeared at 2.7 kb and another at 2.0 kb. These two β subunit mRNA bands have been described previously (Emanuel et al., 1987). After these signals were normalized to those of the 18 S ribosomal RNA band, the signals representing the α and β mRNA levels were enhanced by 313 and 225%, respectively at 2 h following a single dose of corticosterone, when compared to the adrenalectomized signals.

RNA Slot blots

Autoradiographs from slot blots were prepared (Figure 20) and scanned. The values obtained (Figure 21) demonstrate that adrenalectomy decreases NaK-ATPase α and β subunit mRNA levels by a significant 61 and 64%, respectively and that administration of a single dose of corticosterone (1 mg/100 g bw) augmented the α mRNA level within 60 minutes. At this time point the α subunit mRNA level had been returned to that found in kidneys from sham operated rats. The β subunit mRNA level was restored to that of the sham operated value within 30 minutes of the corticosterone administation.

Figure 19. Autoradiographs with kidney total RNA from adrenalectomized rats given the vehicle or corticosterone.

Total RNA (10 μg) from an adrenalectomized rat (A, C, E) and an adrenalectomized rat given a single dose of corticosterone (1 mg/100 g body weight, for 2 h) (B, D, F) was heat denatured, electrophoresed in a denaturing formaldehyde gel and blotted onto nylon. Following UV cross-linking, the blot was hybridized with random primer labeled α cDNA (A and B) then stripped and reprobed with β cDNA (C and D), followed by the DNA coding for an 18 S ribosomal RNA (E and F).

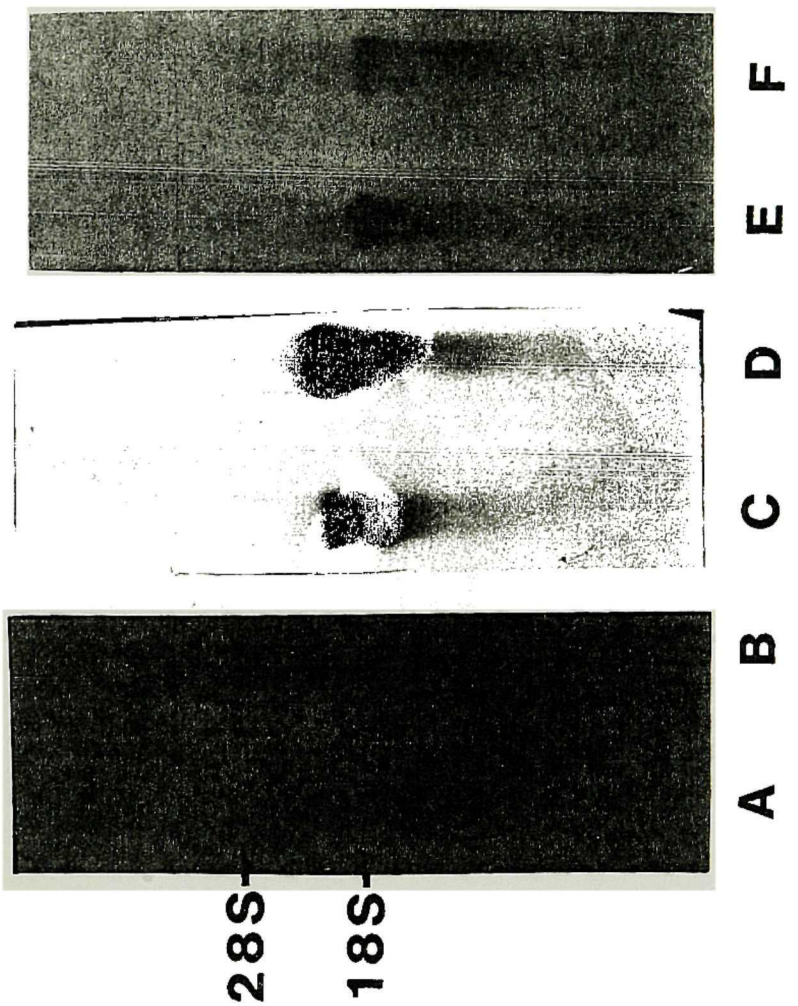

28S
18S
A
B
C
D
E
F

Figure 20. Autoradiographs of NaK-ATPase mRNA in slot blots, probed with random labeled cDNA.

Equal quantities of total RNA from the kidneys of adrenalectomized rats (A), adrenalectomized rats supplemented with corticosterone (B) and sham operated (C) were applied under vacuum to nylon and probed with cDNAs for the NaK-ATPase β subunit. The resulting slots were spectrophotometrically scanned. From top to bottom, the pictured slots contain 1, 0.1 and 0.01 μg total RNA, respectively.

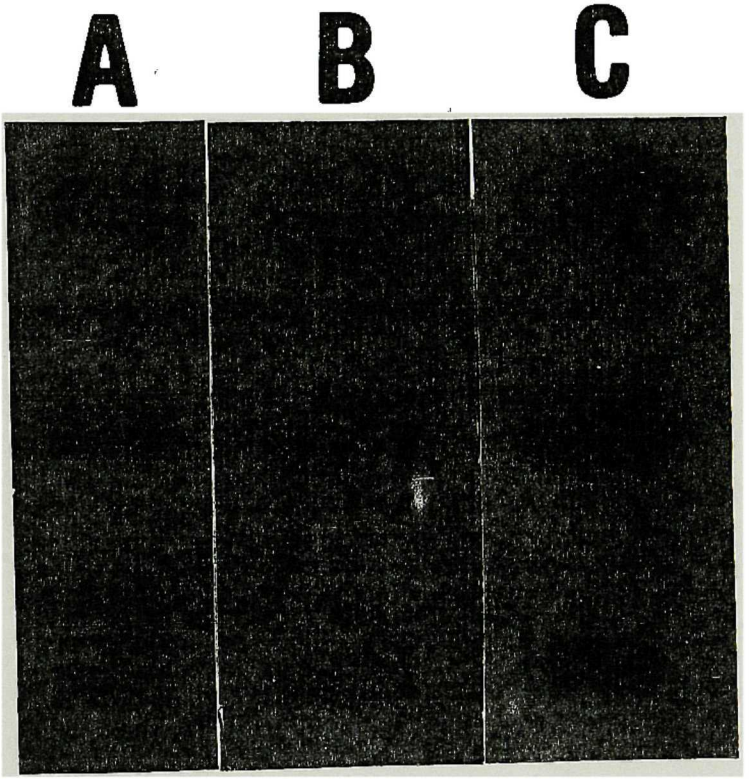
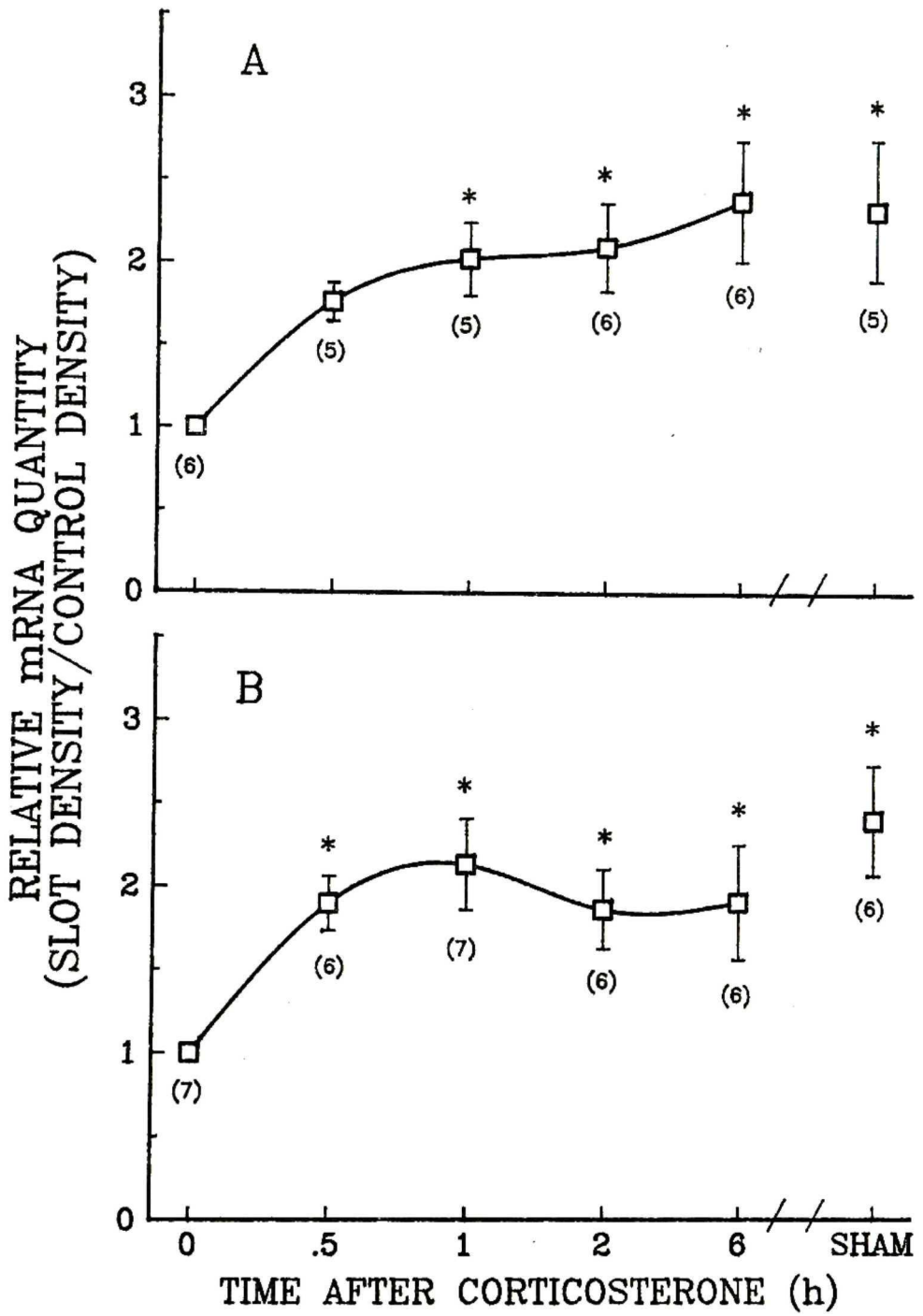

A
B
C

Figure 21. Quantitation of NaK-ATPase subunit mRNA levels with cDNA probed slot blots.

Autoradiograms were prepared from slot blots containing total kidney RNA from adrenalectomized rats. The blots were hybridized with random primer labeled cDNAs. The slots containing 100 ng RNA were scanned with a spectrophotometer and signals from different experiments were normalized to their own control values. The number of observations is in parenthesis. Values are expressed as the mean ± SEM. *P* values are determined with analysis of variance followed by Duncan's multiple range test.

* $P \leq 0.05$ when compared to the control (zero time point) group.


A
B
RELATIVE mRNA QUANTITY
(SLOT DENSITY/CONTROL DENSITY)
0
1
2
3
(6)
(5)
(5)
(6)
(6)
(5)
(7)
(6)
(7)
(6)
(6)
(6)
*
0
.5
1
2
6
SHAM
TIME AFTER CORTICOSTERONE (h)

DISCUSSION

The current work demonstrates that glucocorticoids mediate enhanced levels of the NaK-ATPase subunits and their precursors, in the rat kidney. A body of evidence implicates glucocorticoids in the tissue specific and developmentally specific enhancement of NaK-ATPase activity. This enzyme has been extensively studied in the kidney since the renal medulla has the highest NaK-ATPase specific activity of all mammalian tissues. The NaK-ATPase activity has been shown to rise rapidly in the thick ascending limb of weanling rats (16-20 days), coincident with a surge in serum corticosterone and an increase in urinary concentrating capacity (Rane & Aperia, 1985). Adrenalectomy, prior to this developmental stage, abolished both the enhanced enzyme activity and the renal functional development. Glucocorticoids have been demonstrated to restore both urine concentrating and diluting capacity to patients with Addison's disease (Raisz et al., 1957; Yunis et al., 1964) and to restore NaK-ATPase activity in renal homogenates as well as isolated tubule segments (Rayson & Edelman, 1982). These findings, together with similar NaK-ATPase activity data from other tissues, including the heart and submandibular gland (Klein et al., 1984a; Bartolomei et al., 1983), suggest that glucocorticoids modulate NaK-ATPase activity and that this regulation has critical implications in the study of renal function and whole animal physiology.

The initial experiments in this work were designed to demonstrate regulation of NaK-ATPase activity by glucocorticoids and then to establish whether such changes may be mediated by alterations in Na^+

delivery to NaK-ATPase in the renal tubular epithelial cells. They demonstrate that within 6 h of a single administration of glucocorticoids, NaK-ATPase activity is enhanced in the rat renal cortex and outer medulla (Figure 4) and that there is no effect on sodium delivery to the tubular epithelial cells (glomerular filtration rate, sodium filtration and sodium reabsorption) within the first 24 h (Figure 6). The increase which occurs in renal plasma flow within 12 h of the corticosterone administration may be due to an equivalent dilation of both afferent and efferent arterioles which would not result in an increased GFR. These data support previous findings that glucocorticoids act directly at the renal epithelial cell level and/or indirectly by stimulating the release of other factors which act directly (Fisher et al., 1975; Rodriguez et al., 1981). A direct action by glucocorticoids is supported by investigations with isolated, superfused renal tubules in which the addition of dexamethasone augmented NaK-ATPase activity (Rayson & Edelman, 1982).

Antibodies were used in this project to directly evaluate the effect of glucocorticoids on the number of NaK-ATPase units in kidney microsomes. Previous kinetic studies revealed that dexamethasone administration to adrenalectomized rats produced kidney microsomal NaK-ATPase with an increased *Vmax* value when concentrations of three substrates (Na^+, K^+ and ATP) were varied. These studies also demonstrated that the glucocorticoid had little or no effect on the enzyme's affinity ($K_{½}$) for each of the three substrates (Rodriguez et al., 1980; Sinha et al., 1981). This information, like the phosphorylated intermediate data, also presented by Rodriguez and Sinha et al., is

consistent with the hypothesis that glucocorticoids enhance NaK-ATPase activity through an increase in the number of enzyme units. However, it is also possible that glucocorticoids do not increase the number of enzyme units but act at the plasma membrane surface, which might activate pre-existing NaK-ATPase units. These actions might, for example, involve changes in plasma membrane structure which could alter enzyme conformation or quaternary structure.

There are many similarities between glucocorticoid and thyroid hormone modulation of renal NaK-ATPase activity. The latter is known to increase the synthesis of this enzyme. These factors support a further appraisal of the hypothesis that a glucocorticoid mediated increase in the NaK-ATPase level contributes to the observed rise in enzyme activity. Although thyroid hormones do not appear to participate in the regulation of renal outer medullary NaK-ATPase activity and they require 48 h to produce their peak effect in the kidney, they do act directly at the epithelial cell (Lo & Lo, 1979; Lo & Gerendasy, 1980). When rats, which had been both thyroidectomized and adrenalectomized, were given either or both hormones, the resulting percent increases in renal, as well as myocardial and salivary gland NaK-ATPase activity, suggest that they modulate this enzyme via parallel, independent pathways (Bartolomei et al., 1983; Klein et al., 1984a; Klein et al., 1984b). Thyroid hormones also produce the same type of kinetic and phosphorylated intermediate data as that obtained with glucocorticoids (Lo et al., 1976; Lo & Lo, 1981; Lo et al., 1981). *In vivo* incorporation studies with radiolabeled amino acids and carbohydrates, demonstrated that thyroid hormone enhances renal cortical NaK-ATPase activity through

increased enzyme subunit synthesis without altering the rate of degradation (Lo & Edelman, 1976; Lo & Lo, 1980; Lo et al., 1984). This augmented enzyme synthesis was sufficient to account for the enhanced NaK-ATPase activity seen after supplementation with thyroid hormone. These findings were confirmed when the use of antibodies to quantitate the α and β subunits of NaK-ATPase demonstrated an increased expression in the presence of thyroid hormone (Schmitt and McDonough, 1986). The similarities between modulation of NaK-ATPase activity by both hormones, and the demonstrated increases in NaK-ATPase synthesis following thyroid hormone administration suggest that glucocorticoids stimulate the synthesis of enzyme units.

The antigens used to raise polyclonal antisera (the NaK-ATPase α and β subunits) were initially enriched while in intact enzyme units inserted into plasma membrane vesicles, which permitted them to be monitored with both enzyme assays and SDS-PAGE (Table 1 and Figure 2). Following electroelution of the isolated subunits from the gel, removal of carbohydrate groups from these membrane proteins, especially the highly glycosylated β subunit, might have been useful in decreasing the nonspecific binding to Western blots (see Figure 10).

The aim of the next series of studies was to characterize the polyclonal antisera and assure their selective binding to the NaK-ATPase subunits, prior to their use in quantitative studies. The formation of insoluble antibody-antigen complexes demonstrated that the inoculations were successful in eliciting an immune response against the antigens of interest. The use of Laurell rocket immunoelectrophoresis permitted the monitoring of antiserum titres with the same procedure that provided the

initial characterization of the antisera (Figure 8). Through this monitoring, it was possible to maintain a strong immune response for a year without inducing tolerance. Three different approaches were used to visualize the bound antibodies on Western blots: avidin/biotin/Horseradish Peroxidase complexes (Figure 9), avidin/biotin/fluorescein isothiocyanate complexes (data not shown) and radiolabeled Protein A (Figure 10). All three methods produced signals which led to the same conclusions. The binding of antibodies to Western blotted kidney microsomal proteins having the molecular weights of the NaK-ATPase subunits, suggested that these antibodies were directed against the NaK-ATPase subunits. The appearance of single bands on these blots demonstrated the specificity of the antibody preparations and implied that quantitation of the NaK-ATPase subunits, with these probes, would not be compromised by signals from other proteins.

Functional evidence of antibody binding to the NaK-ATPase subunits was provided by enzyme inhibition studies. The anti-α antibodies, isolated in this work, inhibited NaK-ATPase activity in a dose dependent manner (Figure 11). Similar dilutions of the anti-β and preimmune antibody preparations did not cause this inhibition. High concentrations of the anti-β antibody actually enhanced NaK-ATPase activity. These findings make it unlikely that the ionic composition of the serum inhibits NaK-ATPase activity and support the argument that antibodies in the putative anti-α serum are binding to the NaK-ATPase α subunit. It provides preliminary evidence that a small population of the anti-β antibodies may bind to the enzyme so as to enhance the activity. The use of a highly glycosylated preparation of the β subunit may have

resulted in a major population of antibodies, in this serum, being directed against the carbohydrates rather than the protein regions. This may have resulted in fewer conformational changes than would have otherwise occurred and the weak effect on NaK-ATPase activity caused by this antiserum. Many other laboratories have produced antibodies against both holoenzyme NaK-ATPase preparations and against the isolated subunits (Jorgensen et al., 1973; Koepsell, 1978; Kyte, J. 1974; Ebel et al., 1977; Schenk & Leffert, 1983). These antisera varied widely in their abilities to alter enzyme function. The antibodies in different preparations may bind with varying affinities for active sites or display differing abilities to interrupt substrate binding by producing conformational changes and/or stearic hinderance.

The results of the antibody studies support the hypothesis that glucocorticoid actions enhance NaK-ATPase activity by increasing the number of enzyme units. Slot blot signal intensities were diminished for both subunits following adrenalectomy and augmented following corticosterone administration (Figure 12). In these studies there was no attempt to determine the relative contributions of synthesis and degradation to the production of increased subunit levels. However, glucocorticoids and thyroid hormones are known to act via the same general processes at the cellular level, and have comparable effects on NaK-ATPase activity. A reasonable hypothesis is that the enhanced NaK-ATPase synthesis and the lack of effect on degradative rates seen with thyroid hormones is the same general means by which glucocorticoids enhance NaK-ATPase activity. Evidence for increased NaK-ATPase subunit mRNA levels would lend support to this hypothesis.

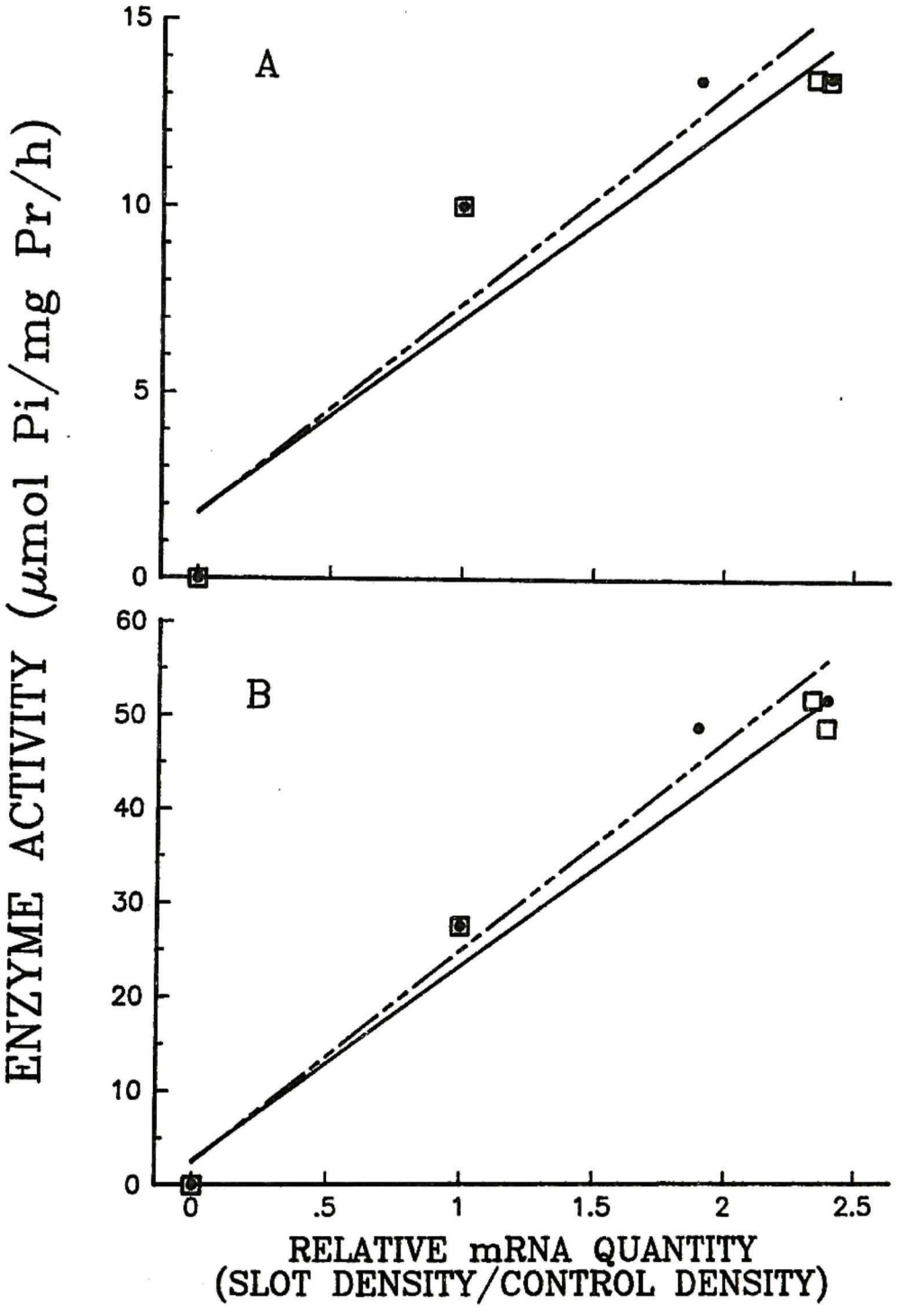
Glucocorticoids are known to enhance gene expression through interaction of DNA binding proteins (activated hormone receptors) with specific gene regulatory regions (Green & Chambon, 1986; Evans, 1988). They have also proven capable of modulating target mRNA stability (Brock & Shapiro, 1983; Paek & Axel, 1987). By one or both of these means, glucocorticoids were seen to enhance NaK-ATPase subunit mRNA levels. Hybridization of the RNA on Northern blots demonstrated that the RNA observed had not been degraded during its preparation and that the cDNAs bound to mRNAs of the appropriate size to code for NaK-ATPase α and β subunits (5.0 and 2.7 kb, respectively) (Figure 19). The Northern blots also provided initial evidence of a glucocorticoid mediated increase in NaK-ATPase subunit mRNA levels. Quantitation by spectrophotometric scanning of signal was more accurately produced with slot blots which permitted the total RNA to be directly applied to a small area. This eliminated the variation that can result from the transfer of RNA from different regions of the gel. Serial dilutions of the RNA were applied to the nylon, permitting identification of the range in which the signal is a linear function of the quantity of RNA. Hybridization of slot blots with the cDNAs demonstrated that NaK-ATPase α and β mRNA levels decrease 61 and 64%, respectively, following adrenalectomy (Figure 21). The α mRNA level rose to the sham level within 60 minutes of a single dose of corticosterone and the β level did so within 30 minutes. When NaK-ATPase activity (Figures 3 and 4) was plotted against relative α and β mRNA levels (Figure 21), a linear relationship [$r = 0.89$, 0.88 for α and β cortex (A), 0.98, 0.97 for α and β medulla (B), respectively] was obtained for the adrenalectomized state, corticosterone treated

adrenalectomized state and the sham operated animals (Figure 22). The results support the hypothesis that the corticosterone dependent increase in NaK-ATPase activity is a result of corresponding increases in the subunit mRNA levels.

By regulating NaK-ATPase activity in renal tubular epithelial cells, glucocorticoids play a critical role in the maintenance of water homeostasis. Future studies should examine finer details of the modulation of NaK-ATPase expression by glucocorticoids. The transcription run-off assay (McKnight & Palmiter, 1979) can be used to quantitate the rate of transcription initiation. It would reveal whether glucocorticoids enhance transcription sufficiently to account for the observed increases in α and β subunit mRNAs or whether mRNA stabilization is involved. Modulation of the rate of NaK-ATPase mRNA degradation by glucocorticoids could be directly observed by inhibiting transcription in cultured cells with actinomycin-D and assessing mRNA levels at various times during the inhibition. The structures of the NaK-ATPase α and β gene upstream regulatory elements could also be studied and interesting interactions between glucocorticoid receptor binding and the binding of other regulatory proteins might be revealed. A large variety of DNA binding proteins, including receptors for hormones, growth factors and viral oncogene products modulate gene expression, often cooperatively, through interactions with such regulatory regions (Evans, 1988). By improving the understanding of how renal NaK-ATPase is modulated by glucocorticoids, these studies should

Figure 22. Correlation between NaK-ATPase activity and subunit mRNA levels.

Slot blot data for NaK-ATPase α (open squares) and β (closed circles) mRNA levels were normalized to control (adrenalectomy + diluent) and plotted against NaK-ATPase activity in the cortex (A) and in the outer medulla (B). The values are means obtained from adrenalectomized rats (Figures 3,4 and 21), those treated with corticosterone for six hours (Figures 3,4 and 21) and sham operated rats (Figures 3 and 21).


A
B
15
10
5
0
60
50
40
30
20
10
0
.5
1
1.5
2
2.5
ENZYME ACTIVITY (μmol Pi/mg Pr/h)
RELATIVE mRNA QUANTITY
(SLOT DENSITY/CONTROL DENSITY)

help clarify the processes which maintain not only water and electrolyte balance, but an array of other physiological processes.

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Laura Elizabeth Klein

1957	Born May 31, 1957 in Queens, New York
1971	Graduated Langley High School, Langley Virginia
1971-1975	Attended Mary Washington College, Fredericksburg, Virginia
1975	Completed B.S. in Chemistry, Mary Washington College, Fredericksburg, Virginia
1981	Lo, C.S., Cheng, W. and L.E. Klein Effect of triiodothyronine on NaK-ATPase and Na^+ + Mg^{++}-dependent phosphorylated intermediate in rat salivary glands. Pflugers Archiv. 392: 134-138, 1981.
1983	Bartolomei, M.S., Klein, L.E., Hsiao, P. and C.S. Lo Effect of corticosterone and triiodothyronine on NaK-ATPase activity in rat submandibular gland. Pflugers Archiv. 399: 255-258, 1983.
1984	Klein, L.E., Hsiao, P. Bartolomei, M. and C.S. Lo Regulation of rat renal NaK-ATPase activity by triiodothyronine and corticosterone. Endocrin. 115(3): 1038-1042, 1984.
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	Klein, L.E., Bartolomei, M.S. and C.S. Lo Corticosterone and triiodothyronine control of myocardial NaK-ATPase activity in rats. Am. J. Physiol. 247: H570-H575, 1984.
1985-1990	Attended graduate program at Uniformed Services University of the Health Sciences
1985	Klein, L.E. and C.S. Lo Thyroidal regulation of rat thymic and splenic NaK-ATPase. Thymus 7: 367-376, 1985.
1988	Bryant, H.J., Eng, S.P. Klein, L.E. and C.S. Lo Effects of triiodothyronine on resting membrane potential of primary cultured rat submandibular gland cells. Cell Biol. International Reports 12(12): 1027-1036, 1988.
1990	Completed Ph.D. in Physiology at Uniformed Services University of the Health Sciences, Bethesda, Maryland.