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TISSUE-SPECIFIC REGULATION OF THE SODIUM POTASSIUM ADENOSINE TRIPHOSPHATASE

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements of the degree of
Doctor of Philosophy.

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TABLE OF CONTENTS

ABSTRACT

The Na,K-ATPase, or sodium pump, is a membrane-associated protein complex comprising two subunits, α and β, both of which can exist as one of several isoforms. It generates and maintains the electrochemical Na^+ and K^+ gradients across the plasma membrane of animal cells. These gradients are the driving force for a variety of ubiquitous and specialized cell functions, such as transport of solutes, as well as maintenance of membrane potential and cell volume. Agents that modulate the kinetic behaviour of the pump enable cells to adapt to changing needs. Distinct substrate activation profiles in various tissues presumably underlie the specialized functions of the sodium pump in these tissues. Although the tissue-specific distribution of various isoforms accounts for some of the differences in kinetic behaviour of the enzyme, other factors are also important determinants of such behaviour. This study describes the characterization of two mechanisms of sodium pump modulation. The first involves alterations in the susceptibility of the enzyme to competitive inhibition by K^+ at Na^+ binding sites. Studies on the $\alpha 1$ and $\alpha 3$ isoform of various tissues and cells have revealed that there exist tissue-specific components that determine the extent to which the two cations compete with each other for cytoplasmic binding sites. Specifically, pumps that are highly susceptible to K^+/Na^+ antagonism also bind and occlude K^+ much more readily on the cytoplasmic site, and this behaviour is abrogated upon fusion of pumps into another membrane environment, that of the red blood cell. The second mechanism of regulation of pump behaviour described in this thesis involves modulation of the apparent affinity of the enzyme for ATP by the γ subunit. This membrane protein had been previously cloned and sequenced, but very little was known about its function. This study shows that the γ subunit is expressed uniquely in kidney tubules, and has a C-terminus-in, N-terminus-out topology. Studies using an antiserum specific for the C-terminus of γ, as well as mammalian cells transfected with the peptide, have revealed that the γ subunit stabilizes the E_1 conformation of the Na,K-ATPase and increases the affinity of the enzyme for ATP. In conclusion, this study describes two novel mechanisms of sodium pump regulation. Both can be reversed and are of potential physiological importance.

RÉSUMÉ

La Na,K-ATPase, ou pompe sodium, est un ensemble protéique associé à la membrane et composé de deux sous-unités, α et β, dont plusieurs isoformes existent. Elle établit et maintient les gradients éléctrochimiques de Na^+ et K^+ à travers la membrane plasmique des cellules animales. Ces gradients fournissent l'énergie nécessaire à plusieurs fonctions cellulaires, qu'elles soient ubiquitaire ou spécialisées, telles que le transport de solutés et le maintien du potentiel transmembranaire et du volume cellulaire. Les agents qui modulent le comportement cinétique de la pompe permettent donc l'adaptation à différents besoins cellulaires. Les fonctions spécialisées de la pompe sodium dans certains tissus dépendent probablement de différentes affinités des pompes de ces tissus pour leurs substrats. Bien que, dans certains cas, la présence dans ces tissus d'isoformes distinctes peut expliquer ces différences, d'autres facteurs peuvent également influencer le comportement cinétique de l'enzyme. Cette étude décrit deux mécanismes de modulation de la pompe sodium. Le premier comprend des altérations de la sensibilité de l'enzyme à une inhibition compétitive par le K^+ aux sites de liaison du Na^+. Des études sur les isoformes α1 et α3 de plusieurs tissus et cellules ont révélé qu'il existe des mécanismes spécifiques aux tissus qui déterminent le degré de compétition entre les deux cations aux sites de liaison cytoplasmiques. Plus spécifiquement, les pompes qui sont fortement sensibles à cet antagonisme Na^+/K^+ réussissent également mieux à lier et occlure le K^+ du côté cytoplasmique, un comportement qui est abrogé suite à la fusion des pompes dans un environement membranaire différent, celui du globule rouge. Le second mécanisme de régulation du comportement de la pompe décrit dans cette thèse comprend la modulation de l'affinité de l'enzyme pour l'ATP par la sous-unité γ. Cette protéine membranaire avait auparavant été clonée et séquencée, mais sa fonction était restée inconnue. Cette étude démontre que la sous-unité γ est exprimée uniquement dans les tubules rénaux et que sa topologie est de type I (partie N-terminale du côté extracellulaire et partie C-terminale du côté cytoplasmique). De plus, des études fonctionnelles utilisant des anticorps reconnaissant la partie C-terminale de cette protéine, ainsi que des cellules rénales transfectées avec la sous-unité γ, ont révélé que ce peptide stabilise la

conformation E_1 de la Na,K-ATPase en augmentant l'affinité de l'enzyme pour l'ATP. En conclusion, cette étude décrit deux nouveaux mécanismes de régulation de la pompe sodium qui sont réversibles et probablement importants au niveau physiologique.

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I reserve my final thanks for my family. A mes parents, qui ont toujours cru en moi, même quand je n'y croyais plus moi-même, and to Dawn, whose love and support have made everything all the more worthwhile... Merci.

ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

1. Cation activation kinetics of the sodium pump are determined by factors other than the primary structure of the enzyme. Characterization of the Na^+- and K^+-activation profiles of identical isoforms of various rat tissues has shown that there exist tissue-specific, isoform-independent differences in kinetic behaviour of the sodium pump. In particular, distinct affinities for Na^+ are due, at least in part, to distinct susceptibilities to competitive inhibition of Na^+ binding by K^+ at cytoplasmic sites (K^+/Na^+ antagonism). These results reconcile many of the discrepancies present in the literature regarding isoform-specific behaviour of the Na,K-ATPase, as well as differences between studies on intact cells *versus* permeabilized membranes.
2. Tissue-specific differences in K^+/Na^+ antagonism correlate with K^+-binding kinetics and are secondary to the membrane environment of the enzyme. Comparison of the affinities of sodium pumps of several rat tissues for K^+ as an inhibitor has shown a correlation between this parameter and calculated rates of K^+ binding and occlusion. Furthermore, fusion of rat kidney pumps into dog red blood cells abrogates the susceptibility of this enzyme to K^+/Na^+ antagonism, demonstrating reversibility and the role of the membrane environment.
3. The γ subunit is a kidney–specific modulator of the conformational equilibria of the Na,K-ATPase. Studies using an antiserum determined to be directed towards the cytoplasmic domain of the γ subunit revealed that this peptide is present only in kidney and stabilizes the E_1 conformation of the enzyme. In addition, the full length sequence of γ was obtained and its topology was determined.
4. The γ subunit reversibly increases the apparent affinity of the Na,K-ATPase for ATP. Transfection of the rat γ subunit into HEK cells increases the affinity of the sodium pump for ATP. Pre-incubation with antiserum specific for the C-terminal 10 amino acids reverses the increase in affinity in transfected cells, and decreases the affinity of the renal enzyme for ATP; presence during pre-incubation of the peptide against which the serum was raised abrogates the latter effect. In addition, expression of a doublet in transfected cells provides evidence for post-translational modification of γ.

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LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 7

LIST OF TABLES

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphoshate
AVP	Arginine vasopressin
BSA	Bovine serum albumin
Ca-ATPAse	Calcium-activated adenosine triphosphatase
cAMP	Cyclic adenosine 3',5'-monophosphate
CCD	Cortical collecting duct
cDNA	Complementary DNA
cGMP	Cyclic guanosine 3',5'-monophosphate
CHAPS	3-(3-cholamidopropyl) dimethylammonio-1-propane sulfonate
CHIF	Channel inducing factor
cRNA	Coding RNA
C-terminus/terminal	Carboxyl-terminus/terminal
DA_1	First type of dopamine receptor
DA_2	Second type of dopamine receptor
DAG	Diacylglycerol
DARPP-32	Dopamine and cAMP-regulated phosphoprotein of 32 kDa
db-cAMP	Dibutyryl-cAMP
DCCD	N,N'-dicyclohexylcarbodiimide
DEAC	4-(diazomethyl)-7-(diethylamino)-coumarin
D-MEM	Dulbecco's modified Eagle's medium
DOG	sn-1,2-dioctanoylglycerol
DPB	12-deoxyphorbol 13-isobutyrate
E^*P	Na,K-ATPase in an intermediate conformation
E_1	Na,K-ATPase in the first conformation
E_1P	Phosphorylated Na,K-ATPase in the first conformation
$E_1P(Na)$	Na^+-occluded form of the Na,K-ATPase
E_2	Na,K-ATPase in the second conformation
$E_2(K)$	K^+-occluded form of the Na,K-ATPase
E_2P	Phosphorylated Na,K-ATPase in the second conformation
ECG	Endogenous cardiac glycosides
EDTA	Ethylenediaminetetraacetic acid
EET	Epoxyeicosatrienoic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N'-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EP	Phosphorylated Na,K-ATPase
H,K-ATPase	Proton and potassium-activated adenosine triphosphatase
H7	1-(5-isoquinolinylsulfonyl)-2-methylpiperazine
HETE	Hydroxyeicosatetraenoic acid
HK	High-K^+ phenotype
HPLP	Human phospholemman-like protein
I1	Inhibitor 1

IBMX	3-isobutyl-1-methylxanthine
IC_{50}	Concentration of inhibitor causing 50% inhibition
$K_{0.5}$	Concentration of substrate causing half-maximal activation
kb	One thousand base pairs
kDa	kiloDaltons
KLH	Keyhole limpet hemocyanin
LK	Low-K^+ phenotype
Mat-8	Mammary tumor-associated 8 kDa protein
MES	4-morpholineethanesulfonic acid
mRNA	Messenger RNA
mTAL	Medullary thick ascending loop of Henle
Na,K-ATPase	Sodium and potassium-activated adenosine triphosphatase
NO	Nitric oxyde
N-terminus/terminal	Amino-terminus/terminal
OAG	L-1-oleoyl-2-acetyl-sn-3-glycerol
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCT	Proximal convoluted tubules
PDA	Phorbol 12,13-diacetate
PDB	Phorbol 12,13-dibutyrate
PG	Prostaglandin
PGE	Prostaglandin E
P_i	Inorganic orthophosphate (PO_4)
PKA	cAMP-activated protein kinase
PKC	Calcium-activated protein kinase
PKG	cGMP-dependent protein kinase
PLA_2	Phospholipase A_2
PLM	Phospholemman
PMA	Phorbol 12-myristate 13-acetate
PP1	Protein phosphatase 1
PP2B	Protein phosphatase 2B
PTH	Parathyroid hormone
PVDF	Polyvinylidene difluoride
RACE	Rapid amplification of cDNA ends
RIC	Regulated ion channel homolog
S.D.	Standard deviation
S.E.M.	Standard error of the mean
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SERCA	Sarcoplasmic/endoplasmic reticulum Ca-ATPase
SHE	Sucrose-histidine-EDTA
T3	Triiodothyronine
TM	Transmembrane domain
TPA	12-O-tetradecanoylphorbol-13-acetate
TX	Thromboxane

CHAPTER 1

INTRODUCTION

1.1 PREFACE

In 1997, The Nobel Prize in Chemistry was awarded to Danish researcher Jens C. Skou for the discovery of the sodium potassium adenosine triphosphatase (Na,K-ATPase; see ref. 462). Although the existence of an active "sodium pump" had been suspected for years, Skou was the first to suggest a link between transport of sodium ions (Na^+) and potassium ions (K^+) across the plasma membrane and a Na^+- and K^+-activated ATPase activity (463). The significance of this discovery is underscored by the publication, each year, of thousands of reports relevant to various aspects of Na,K-ATPase structure and function. Indeed, the enzyme generates and maintains the electrogenic Na^+ and K^+ gradients on which most transport systems depend, and is the main utilizer of cellular adenosine triphosphate (ATP; see, for example, ref. 252). Although much has been discovered about the pump in the years since its discovery, still very little is known about several aspects of the enzyme, especially with regards to its regulation, structure-function relationships, and the role of the various isoforms.

The focus of this thesis is on regulation of the Na,K-ATPase. As such, the Introduction provides a brief overview of the structure, function and mechanism and focuses mainly on pump regulation, with emphasis on aspects not extensively covered in recent review articles, and those particularly relevant to the work presented in this thesis.

This thesis is manuscript-based, in accordance with Section C of the Faculty of Graduate Studies and Research Guidelines for Thesis Preparation. Chapters 2, 4 and 5 are published manuscripts, while chapter 3 is in press. As such, Chapters 2 to 5 contain their own reference lists, and Chapter 8 contains references for the Introduction, General Discussion and Appendices. In addition, in compliance with section C, paragraphs 2 and 5, Chapters 2 to 5 contain a preface which includes "connecting texts that provide logical bridges between the different papers", as well as an "explicit statement ... as to who contributed to (the) work and to what extent".

1.2 THE NA,K-ATPASE

1.2.1 ATP-driven ion pumps

The Na,K-ATPase, also known as the sodium pump, is one of hundreds of enzymes that utilize the energy generated from ATP hydrolysis to transport ions across cell membranes. Such pumps are grouped into one of three families, called V-type, F-type and P-type ATPases (for review, see ref. 60).

V-type pumps are commonly found in the various intracellular compartments and vesicles present in all cells, and serve to drive the transport of protons against their concentration gradient (reviewed recently in ref. 169). Such pumps serve to acidify the lumen of intracellular organelles or, in the case of some prokaryotes, the extracellular environment. F-type ATPases, on the other hand, are found in the plasma membranes of bacteria and, in eukaryotes, in the inner membrane of mitochondria and chloroplasts (for recent reviews, see refs. 146, 255). Although called ATPases, they generally function physiologically as ATP synthases in that they utilize proton (or Na^+) gradients to synthesize ATP, rather than break down ATP to create a proton (or Na^+) gradient.

The P-type family of ATPases comprises over fifty members, including the Na,K-ATPase (for reviews, see refs. 313, 318, 342). The name is derived from the fact that these pumps are transiently phosphorylated, or form a phosphoenzyme intermediate, as part of their normal enzyme cycle. Much of what is known about the functional properties of P-type ATPases was discovered by studying the Na,K-ATPase, and the details will be discussed below (Section 1.2.3). However, a brief overview of the structure of P-type ATPases follows. Overall, the primary sequence of these proteins predicts several transmembrane domains which presumably contain the cation binding sites, as well as a major and a minor cytoplasmic loop (313, 318). Specific structural motifs include (i) the putative ATP binding site (TGDN), the sequence containing the aspartate residue that becomes transiently phosphorylated during the catalytic cycle (DKTGS/T) and the "hinge" region that connects the large cytoplasmic loop with the transmembrane segments (GDGXNDXP), all found in the major cytoplasmic loop, and (ii) a flexible

TGES/A motif found in the minor cytoplasmic loop. Proteins which contain these sequences are classified into 2 sub-families, P_1 and P_2 (342, 383). P_1 pumps are believed to be heavy metal transporting ATPases such as the bacterial cadmium and copper transporters, and the mammalian copper transporters, all of which contain long cytoplasmic N-terminal tails that have 1 to 6 cysteine-rich metal-binding motifs. P_2 ATPases include the Na,K-ATPase and H,K-ATPase (subgroup a), and the Ca- , Mg- and H- ATPases (subgroup b).

Contrary to V-type and F-type ATPases which are aggregates of several separate polypeptides, P-type enzymes are generally composed of a single protein, although pumps of type 2a have two essential components, called the α and β subunits (318). All catalytic functions are generally ascribed to the α subunit, while the β subunit is necessary for proper targeting and/or protein stability of the pump complex (107).

The P type ATPases whose structures are most similar to that of the Na,K-ATPase (>60% sequence homology) are the H,K-ATPases of the stomach and other organs (reviewed in ref. 495). In addition to similarities in primary structure, these pumps are also the only known P-type ATPases that contain a β subunit (107). The gastric pump functions in much the same way as the sodium pump whose functional characteristics are described later in this chapter. The role of this H,K-ATPase is to maintain the low pH found in the lumen of the stomach which it does by pumping protons in exchange for potassium ions. Non-gastric H,K-ATPases have been found on the apical membranes of kidney, small intestine and colon, where they probably function to secrete H^+ into the lumen of their respective organs (119, 203, 238).

The Ca-ATPases of the plasma membrane and sarcoplasmic/endoplasmic reticulum (SERCA) both function to keep the cytoplasmic Ca^{++} concentration low (for reviews, see refs. 339, 343). Ca^{++} has a major role in intracellular signalling, thus the importance of maintaining Ca^{++} concentrations at sub-micromolar levels. The calcium pumps are therefore required to maintain very large gradients of Ca^{++} across the membrane (up to 10^5). In muscle, these pumps serve to clear the cytoplasm of Ca^{++} after

muscle contractions; the efficacy of the pump in this regard determines in part the intensity of the next contraction.

In addition to those mentioned above, other P-type ATPases include mammalian copper pumps, mutations in which are believed to be the cause of Wilson and Menkes diseases in humans; copper, cadmium and magnesium ATPases of bacteria, which are involved in uptake or extrusion of these heavy metals; and proton pumps of plants, fungi and protozoans, which generate H^+ gradients essential for a variety of secondary transport mechanisms (342).

1.2.2 Structure of the Na,K-ATPase

1.2.2.a The α subunit

The minimal Na,K-ATPase model comprises a membrane-associated complex of two peptides, subunits α and β, which are present at a 1:1 ratio (see, for example, 252). The largest of the two is the catalytic α subunit, which migrates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis at around 112 kDa. The rat cDNA sequence predicts a protein of 1023 amino acids (α1 isoform; c.f. section 1.2.4 below), but in the mature protein, the first 5 amino acids are cleaved (316). The sequence also predicts several transmembrane domains, ten being the most plausible (for example, see refs. 263, 268, 457), with both amino- (N-) and carboxy- (C-) termini on the cytoplasmic side (498). The α subunit is not considered to be a glycoprotein, although there has been a report of intracellular glycosylation (389). The α subunit was first cloned in the sheep (457), and the gene was subsequently cloned in several other species (reviewed in ref. 498). The intra-species homology of similar α subunit isoforms (c.f. section 1.2.4) is quite high, at approximately 90% (52).

1.2.2.b The β subunit

The β subunit is a glycoprotein with a protein core of approximately 35 kDa that migrates at 45-55 kDa by SDS-PAGE analysis (for an extensive review on the β subunit, see ref. 107). Its topology is that of a type II membrane protein, with a large extracellular

C-terminal domain and a shorter cytoplasmic N-terminus. The sequences of the various β subunits are much less conserved than those of the α subunit, ranging from 15% to 65% among different species and isoforms (52). The protein is glycosylated at three to eight different sites, depending on the species and the isoform (52). The role of the carbohydrate moiety is unclear, since pumps whose β subunits lack oligosaccharides are still properly processed, and can still hydrolyze ATP and bind ouabain (28, 533). In addition to glycosylation, the β subunit contains six conserved cysteines that form three disulfide bonds in the mature protein (277). The main role of the β subunit is thought to be in stabilization and maturation of the Na,K-ATPase protein complex, as evidenced in part by the observation that cleavage of disulfide bonds in the β subunit is sufficient for total abrogation of all enzymatic activity associated with the sodium pump (269). A functional role of the β subunit has been proposed, however, and is described in more detail in section 1.3.4.

1.2.2.c Tertiary and Quaternary structure of the Na,K-ATPase

The minimal structural model of the Na,K-ATPase is shown in Fig. 1-1. Association between the α and β subunits occurs in the endoplasmic reticulum, and is necessary for expression of a functional enzyme (3). Using a variety of molecular techniques, Fambrough and his colleagues (112, 212) have determined that (i) the site of interaction on the β subunit appears to be in the 96 residue domain on the immediate extracellular side of the transmembrane domain, while (ii) the residues important for α/β interaction on the α subunit are in the loop between transmembrane domains 7 and 8 (TM7 and TM8). Recently, Shimon *et al.* (454) have used Cu^{++}-catalyzed cleavage to confirm interactions between the TM7/TM8 loop of the α subunit and extracellular sites in the β subunit.

The difficulty in obtaining high resolution data from crystals of the sodium pump has precluded detailed knowledge about the three-dimensional structure of the enzyme. Nevertheless, analysis of the purified membrane-embedded protein has provided insight into the nature of the interactions of the various transmembrane domains of the α and β

subunits in the membrane (374, 375). Such structural data are consistent with the recent low-resolution (8 Å) crystal structures obtained for the SERCA pump (537).

There exists considerable controversy regarding the oligomeric structure of the Na,K-ATPase (for example, see ref. 416). It has been demonstrated that the αβ heterodimer is capable of catalyzing the complete reaction cycle (505), and that this is the naturally occuring form, at least in the red blood cell (324, 427). However, there exists a body of evidence supporting the idea that, in other systems, the enzyme functions as a $(\alpha\beta)_2$ heterodimer, or indeed, possibly as a $(\alpha\beta)_4$ tetramer (see, for example, refs. 49, 250, 305, 465, 491). Accordingly, it is possible that the nature of αβ dimer oligomerization is a tissue-specific phenomenon.

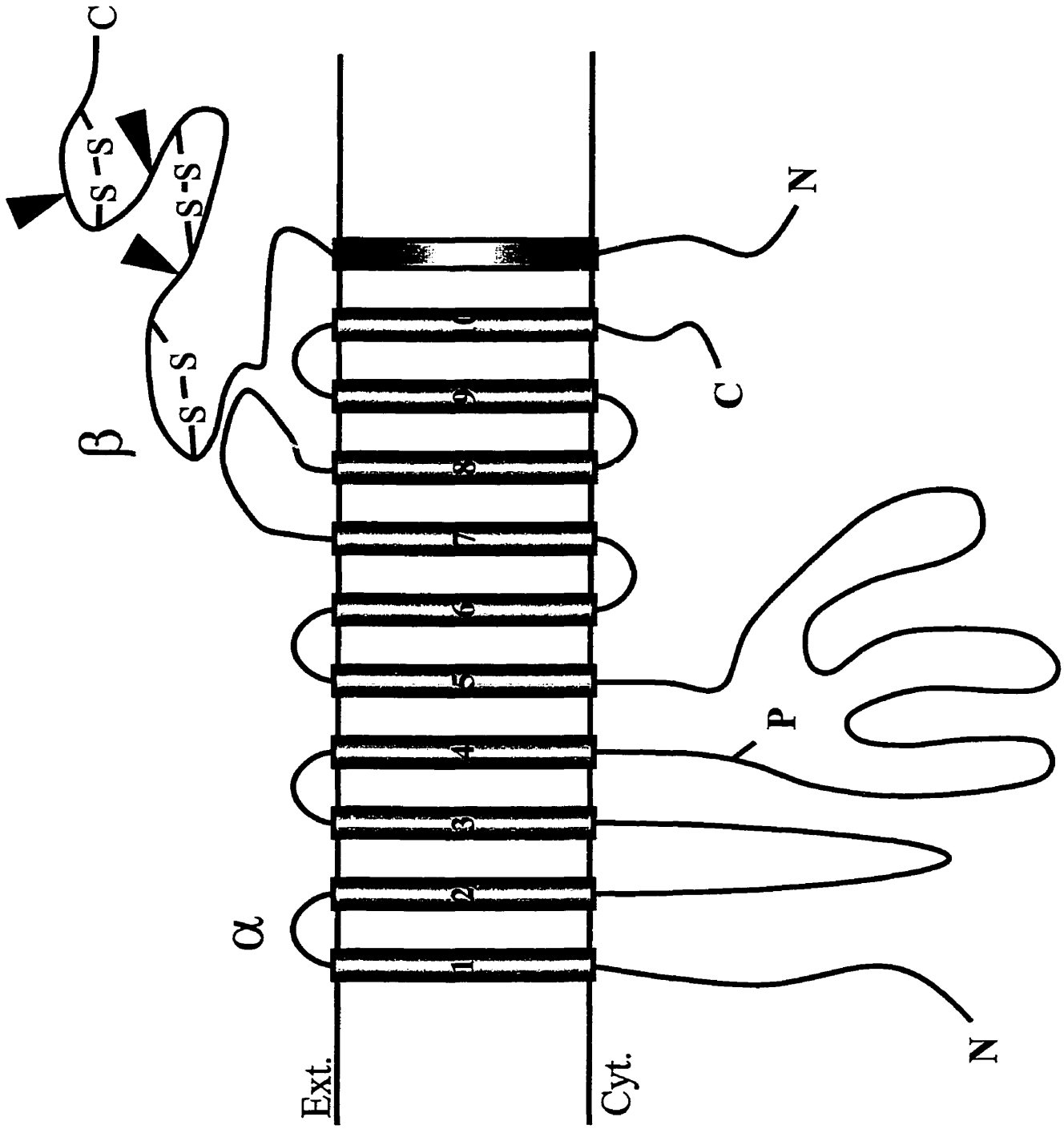
1.2.3 Function of the Na,K-ATPase

1.2.3.a Enzyme cycle

The Na,K-ATPase reaction cycle is based on the original work of R.W. Albers (6) and R.L. Post (405), and is therefore referred to as the Albers-Post model. Its current version includes additional information obtained in numerous other laboratories, however, and is still challenged and altered to account for new data. A generally accepted minimal model, including the normal 'physiological' pathways in bold and altered pump modes described below, is shown in Fig. 1-2. The normal physiological reaction cycle is as follows. The sodium pump (in the E_1 conformation; see below) first binds three Na^+ ions and one molecule of ATP with high affinity at the cytoplasmic side. The pump then becomes phosphorylated through transphosphorylation from an ATP molecule, and the sodium ions become occluded, or buried, within the protein. The enzyme then undergoes a conformational change (to E_2) whereupon its cation binding site becomes accessible from the extracellular medium. The affinity of the pump for Na^+ is very low in the E_2 state, resulting in release of these cations and subsequent binding of two K^+ ions. Dephosphorylation of the enzyme and occlusion of the K^+ ions is followed by an ATP-accelerated deocclusion of K^+ and a return of the enzyme to its original E_1 conformation, with release of K^+ at the cytoplasmic side. The Na,K-ATPase is then

Fig. 1-1 **Tertiary structure of the Na,K-ATPase.**

Current accepted topology of the rat α and β subunits and probable interaction site between the two subunits. The aspartate that is transiently phosphorylated during the catalytic cycle is indicated (-P), as are the sites of glycosylation (▼) and the disulfide bridges (-S-S-) of the β subunit. In addition, the transmembrane domains (M1-M10) of the α subunit are identified.


C
S-S
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Ext.
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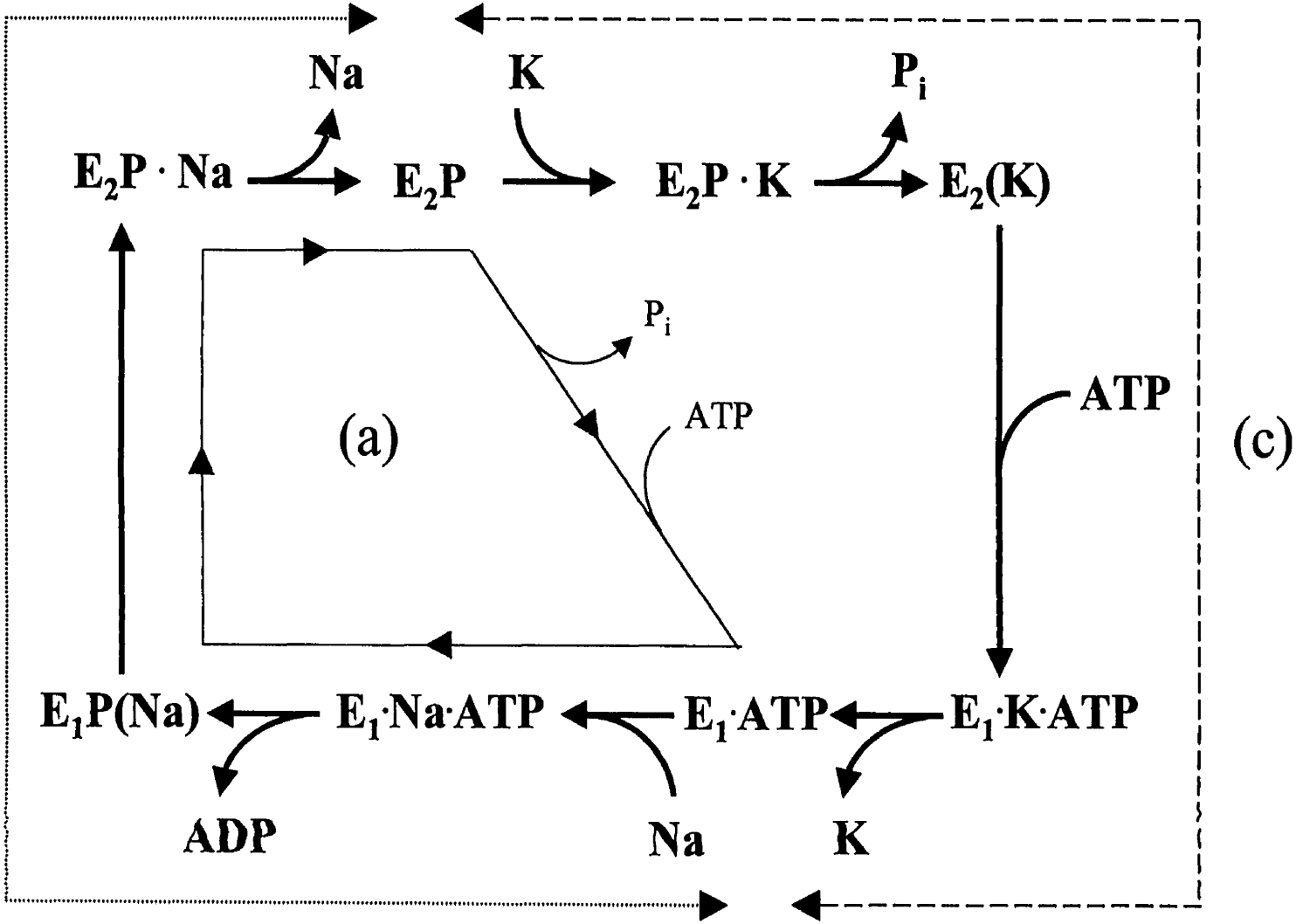
N

Fig. 1-2 **Albers-Post model of Na,K-ATPase enzyme cycle.**

Occluded cations are represented by brackets. Thick lines represent the "normal", physiological cycle. Non-physiological reactions include a) $Na^+/0$ flux (thin lines), b) Na^+/Na^+ exchange (dotted lines), and c) K^+/K^+ exchange (broken lines). See text for details.


Na
K
P_i
$E_2P \cdot Na$
E_2P
$E_2P \cdot K$
$E_2(K)$
P_i
ATP
(b)
(a)
(c)
ATP
$E_1P(Na)$
$E_1 \cdot Na \cdot ATP$
$E_1 \cdot ATP$
$E_1 \cdot K \cdot ATP$
ADP
Na
K

poised for another round of pumping. Under normal circumstances, the turnover rate of the enzyme is ≈10,000 min^{-1} (see for example, ref. 252). Evidence for the individual steps of the cycle is presented below.

1.2.3.b Conformational states

During its enzyme cycle, the sodium pump exists in one of two major conformational states, E_1 and E_2. While in the E_1 state, the enzyme has a high affinity for Na^+ and ATP, and a low affinity for K^+, and its cation binding pocket is accessible from the cytoplasmic side. Conversely, the E_2 form has a high affinity for K^+, a low affinity for both Na^+ and ATP, and its cation binding pocket is open to the extracellular side. Both E_1 and E_2 conformations can exist in either the phosphorylated or unphosphorylated form, the former being referred to as E_1P and E_2P.

Evidence for the existence of at least two conformational states comes from the observation that the Na^+-bound and K^+-bound forms (E_1 and E_2, respectively) are differentially cleaved by trypsin and chymotrypsin (251, 253). In addition, changes in fluorescence, either intrinsic (265) or from bound fluorescent probes (218, 262), have been associated with ligand-dependent conformational changes.

Recent studies have identified interactions between distinct domains of the sodium pump that are specific to the two main conformations. Taking advantage of the ability of heavy metal cations to cleave peptide bonds in the presence of peroxide, Goldshleger *et al.* (195) have shown that iron-catalyzed cleavage of the sodium pump occurs at several distinct sites when the enzyme is in the Na^+-bound E_1, but not in the K^+-bound E_2 conformation. These studies have allowed for the identification of several domains in the first and second cytoplasmic loops which are in close proximity in E_1, and separate in E_2.

While most of the available data point towards two main conformational states, there is evidence that additional subconformations exist. The evidence comes from studies on the reactivity of the phosphoenzyme to adenosine diphosphate (ADP) and K^+.

Thus, in accordance with the Albers-Post model, enzyme in the E_1P state becomes dephosphorylated in the presence of ADP, while enzyme in E_2P becomes dephosphorylated in the presence of K^+. Accordingly, the fraction of phosphoenzyme that reacts with either of these two reagents is a measure of the conformational equilibrium between E_1P and E_2P. In their studies, Yoda and Yoda (530) identified a fraction of phosphoenzyme that is sensitive to both ADP and K^+, and concluded that an intermediate phosphorylated conformational state, E^*P, exists in equilibrium with E_1P and E_2P.

1.2.3.c Rate-limiting steps

Under conditions of saturating concentrations of substrates and at neutral pH, the major rate-limiting step of the reaction is the K^+ deocclusion step. Much of the evidence comes from experiments done by Karlish and Yates (265). Taking advantage of the differences in intrinsic fluorescence of the two conformational pump states (E_1 and E_2), they showed that the rate of the $E_2(K) \rightarrow E_1$ transition was fast enough to be a part of the overall cycle, but slow enough to be one of the rate-limiting steps of the reaction. Forbush obtained similar results using his rapid filtration apparatus to measure the rate of breakdown of $E_2(K)$ (165).

Forbush also showed that pH can affect the nature of the rate-limiting step. While the rate of deocclusion increases linearly with pH between 6.4 and 9, ATPase activity starts decreasing steadily above pH 8, showing that some other step in the reaction must become rate-limiting at high pH (165). More recently, using a bound fluorescent probe and measuring the rate of fluorescence change associated with the E_1 to E_2 conformational change, Forbush and Klodos (168) showed that at high pH (>7.5), the E_1 to E_2P reaction becomes inhibited to levels where it becomes the major rate-limiting step of the overall reaction. The consensus, therefore, is that the rate-limiting step changes from being the K^+ deocclusion step ($E_2(K) \rightarrow E_1$) at low pH to being the $E_1 \rightarrow E_2P$ transition at high pH, with the switch-over point being around pH 7.5.

1.2.3.d Binding of ATP and enzyme phosphorylation and dephosphorylation

ATP is known to bind to the Na,K-ATPase with either low or high affinity (419). ATP binds with low affinity to drive the K^+-deocclusion reaction ($E_2(K) \rightarrow E_1K$), and this step does not require hydrolysis of the nucleotide. However, hydrolyzable ATP is required to bind with high affinity to catalyze the Na^+-occlusion reaction ($E_1 + Na^+ + ATP \rightarrow E_1P(Na)$), which is associated with hydrolysis of ATP and phosphorylation of the enzyme. The site to which ATP binds on the enzyme is not known precisely, but several residues appear to be involved including Asp^{376}, Lys^{487}, Lys^{507}, Cys^{663}, Asp^{716}, Asp^{720} and Lys^{725} (of the rat α1 sequence, counting the five cleaved N-terminal amino acids), all of which can be labeled by fluorescent ATP derivatives in an ATP-protected fashion (reviewed in refs. 141, 387). That residues outside the cytoplasmic loop are not involved in ATP binding was unequivocally demonstrated recently. Thus, expression of the large cytoplasmic loop in *E. coli* resulted in a protein with full ATP-binding capacities (183, 364).

As is the case for all members of the P-type family of ATPases, the Na,K-ATPase becomes transiently phosphorylated as part of its normal catalytic cycle. A phosphorylated intermediate of the Na,K-ATPase has been isolated from several tissues (59, 406, 461). The residue that becomes phosphorylated is Asp^{376} (rat nomenclature), part of the highly conserved consensus sequence SDKTGTLT in the major cytoplasmic loop found between transmembrane domains 4 and 5. That an aspartate residue is the target of phosphorylation was first demonstrated by Post and Kume (404), and the specific localization of the residue was obtained by the combination of analysis of tryptic fragments of the phosphorylated enzyme (92) and molecular cloning and sequencing of the protein (457). Further confirmation came from studies where the aspartate residue was mutated to a variety of other amino acids, resulting in inactive enzyme (287, 390).

Binding of K^+ to the Na,K-ATPase induces dephosphorylation of the phosphoenzyme and occlusion of the cation. Evidence that there is a phosphatase activity associated with the enzyme comes from observations that the sodium pump can

hydrolyze a phosphate group from an exogenously added phosphorylated substrate, such as ortho-methylfluorescein phosphate (228) or para-nitrophenyl phosphate (378). Consistent with predictions based on the Albers-Post model, it appears to be the $E_2(K)$ form of the enzyme that catalyzes this activity (137). In support of this, reagents that stabilize the E_2 conformation, such as Mg^+, K^+, and dimethyl sulfoxide, stimulate phosphatase activity while those that promote conversion to E_1, for example Na^+ and oligomycin, inhibit this activity (418).

1.2.3.e Binding and occlusion of Na^+ and K^+ ions

After the Na,K-ATPase binds Na^+ in the E_1 conformation or K^+ in the E_2 conformation, the cations become buried, or occluded, in the protein such that they become inaccessible from either the cytoplasmic or extracellular side (for reviews, see 164, 194). Evidence for the occlusion of Na^+ ions in the normal pathway is indirect, since it has thus far been impossible to isolate a Na^+-occluded form of the uninhibited enzyme (164). The Na,K-ATPase can be isolated as $E_1P(Na)$ in circumstances where the E_1P to E_2P transition is blocked, such as following pre-treatment of the enzyme with N-ethylmaleimide or α-chymotrypsin (191), or by including oligomycin, a specific inhibitor of $E_1P \rightarrow E_2P$ (147), in the reaction medium. Indeed, it is possible to form a dephosphorylated, Na-occluded form (presumably $E_1(Na)$) in the presence of oligomycin (147), supporting the idea that Na^+ occlusion may occur before phosphorylation in the normal enzyme cycle.

The existence of a K^+-occluded form of the enzyme was much more readily observed. Binding of K^+ ions to the E_2 form of the phosphoenzyme (E_2P) leads to spontaneous dephosphorylation of the pump, and occlusion of the potassium ions. The first evidence of K^+ occlusion was obtained indirectly by Post *et al.* (403), who showed that phosphoenzyme that had been dephosphorylated by incubation in the presence of K^+ or Rb^+ showed a time-dependent lag in its ability to rephosphorylate, even after diluting out the Rb^+ or K^+ ions. The pump apparently "remembered" its exposure to these cations, which was interpreted to mean that the cations remained tightly bound, or occluded.

The experiments by Post *et al.* (403) describe binding of K^+ ions to a phosphorylated form of the enzyme, leading to dephosphorylation and occlusion of the ions. It was later shown that a K^+-occluded form could also be formed by incubating dephosphorylated enzyme in K^+-containing, Na^+-free media (26, 266). Karlish *et al.* (266) suggested that this K^+-occluded form was identical to that identified by Post *et al.*, on the basis that both forms undergo a large increase in the rate of conversion to E_1 upon binding of nucleotides to a low affinity site. Forbush later confirmed these observations (165) and also showed that K^+ is released from its occluded state to the extracellular medium upon addition of P_i (166), providing evidence that the K^+-occluded state corresponds to $E_2(K)$ of the Albers-Post model. There are therefore two routes for forming $E_2(K)$: (i) the physiological, or indirect route, where K^+ binds to the phosphorylated form of the enzyme (E_2P) and becomes occluded after catalyzing dephosphorylation, and (ii) the direct route, where K^+ binds to the dephosphorylated form leading to occlusion. Karlish and co-workers (266) showed that this latter pathway involves binding of K^+ to the E_1, and not the E_2, form of the enzyme.

In the last several years, much effort has gone into determining the nature of the specific residues of the enzyme that can bind and occlude Na^+ and K^+ ions. Most of the information available was obtained first from studies using biochemical modifications, and later mutagenesis, of specific residues. Karlish and co-workers first showed that a 19-kDa protein complex corresponding to most of the C-terminal end of the α subunit, and formed upon extensive tryptic digestion of Rb^+-occluded enzyme, retains Na^+ and K^+ occlusion capacities (264). Experiments by Shani-Sekler *et al.* (451) showed that labeling of the Na,K-ATPase with DCCD, a carboxyl-specific reagent, can be prevented by either Na^+ or Rb^+, suggesting that the residue(s) modified by DCCD are involved in binding of both cations. Furthermore, the residues labeled by DCCD, presumed to participate in cation binding, have been suggested to be Glu^{953} and Glu^{327} in the pig enzyme (196). Using another carboxyl group-reactive reagent, DEAC, Argüello and Kaplan (16) have suggested the involvement of Glu^{779} of the dog enzyme in cation binding.

Mutagenesis experiments have identified several residues that may participate in

cation binding. For example, mutations of residues in the TM4 (Glu^{329} of rat $\alpha 1$ and Glu^{327} of rat $\alpha 2$) have significant effects on both Na^+ and K^+ affinities, as do mutations in the TM5 (Glu^{779} or Glu^{781}, both equivalent), TM6 (Asp^{804}, Asp^{808}, Thr^{809}) and TM8 (Asp^{925}) (150, 245, 286, 360, 503, 504). In particular, it has been suggested that TM5 and TM6 form a hairpin loop ("M5-M6 loop") that has functional importance in the occlusion and transport of K^+ (312), although that claim has been disputed (449). In addition to residues that lie in the transmembrane domains, cytoplasmic regions may contain important determinants of cation binding and occlusion (recently reviewed in ref. 63). For example, Ser^{775}, a residue suggested to flank TM5 on the cytoplasmic side, is important for K^+, but not Na^+ binding (17). In addition, insertion of the N-terminal part of the major cytoplasmic loop of the gastric pump into the Na,K-ATPase can affect cation selectivity (64), and residues in the N-terminus (121, 520) as well as Glu^{233} in the first cytoplasmic loop (120) have been implicated in the K^+ deocclusion limb of the cycle.

In general, it has been difficult to distinguish between effects of mutations or chemical modifications directly on cation binding and indirectly, through effects on the conformational equilibrium of the enzyme. Most affinity changes secondary to the mutations mentioned above are modest, with the exception of Ser^{775} which effects a 30-fold decrease in affinity for K^+. Recently, Blostein and co-workers (66) showed that mutations of this particular residue result in direct effects on cation binding, since several kinetic parameters of the mutant enzyme were not consistent with an altered conformational equilibrium. Another difficulty encountered by studies using site-directed mutagenesis is the possibility that mutations of residues that are relevant to cation binding result in inactive or degraded enzyme. Expressing mutated Na,K-ATPase in yeast can circumvent this problem since these organisms are capable of expressing even inactive or unstable sodium pumps. Using this system, Jørgensen and co-workers have provided strong evidence that Glu^{327}, Glu^{779}, Asp^{804} and Asp^{808} are critical for binding and/or occlusion of K^+ (360).

1.2.3.f Electrogenic properties

The Na,K-ATPase is an electrogenic pump in that one enzyme cycle results in the net transport of one positive charge to the outside of the cell (or one negative charge to the inside). As such, its function is affected by membrane potential (177, 197, 444). Studies on the voltage-dependence of sodium pump activity have led to the conclusion that Na^+ translocation is the electrogenic step. It has therefore been suggested that two negative charges in the protein are responsible for binding to two K^+ or two Na^+, neutralizing their charges, while the third Na^+ binds to a neutral, albeit polar, residue (197). More recent reports have shown that neither Na^+ binding and occlusion nor the subsequent phosphorylation are responsible (74), but rather that release of Na^+ to the extracellular side carries the charge (178, 222, 355).

1.2.3.g Altered pump modes

In addition to the physiological mode of pumping described above, the sodium pump can catalyze several other modes of transport, at least under specific conditions. First, in a reaction reminiscent of F-type ATPases, the sodium pump is able to catalyze the synthesis of ATP from ADP and P_i (182) in a mechanism involving the exact reversal of the normal physiological reaction. The sodium pump is also able to catalyze two types of Na^+/Na^+ exchanges, one electrogenic, the other electroneutral. Electrogenic Na^+/Na^+ exchange, first described in the red cell (296), involves Na^+ acting as a surrogate of K^+ (62, 118) with a mechanism otherwise identical to the physiological one (62). Electroneutral Na^+/Na^+ exchange is illustrated in Fig. 1-2 (pathway (b)) and involves the transport of three Na^+ ions to the extracellular side followed by the binding and translocation of three Na^+ back to the cytoplasm (reviewed in ref. 116; see also refs. 122, 192). A third type of non-physiological Na^+ transport has been observed in the absence (or presence at very low concentrations) of extracellular Na^+. This pump mode is thought to occur in the absence of counterion transport, and is therefore referred to as $Na^+/0$ flux (193). As illustrated in Fig. 1-2 (pathway (a)), the pump presumably transports Na^+ to the extracellular side, and having no other option in the absence of extracellular cations,

undergoes a slow, spontaneous dephosphorylation and conformational change back to E_1, only to bind ATP and Na^+ on the cytoplasmic side and start the cycle over. In addition to these various altered pump modes involving Na^+, the Na,K-ATPase has been shown to catalyze K^+/K^+ exchange in the absence of cytoplasmic Na^+, and the presence of cytoplasmic P_i, as shown in Fig. 1-2, pathway (c) (460).

Another non-physiological mode of cation pumping catalyzed by the sodium pump occurs at low pH and in the absence of either cytoplasmic Na^+ or extracellular K^+. Under these conditions, the enzyme can utilize protons instead of Na^+ or K^+ and become, in effect, an H,K-ATPase or an Na,H-ATPase (400). With low Na^+, the Na,K-ATPase can pump protons and Na^+ together, altering the Na^+ to K^+ stoichiometry (65). Interestingly, it has also been shown that the H,K-ATPase can transport Na^+ instead of H^+ if the pH and Na^+ concentration are sufficiently high (401).

1.2.3.h Inhibitors

Several molecules have been shown to inhibit Na,K-ATPase activity and Na^+ pump-catalyzed cation transport, some specifically, others not. The most specific inhibitors of the sodium pump are the cardiac glycosides such as ouabain and digoxin. Such reagents bind specifically to the extracellular surface of the enzyme (393), in a manner that is antagonized by K^+ (5). The affinity of the enzyme for cardiac glycoside binding is determined, at least in part, by residues in the first extracellular loop, namely those that flank the first two transmembrane domains (TM1 and TM2). Thus, mutating Gln^{111} and Asn^{122} of the ouabain-sensitive sheep enzyme to the corresponding ones of the relatively ouabain-resistant rat enzyme (Arg and Asp) results in a pump with dramatically lowered affinity for ouabain (408). A recent report has cast doubt on to the importance of these two residues in the direct binding of ouabain, however (115). In addition, other parts of the enzyme have been implicated in ouabain-pump interactions. For example, mutations in the first and second transmembrane domains influence ouabain binding (85, 443), as does the nature of the C-terminal half of the enzyme, as determined by studying the ouabain binding properties of a Na,K-ATPase/H,K-ATPase chimera (67).

There exist several compounds that inhibit the P-type family of ATPases, including the Na,K-ATPase. Of particular note are vanadate and oligomycin. Inorganic orthovanadate binds to the cytoplasmic surface and is believed to act as an analog of orthophosphate, by binding tightly to the E_2 conformation (84). Oligomycin, on the other hand, is believed to act by increasing the affinity of the enzyme for Na^+, and thus locking the enzyme in the $E_1P(Na)$ conformation (399).

1.2.4 Isoforms of the Na,K-ATPase

1.2.4.a Evidence for pump isoforms

Both the α and β subunits of the sodium pump exist as several genetically distinct isoforms, or isozymes. The earliest evidence for more than one isoform of the catalytic α subunit consists of observed differences in cardiac glycoside sensitivity when comparing Na,K-ATPase activity of the rabbit brain and kidney enzymes (464). The first evidence for the existence of two functionally distinct isoforms in the same tissue was reported in 1978. Marks and Seeds (323) showed that mouse brain enzyme contains a ouabain-sensitive ($IC_{50} \sim 10^{-7}$ M), and a ouabain-resistant ($IC_{50} \sim 10^{-3}$ M) component, whereas only a ouabain-resistant activity was detected in a tissue (kidney) which we now know contains a single major isoform (α1). Structural evidence for the existence of at least two isoforms was independently reported in the brine shrimp (394) and in mammals (478). In both cases, the α subunit was seen to migrate as two distinct bands on SDS-PAGE, both of which could be phosphorylated in a K^+-sensitive fashion. Definitive evidence that these two bands represent distinct Na,K-ATPase isoforms was provided by Sweadner (478). She showed that the α subunit migrates as two bands in brain of dog, mouse, rat and frog, but as a single band in kidney or eel electric organ, and that both bands of brain can be phosphorylated in a Na^+-stimulated, K^+-inhibited fashion. Inhibition of phosphorylation by strophanthidin (a membrane-permeable analog of ouabain) occurred with high affinity for the slower-migrating band, and with low affinity for the faster-migrating band. Thus, a structural basis for the high and low ouabain affinities detected earlier in functional assays of Na,K-ATPase activity (323) was demonstrated. Since the

faster-migrating band corresponded to the kidney band both in terms of strophanthidin sensitivity and migration rate, Sweadner called this band α, and the slower-migrating, ouabain-sensitive one, α+ . The α and α+ isoforms were subsequently found to be different in terms of their sensitivities N-ethylmaleimide (494) and pyrithiamine (328), strengthening the hypothesis that they comprised structurally different proteins. This was unequivocally demonstrated by Lytton (316) who showed that α and α+ have different N-terminal sequences, suggesting that they are the products of different genes. The advent of molecular biological techniques have since shown this hypothesis to be correct.

In 1985, the Na,K-ATPase α subunits of sheep (457), *Torpedo californicus* (268), and rat (439) were independently cloned, providing probes that were subsequently used in the search for other α isoform genes. In 1986, Shull *et al.* (456) cloned three distinct isoforms of the α subunit from rat brain, and observed that two of them had N-termini identical to the sequences published previously by Lytton (316). The proteins were identified as the previously described α and α+ isoforms, and the third was named αIII. The three brain isoforms of α are now known as α1, α2 and α3, respectively. Homologs of these isoforms have now been found in several species, including human, pig, rat and chicken. In addition, a fourth isoform, α4, has recently been cloned from rat testis (450) and detected by immunological methods in this tissue (522). There is evidence that α isoforms exist in all mammals, and probably occur widely in vertebrates (485), underscoring their probable physiological importance.

In addition to α subunit isoforms, there is recent evidence for the existence of multiple β isoforms. The β1 isoform was first cloned in the rat in 1986 (335), and the β1 cDNA was used in a low-stringency screening of a human liver cDNA library to identify the β2 isoform (326). A third isoform of β, β3, has been cloned and sequenced in amphibians (198) and more recently, in mammals (319). Since these initial reports, the β isoforms of several species have been cloned and sequenced (reviewed in ref. 52).

1.2.4.b Structure and tissue distribution of isoforms

The sequences of α isoforms of several species have been deduced from the

cDNAs encoding the polypeptides. A comparison of similar isoforms among different species shows very high identity, ranging from about 92% for α1 and α2, to greater than 96% for α3. Compared to each other, the α1, α2 and α3 isoforms are approximately 87% identical, while α4 is slightly less homologous, sharing 78% identity with α1. Most of the structural differences are found in the N-terminus and in the large cytoplasmic loop, specifically between residues 400 and 500 (52).

A comparison of the amino acid sequences of β isoforms shows that they are much less conserved than the catalytic isoforms. In rat, for example, the β2 and β3 isoforms are only 34% and 39% identical to the β1 isoform, respectively. Identity between the β2 and β3 isoforms is 49%. Across the species, individual isoforms are approximately 60% conserved (52). In addition to differences in primary sequence, the β isoforms differ in their capacity for N-linked glycosylation and sulfhydryl bridge formation (reviewed in ref. 107). While all β isoforms are heavily glycosylated, the β1 isoform has three sites for N-linked glycosylation, the β2 isoform, four to nine, depending on the species, and the β3 isoform, two (18). Similarly, the β1 isoform is known to contain three disulfide bridges, which are necessary for normal function, while the existence of such bridges in other isoforms has not been ascertained.

Both α and β isoforms have been found to be expressed in a tissue-specific fashion (for reviews, see refs. 52, 298). While the α1 isoform seems to be expressed ubiquitously, α2, α3 and α4 isoform expression is much more restricted. The α2 isoform is a major component of skeletal muscle, and is found in smaller amounts in lung and adult heart, while α3 is the main source of Na,K-ATPase activity in brain and pineal gland, and is also found in fetal and neo-natal heart (230, 376, 459). Detailed Western blot analysis of α isoform expression in the central nervous system has revealed that all three isoforms are expressed in a complex cell-specific manner, and that most cells in this organ can express more than one isoform (330). Both α2 and α3 have been detected in the kidney, but by most accounts, the major isoform of this organ is α1 (310). α4 has thus far only been detected in testis (450, 522). β isoforms are also restricted in their expression. The β1 isoform is found in most tissues, with a few exceptions; β2 has been

detected in skeletal muscle (292) and neuronal tissues (392), including pineal gland (459); and β3 is present in testis, retina, liver and lung (18, 319). It is interesting to note that in the red cell, the classical model for studies of the sodium pump, message for β2 and β3, but not β1 is detected (471).

1.2.4.c Kinetic properties of isoforms

One of the characteristics of the α isoforms is that they appear to have distinct kinetic behaviour. Several groups have attempted to define the enzymatic properties of each isoform, specifically with regards to their affinities for Na^+, K^+ and ATP. These studies can generally be classified into two categories: (i) those that compare the kinetics of tissues containing a preponderance of one catalytic isoform or the other, and (ii) those that compare the behaviour of exogenously-expressed isoforms in various cellular systems.

Many of the earlier studies on isoform-specific differences in kinetic behaviour involved the comparison of tissues containing mainly the α1 isoform, usually kidney, with tissues that express a large amount of the α3 isoform, brain or axolemma at first, and more recently, pineal gland. Such studies have generally shown that the kidney enzyme has a lower affinity for Na^+ and a higher or equal affinity for K^+ than the enzyme of brain (464, 494), axolemma (476) or pineal gland (459).

More recently, many groups have used systems whereby the different α isoforms are exogenously expressed in an attempt to define their kinetic behaviours. A major problem has been to distinguish endogenous from exogenous Na,K-ATPase activities. One strategy has been to take advantage of the large difference in ouabain-sensitivity of the rat or mouse α1 enzyme. Accordingly, the ouabain-sensitive α2 and α3 isoforms were expressed in a mouse cell line and the ouabain-sensitivity properties were found to be biphasic; the first phase was ascribed to the exogenous isoforms, and the second, to the endogenous, ouabain-resistant α1 isoform (213, 279). Unfortunately, the low activity of the former precluded a detailed kinetic analysis using this system. A different strategy was devised by Jewell and Lingrel (244) when they observed that the α2 and α3 isoforms

of the rat enzyme could be rendered ouabain-resistant by mutation of the two residues that flank the first two transmembrane domain, similar to what had previously been observed for the sheep enzyme (408). The mutated α2 and α3 isoforms were referred to as α2* and α3* (244). The rat α1, α2* and α3* isoforms were expressed in HeLa cells and individual clones were selected in the presence of 1 μM ouabain, allowing for isolation of high-expressing cells. Their results indicated that α1 and α2* have similar affinities for the Na^+, K^+ and ATP, but that α3* has a lower affinity for Na^+ and a higher affinity for K^+ and ATP compared to the other isoforms. A series of α1/α3 chimeras failed to elucidate the specific regions responsible for the differences in Na^+-affinities (243). Munzer *et al.* (351) subsequently observed similar, albeit more dramatic differences using the same transfected cells in Rb^+ transport assays. These authors also reported a higher Na^+-affinity of the enzyme of rat kidney compared to enzyme of axolemma after these pumps had been fused into dog red blood cells. In more recent experiments using α1 and α2 isoform-transfected HeLa cells, isoform-specific differences in the conformational equilibrium of the Na,K-ATPase have been shown (121). Another approach has been to use a system that lacks endogenous Na,K-ATPase activity. In a series of experiments, Blanco and co-workers have taken advantage of the low level of Na,K-ATPase activity in insect cells to investigate the isoform-specific kinetic behaviour of the sodium pump (reviewed in ref. 52). Using bacculovirus-infected Sf-9 cells, their experiments have shown that the order of affinities of the different isoforms is α2>α1>α3 for Na^+, α1>α2>α3 for K^+, and α2≈α3>α1 for ATP.

The kinetic properties of the testis-specific rat α4 isoform have recently been investigated. It was found that this isoform has a high affinity for ouabain (approximately equivalent to that of the rat α2), and that it can bind both Na^+ and K^+ with affinities similar to those of the sheep α1 isoform (522).

Clearly, the kinetic properties of Na,K-ATPase depend on factors other than the nature of the α subunit isoform. As described in Section 1.3, sodium pump activity can be modulated by the membrane and cytoskeletal environment, as well as the nature of the β subunit isoform. Nevertheless, the aforementioned studies show that with all other

variables held constant, the α3 isoform appears to have a lower affinity for Na^+, and α1, a lower affinity for ATP, compared to other α isoforms, while a consensus cannot be reached regarding K^+-affinity of the enzyme.

1.2.4.d Physiological role of isoforms

The physiological basis for the existence of several α isoforms is the subject of some controversy (for recent discussions, see refs. 52, 298). The α1 isoform, being ubiquitously expressed, probably has a "housekeeping" role in that it maintains the electrochemical gradient across biological membranes in most tissues. While the roles of the α2, α3 and α4 isoforms are less clear, the high degree of inter-species homology suggests that they have an important physiological role. A recent report has provided unequivocal evidence in favor of a non-redundant role of Na,K-ATPase isoforms. In a study using mice in which the gene for either the α1 or α2 isoforms were knocked out, James *et al.* showed that both isoforms are necessary for survival (240). Specifically, α1 knock-outs did not survive to birth, while α2 knock-outs were born, but died within a day.

The distinct enzymatic properties of α3 makes it particularly tempting to suggest a specific physiological role for this isoform. In particular, its expression in neurons may be related to a role in re-establishing the Na^+ and K^+ gradients across the membrane of these cells following action potential-induced depolarization. The α3 isoform may act as a latent pool of Na,K-ATPase activity in these cells which is activated upon the entry of Na^+ which follows depolarization. The low affinity of this isoform for Na^+, as well as its high affinity for ATP, make it particularly suited for such a role, since one might expect ATP levels to fall rapidly in this situation.

The isoform-specific differences in cardiac glycoside affinity may also have physiological relevance. Although the near 1000-fold difference in ouabain sensitivity typical of rat α1 versus α2 and α3 isoforms is not duplicated in most other species, small differences do exist even in non-rodents (204). It is possible that the role of endogenous cardiac glycosides (see Section 1.3.6) is to modulate Na,K-ATPase of organs that express

isoforms other than α1, such as nerves and muscles, without affecting the enzyme activity of α1-expressing organs, such as the kidney. In support of this notion is the aforementioned report by James *et al.*, whose studies using knock-out mice allowed the identification of α2 as the target isoform for cardiac glycoside inhibition of Na,K-ATPase in the heart (240). Other recent evidence points towards a role of Na,K-ATPase isoforms in cardiac and vascular function. Thus, while the α1 isoform is uniformly distributed in these tissues, other isoforms are localized to areas of the plasma membrane that interact with the sarcoplasmic/endoplasmic reticulum (areas called "plasmerosomes"). These functional units are thought to mediate the Na,K-ATPase-dependent regulation of cytoplasmic Ca^{++}, and therefore contractility of vascular and cardiac muscles (57, 58; see also section 1.3 below).

Finally, the role of distinct sodium pump isoforms may be reflected in isoform-specific regulation of the pump, rather than in isozyme-specific kinetic properties. A specific example involves the α2-specific recruitment of pumps by insulin in skeletal muscle (see section 1.3.7.b). In addition, there are several other examples of isoform-specific regulation by a variety of hormones, as described in section 1.3.7.

1.3 REGULATION OF THE NA,K-ATPASE

1.3.1 Physiological rationale for regulation of the sodium pump.

The basic function of the sodium pump is to maintain the high Na^+ and K^+ gradients across the plasma membrane of animal cells. As such, it has an important role in regulating cell volume, cytoplasmic pH and Ca^{++} levels through the Na^+/H^+ and Na^+/Ca^{++} exchangers, respectively, and in driving a variety of secondary transport processes such as Na^+-dependent glucose and amino acid transport. Since the sodium pump is the main determinant of cytoplasmic Na^+ concentration, which, in turn, determines the activity of the above cellular processes, it is the target of multiple regulatory mechanisms activated in response to changing cellular needs. The requirement for modulators of the Na,K-ATPase is especially great in tissues where the

enzyme has specialized roles in addition to the cellular functions mentioned above (see below for specific references). In such tissues, sodium pump activity must adapt to changing physiological stimuli such as nerve impulse propagation, exercise and changes in diet. In some cases, expression of various isoforms of the sodium pump appears to fulfill some of the requirements for altered pump behaviour (see Section 1.2.4 on isoforms), but in others, direct modulation of the enzyme may be the mechanism for altered pump activity.

One of the primary needs for sodium pump adaptation comes from changes in dietary Na^+ and K^+. The mediators of natriuresis and diuresis, that is hormones that control the volume and ionic composition of blood and urine, often act directly on the sodium pump of the kidney and intestine. Since the role of the pump in these tissues, in addition to the 'classical' roles mentioned above, is for absorption or reabsorption of Na^+ and K^+, as well as other solutes, tight regulation of the enzyme is necessary to maintain normal levels of Na^+ and K^+ during altered salt intake (for reviews, see refs. 136, 224). In addition, since water and Na^+ transport across epithelia are often linked events, the sodium pump has an additional role in maintaining water absorption in the intestine and reabsorption in the kidney. Illustrating this, is the observation that diseased states resulting from altered salt and water homeostasis such as hypertension (234) and chronic diarrhea (163), are often the result of abnormal control of the sodium pump in kidney and intestine, respectively.

In excitable tissues such as neurons (190), skeletal muscle cells (108) and pacemaker fibers of the heart (483), the sodium pump must re-establish the electrical potential across the plasma membrane following excitation-induced depolarization of the cell. Although part of this function is undoubtedly fulfilled by the presence and distinct kinetics of the $\alpha 3$ isoform in neurons, regulatory events are likely to be involved as well, as evidenced by the multiple effects of various hormones on Na,K-ATPase activity in these tissues. In particular, regulation of skeletal muscle sodium pump activity has widespread physiological implications. Since continuous stimulation of muscle fibers during exercise leads to dissipation of the cation gradient necessary for muscle

contraction, resulting in some cases in hyperkalaemia due to excessive release of K^+ from the muscle cells, upregulation of the sodium pump under these conditions is necessary to delay the onset of muscular fatigue and lower potentially life-threatening levels of plasma K^+.

Another important role of the Na,K-ATPase is observed in smooth muscle of the heart and blood vessels, where the enzyme acts as an indirect regulator of contraction (56). Thus, the sodium pump controls the steady-state cytoplasmic Na^+ concentration which, via the Na^+/Ca^{++} exchanger, is a determinant of Ca^{++} concentration. In turn, this cation is taken up into the sarcoplasmic reticulum by the SERCA pumps, which results in a greater Ca^{++} gradient across the membrane of this organelle, directly impacting muscle contraction. Regulation of the sodium pump in these tissues is therefore paramount in determining the set-point for cardiac muscle contraction and the steady-state contraction of vascular smooth muscle. Based on this principle, the cardiac glycoside inhibitors of the Na,K-ATPase are used extensively in the treatment of cardiac insufficiency (488).

Clearly, mechanisms for modulating the sodium pump in a tissue-specific manner are of great physiological importance. Whether reversible or irreversible, through interactions with intrinsic membrane components or as the result of highly complex signalling cascades, regulation of the Na,K-ATPase plays an important role in adaptation of the organism to various external and internal stimuli. The major mechanisms by which the Na,K-ATPase is regulated are discussed in the following sections.

1.3.2 Substrate concentrations

The simplest and most straightforward determinants of pump activity are the concentrations of substrates. The sodium pump is activated by Na^+ and ATP at cytoplasmic sites, and by K^+ at extracellular sites. The most dramatic effects involve variations in cytoplasmic Na^+ concentration, since half-maximal activation of the enzyme by Na^+ occurs at concentrations of $\approx$10 to 40 mM, depending on the tissue, and the Na^+ concentration is often below these values (for example, see ref. 468). Accordingly, small changes in the cytoplasmic Na^+ concentration secondary to activation of various Na^+-

dependent transporters or Na^+ channels can have dramatic effects on sodium pump activity. In fact, as described below, many effectors of sodium pump stimulation act on the activity of these secondary transport mechanisms. Changes in Na^+-stimulation of enzyme activity can also occur without changes in the cytoplasmic concentration of the cation. As described below, some hormones appear to alter sodium pump activity by changing its apparent affinity for Na^+. Aside from its direct effects on the Na,K-ATPase, Na^+ has been shown to induce other mechanisms of upregulation of the sodium pump. For example, Na^+-influx is thought to be the first signal leading to upregulation of sodium pump activity in aldosterone-mediated short-term regulation (see section 1.3.7.c).

Variations in K^+ concentration can also have dramatic effects on Na,K-ATPase activity, not at extracellular sites, as would be expected, but rather at cytoplasmic Na^+ binding sites. Thus, while the high affinity of the enzyme for K^+ at activating sites generally precludes an effect of variations in extracellular K^+ concentrations on sodium pump activity, at least in non-neuronal tissues (see, for example, ref. 481), K^+ has been shown to act as a competitive inhibitor of Na^+ binding (180). Therefore, variations in cytoplasmic K^+ concentration, or more likely, in the affinity of the enzyme for K^+ at cytoplasmic binding sites, are two mechanisms by which the sodium pump may be regulated, as discussed in Chapters 2 and 3.

Since the $K_{0.5}$ of the Na,K-ATPase for ATP is between 300 and 800 μM (469), the ATP concentration in most cells is saturating for the enzyme. However, in some tissues and under certain conditions, ATP levels may fall to subsaturating levels. For example, cells of the kidney medulla are known to function under near anoxic conditions (80), and such conditions can lead to dramatic drops in ATP levels (469). Thus, variations in ATP concentration or in the affinity of the sodium pump for ATP may be physiologically relevant mechanisms of pump regulation in this tissue. Of relevance to this, as discussed in Chapters 4 and 5, the γ subunit can regulate the apparent affinity of the Na,K-ATPase for ATP in kidney medullary tubules.

1.3.3 Membrane and cytoskeletal components

1.3.3.a Lipids

The nature of the membrane lipids that surround the pump is an important determinant of Na,K-ATPase activity. The main effects of lipids appear to be related to membrane fluidity and thickness. In general, lipids that promote bilayer formation of physiological thickness and increased fluidity tend to promote optimal Na,K-ATPase activity (246, 275, 321), as do negatively-charged lipids, such as phosphatidylserine and phosphatidylglycerol (276). The effects of cholesterol on enzyme activity are often also related to membrane fluidity (189), although specific effects of cholesterol on the sodium pump have been reported (529). Free fatty acids present in the membrane or as the products of phospholipase A_2-dependent regulatory pathway tend to inhibit the Na,K-ATPase (368), as described in Section 1.3.8.e.

1.3.3.b L_p antigen

One of the most widely studied membrane-associated modulators of the Na,K-ATPase, indeed the only known protein regulator of the enzyme, other than the β and γ subunits, is found in low-K^+ (LK) erythrocytes of sheep, which have typically low cytoplasmic K^+ and high cytoplasmic Na^+ concentrations. The molecular basis for this abnormal ionic distribution is an inhibitor of the Na,K-ATPase known as the L_p antigen, so-called because of its association with the L blood group antigen and its highly specific effects on the sodium pump (reviewed in ref. 140). Evidence for the existence of this inhibitor comes from studies on the effects of an antiserum specific for the L_p antigen; treatment with this antiserum stimulates Na,K-ATPase of low-K^+, but not of high-K^+ (HK), erythrocytes (145). In addition, trypsin reverses the functional effects of anti-L_p antigen antiserum (291), providing evidence that the inhibitor is a peptide distinct from the sodium pump itself and that the anti-L_p epitope is removed upon trypsin treatment. Experiments using anti-L_p and trypsin have led to a model of L_p-mediated inhibition of Na,K-ATPase whereby the antigen inhibits sodium pump activity in two distinct ways. One is secondary to an L_p antigen-induced increase in the susceptibility of pumps to

noncompetitive inhibition by K^+ (138), and the other, to a large decrease in pump number (524). In addition, the L_p antigen-mediated changes in sodium pump kinetics appear during maturation of erythrocytes. Indeed, the kinetic behaviour of sodium pumps of LK reticulocytes is indistinguishable from those of HK reticulocytes (139), but presence of the L_p antigen at this immature stage leads to the acquisition by the enzyme of the characteristic noncompetitive inhibition by K^+ and loss of surface sodium pumps. Evidence that the presence of L_p at the reticulocyte stage is required comes from evidence that anti-L_p and trypsin treatment of such cells abolishes maturation-acquired modulations in pump behaviour (525) and expression (524). In addition, rat kidney pumps fused into LK red blood cells by the method developed by Munzer *et al.* (352) were stimulated by anti-L_p only after fusion at the reticulocyte stage and subsequent maturation *in vitro* (526). This latter experiment also provided further evidence that the L_p antigen is a molecular entity distinct from the sodium pump.

1.3.3.c Cytoskeleton

Interaction of the Na,K-ATPase with components of the cytoskeleton of cells are well documented. Specific cytoskeletal proteins thought to interact with the sodium pump, either directly or indirectly, include spectrin (267), actin (281), adducin (490), pasin (285) and ankyrin (357). Generally, ankyrin appears to mediate associations between the sodium pump and other cytoskeletal proteins, although direct associations of the enzyme with pasin and actin have also been observed. The two specific domains of the sodium pump that interact with ankyrin have been recently identified (130, 538). Of these, residues in the first cytoplasmic domain (142-166 of the rat α1 isoform) are especially intriguing as this region is highly conserved in all sodium pump isoforms and in H,K- and Ca-ATPases, suggesting interactions of these various membrane proteins with ankyrin. Ankyrin binding to the second cytoplasmic loop is likely mediated by a 4-residue motif (ALLK) which has homology to a sequence of the anion exchanger, another ankyrin-binding transporter (249).

The main consequence of interactions between the Na,K-ATPase and

cytoskeleton is believed to be the correct processing and targeting of sodium pumps to the appropriate membrane compartment. For example, disruptions in the cellular distribution of Na,K-ATPase, induced either by ATP-depletion or hypoxia, are linked to alterations in cytoskeletal proteins (341, 380), and a spectrin/ankyrin complex is required for transport of pumps from the endoplasmic reticulum to the Golgi apparatus (131). Recently, a role for cytoskeletal proteins in regulating sodium pump activity has been suggested. For example, monomeric, but not polymerized, actin has been shown to activate the sodium pump by a mechanism mediated by cyclic adenosine triphosphate (cAMP)-dependent protein kinase (PKA) (82, 83; see also Section 1.3.8.a on regulation by PKA). In addition, mutant forms of adducin have been shown to stimulate Na,K-ATPase activity in transfected NRK-52E cells (490).

1.3.4 The β subunit

As described in Section 1.2.2.b, the major role of the β subunit is the correct membrane insertion and stability of the Na,K-ATPase protein complex (107). However, a role for the β subunit in modulating the kinetic behaviour of the enzyme has also been described.

The first evidence for a functional role of the β subunit was provided by Kawamura and Nagano (270), who showed that Na^+ or K^+ can protect the sodium pump from inactivation by β subunit disulfide bond reduction. Subsequent experiments on the H,K-ATPase enzyme showed that the lability of the β subunit disulfide bridges is related to the conformation of the enzyme, specifically that the cysteine bonds are protected while the enzyme is in the $E_2(K)$ conformation (106). Using a different approach, Capasso *et al.* (86) also provided evidence for the involvement of the β subunit in K^+ binding. They observed that a nearly intact β subunit is a major component of so-called '19 kDa membranes', formed by extensive trypsin digestion of Rb^+-occluded enzyme.

Molecular techniques have further emphasized the role of the β subunit in modulating K^+ binding or the conformational steps associated with K^+. The first genetic evidence came from experiments showing that the *Bufo marinus* enzyme comprising α1

and β1 Na,K-ATPase isoforms expressed in *Xenopus* oocytes has a higher affinity for K^+ than the enzyme comprising either β3 or the β subunit of the H,K-ATPase (237, 239). Similarly, Schmalzing *et al.* (438) showed that the mouse β2 isoform confers a lower affinity for K^+ to the Torpedo enzyme than the β1 isoform. The β subunit has also been found to modulate the affinity of the enzyme for K^+ in α1- and α3-transfected yeast cells (142) and Sf-9 cells (531). In addition to modulating the Na,K-ATPase affinity for K^+, there is evidence that the β subunit has a role in determining the affinity of the enzyme for its other cationic substrate, Na^+. Using Bacculovirus-infected Sf-9 cells, Blanco and co-workers showed that the rat α2 and α3 enzymes co-expressed with β2 have a higher affinity for Na^+ than those co-expressed with the β1 isoform (50, 53). In addition, this group showed that the nature of the β isoform has no effect on K^+, ATP or ouabain affinities in this system. Similar results were obtained with the human α1 isoform co-infected in Sf-9 cells with either the β1 or β3 isoforms, although differences in K^+ and ouabain affinities were observed in this case (531). In studies in yeast, α1 pumps were found to have a higher affinity for Na^+, ATP and ouabain in the presence of a Na,K-ATPase/H,K-ATPase chimeric β subunit compared to the wild type Na,K-ATPase β subunit (143). In addition, recent experiments in Sf-9 infected cells suggest that the β subunit has a role in determining the catalytic turnover of the enzyme, and that this may be the basis for the much lower turnover of the H,K-ATPase compared to the Na,K-ATPase (278).

Using chimeras of the H,K- and Na,K-ATPase β subunits, two groups have independently shown that the region of the β subunit that alters Na,K-ATPase function is the extracellular domain (142, 242). However, a recent report shows that the N-terminus, while having no direct effects on function, can affect the conformation of the extracellular domains, and thus alter both Na^+ and K^+ affinities (216).

1.3.5 The γ subunit

1.3.5.a Structure and expression of the γ subunit

The γ subunit is a small transmembrane protein that specifically associates with

the Na,K-ATPase in some systems. While its existence had been previously suggested (417), it was Forbush and co-workers (167) who first demonstrated that this small hydrophobic peptide is specific to the sodium pump by showing that it is specifically labeled, along with the α subunit, by a photoactive derivative of ouabain. Although the peptide was at first thought to represent a third component of the Na,K-ATPase, recent evidence suggests that it is not an integral part of the enzyme complex, but rather acts as a modulator of Na,K-ATPase activity (see below and Chapters 4 and 5).

Following the studies of Forbush *et al.* in pig kidney, experiments using various ouabain derivatives resulted in the identification of a small sodium pump-associated proteolipid in several membrane preparations (210, 309, 421, 423). This peptide was initially referred to as 'γ component' or 'γ subunit' (415), and appears to be present in approximately equimolar amounts compared to the α and β subunits (110, 214). The first structural data for the γ subunit came from HPLC analysis of the peptide of lamb kidney (415). Initial reports suggested that γ might be a proteolytic cleavage fragment of the α or β subunits (110, 215), a hypothesis that has been since shown to be incorrect. Initial attempts at direct sequencing of γ were unsuccessful due to its apparently blocked N-terminus (111). To get around this problem, Collins and Leszyk (111) sequenced two overlapping proteolytic fragments of γ for a total of 33 consecutive amino acids, and deduced from previous amino acid composition data that the peptide comprised 68 amino acids, with a molecular weight of 7675 Da. The subsequent molecular cloning of the γ subunits of rat, mouse, cow and sheep agreed with the data obtained from N-terminal sequencing: the γ subunits of these species was shown to consist of 58 amino acids and have a molecular weight of ≈6500 Da (334), although an altered sequence for the rat protein (66 amino acids, 7237 Da) has since been published (see Chapter 4, and ref. 338). Since then, the human (274) and *Xenopus laevis* (31) γ subunits have also been cloned and sequenced, and a comparison of the sequences shows that γ subunits of different species are approximately 75% homologous. If only mammalian sequences are compared, the homology increases to 93%. Further structural analysis has revealed that the γ subunit contains a single transmembrane domain, and has a C-terminus-out, N-terminus-in

topology (see Chapter 4 and ref. 31).

One of the intriguing structural aspects of the γ subunit is that it is detected as two bands or peaks using various protein separation techniques (for examples, see refs. 110, 309, 334). Early evidence suggested that the larger peptide might be an aggregate of the smaller one, since the individual γ species, referred to as γ1 and γ2 and separated by chromatography on a Sephadex LH-60 column, yielded both species after a second round of chromatographic separation (110). In addition, γ1 and γ2 had similar amino acid compositions. It was subsequently shown that the two γ subunit bands detected on Western blots are the product of a single mRNA species (334). Recently, Béguin and co-workers (31) have shown that in *Xenopus*, the presence of two bands of γ is secondary to alternate usage of two distinct start codons in the γ subunit message, although only the first appears to be relevant *in vivo* in this species. However, a second starter methionine has not been detected in the mammalian sequences (see Chapter 5 and refs. 274, 334), and the nature of the two bands therefore remains unclear in these species.

The expression of γ subunit mRNA has been investigated by Northern blot analysis in the rat, human and *Xenopus laevis*, and it was shown that the peptide is expressed in a tissue-specific manner in these species. Thus, in humans, γ subunit mRNA was detected in kidney, pancreas and fetal liver (274), and in *Xenopus*, mainly in kidney and stomach, with trace amounts in heart, skin and oocytes (31). In rat, the situation is more complex, as two distinct mRNA species were detected using the rat γ cDNA as a probe (334). The larger of the two, at 1.5 kb, corresponds in size to the *Xenopus* mRNA, and was detected mainly in kidney and spleen, with lower amounts in lung, heart and brain. The smaller transcript migrated at 0.8 kb, a size similar to human γ message, and was detected at high levels in the kidney, and at very low levels in the spleen, lung and heart.

Most available data indicate that the γ subunit is co-expressed with the Na,K-ATPase. For example, γ is expressed at the surface of *Xenopus* oocytes only upon co-injection of cRNA for the α and β subunits (31), and immunocytochemistry has shown

that the expression patterns of α and γ are identical in renal proximal tubules and collecting ducts (334). In addition, co-immunoprecipitation of the γ subunit with both the α and β subunits has been demonstrated (334). On the other hand, Jones and co-workers (247), in their study on the role of the γ subunit in mouse blastocyst development, have shown that the γ subunit is expressed at high levels at the apical membrane, while the α and β subunits are present only at the baso-lateral membrane.

1.3.5.b Function of the γ subunit

The functional role of the γ subunit has only recently begun to be investigated. The first attempts at defining a functional role for the γ subunit indicated that this peptide is not necessary for normal enzyme function. For example, Hardwicke and Freitag (214) were able to show that separation of the γ peptide from the α/β complex by solubilization of shark rectal gland and avian salt gland membranes with a non-ionic detergent, had no effect on Na,K-ATPase activity. More recently, it has been shown that the presence of the γ subunit is not necessary for functional expression of the sodium pump in insect cells (129), *Xenopus* oocytes (31) and yeast (437). In the latter system, the γ subunit was shown to have no effect on either ouabain-sensitive Na,K-ATPase activity or ^{86}Rb influx. The failure to detect γ subunit mRNA in many tissues also supports the notion that the γ subunit is not an essential component of the Na,K-ATPase (31, 274, 334).

In contrast, more recent experiments have shown that γ has an important functional role in some systems. Treatment of mouse blastocysts with γ subunit antisense oligodeoxynucleotide reduced the amount of expressed γ subunit, and caused a reduction in ouabain-sensitive $^{86}Rb^+$ transport, as well as delayed blastocoel formation (247). In experiments on cRNA-injected *Xenopus* oocytes, the γ subunit has been shown to influence the apparent affinity of the Na,K-ATPase for K^+ in a complex Na^+- and voltage-dependent fashion (31), although the interpretation of these results remains unclear. Thus, the presence of the rat γ subunit caused (i) a voltage-independent decrease in the affinity of the enzyme for K^+ in the absence of Na^+, and (ii) an increase in K^+-affinity at negative, and decrease at positive, potentials in the presence of Na^+. Presence of the *Xenopus* γ

subunit had no effect on K^+-affinity in the absence of Na^+, and increased the affinity of the pump for the cation at negative potential in the presence of Na^+. A role of the γ subunit in interactions of the Na,K-ATPase with K^+ had previously been suggested by Or *et al.* (373), who showed that the γ subunit is a component of the protein complex found in so-called '19 kDa membranes'. As discussed in Section 1.2.3.e, such membranes are formed by tryptic digestion following occlusion of K^+ or Rb^+ by the enzyme to form $E_2(K)$. In a more recent report, the γ subunit has been shown to induce ouabain-independent ion currents in injected *Xenopus* oocytes, and ^{86}Rb and ^{22}Na influx in bacculovirus-infected Sf-9 cells (338). This current-inducing role of the γ subunit has been proposed to be the basis for the aforementioned γ subunit-mediated alterations in fluid transport and blastocoel formation in the mouse blastocyst (247).

Clearly, the precise role of the γ subunit remains poorly understood. What functional data exist come from studies on bacculovirus infected Sf-9 cells and cRNA-injected *Xenopus* oocytes, and may not be relevant to mammalian systems. Nevertheless, the data is consistent with the idea that the γ subunit is not necessary for Na,K-ATPase function, but rather is a tissue-specific modulator of the enzyme.

1.3.5.c The γ subunit as a member of a family of proteins.

In recent years, several small single-transmembrane-domain peptides with high sequence homology to the γ subunit have been identified. As such, studies on these peptides may reveal interesting information on the role of the γ subunit in modulating cation transport. To-date, in addition to γ itself, three members of this family have been cloned: phospholemman (PLM) (381), channel inducing factor (CHIF) (19) and Mammary tumor-associated 8 kDa protein (Mat-8) (347). Cloned sequences of these peptides include PLM of mouse (70), dog (381), rat and human (97), CHIF of rat (19), and Mat-8 of human (348) and mouse (347). Two additional sequences with homology to the γ subunit family of proteins are known, namely a "phospholemman-like protein" in humans (HPLP; ref. 22), and a "regulated ion channel homolog" (RIC; ref. 173) in mouse. The amino acid sequences of the rat γ subunit, CHIF, PLM, and mouse Mat-8 are

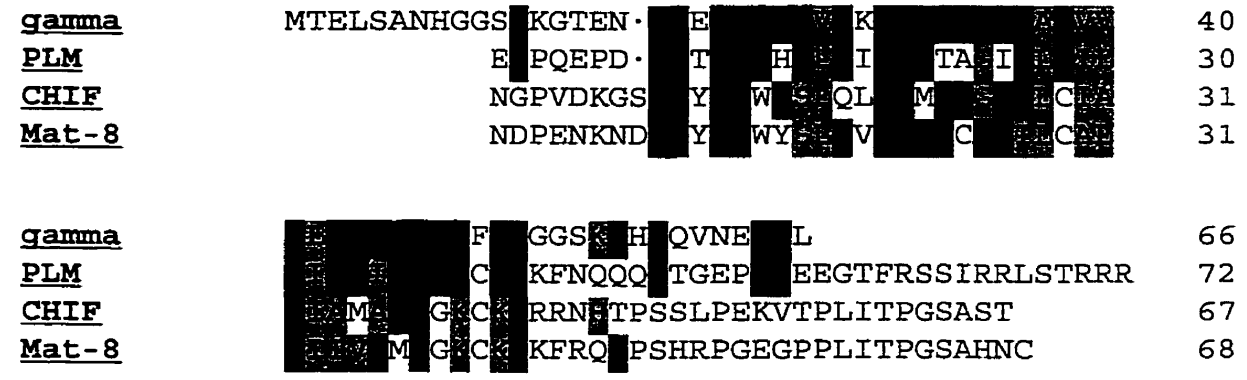
compared in Fig. 1-3. For the rat γ subunit, the revised sequence is shown (see chapter 5 and ref. 338), while for PLM, CHIF and Mat-8, the sequence for the mature proteins, after cleavage of their putative signal peptide (see below), are shown. As illustrated in the Figure, the latter three proteins have 38-43% homology with the γ subunit, and this value increases to 74-80% in the transmembrane domain and immediate flanking sequences (P_{18} to C_{52} of the rat γ subunit). There are several highly conserved motifs present in most of the known sequences of this family of proteins. Using the numbering for the rat γ subunit, these include (i) P_{18}FXYD in the extracellular domain; (ii) G_{29}G in the transmembrane domain; and (iii) S_{47}X(R/K)C(R/K)C, flanking the transmembrane domain on the cytoplasmic side. It should be noted, however, that in the γ subunit, motif (iii) contains a Phe residue instead of the first Cys. Interestingly, Gly^{30}, Gly^{41} and Ser^{47} are 100% conserved among all known sequences. Of these, Ser^{47} is especially intriguing, since the nearby presence of positive charges (either K or R) make it a possible target for phosphorylation by calcium-dependent protein kinases (PKC).

Phospholemman is perhaps the most widely studied member of this family of proteins. It was first identified as a 15-17 kDa protein in cardiac sarcolemmal vesicles (248) and subsequently in liver (114), skeletal (508) and smooth (429) muscle, and adrenal tumor cells (518). It is highly phosphorylated by both PKC and PKA, following activation of these kinases by α- and β-adrenergic agents, respectively (302). The precise residues phosphorylated by these protein kinases have been identified: Ser^{63} is phosphorylated by PKC, while Ser^{68}, by both PKC and PKA (507). Dog phospholemman was sequenced by Edman degradation and was also the first member of this family to be cloned (381). Direct sequencing of the protein combined with its genetic cloning has allowed identification of a cleaved amino-terminal signal peptide in this protein. Because of the high homology in this region between phospholemman, CHIF and Mat-8, but not the γ subunit, it is assumed that this signal peptide is cleaved in the other two proteins as well, and the sequences shown in Fig. 1-3 are those of the mature proteins for these three γ subunit homologs.

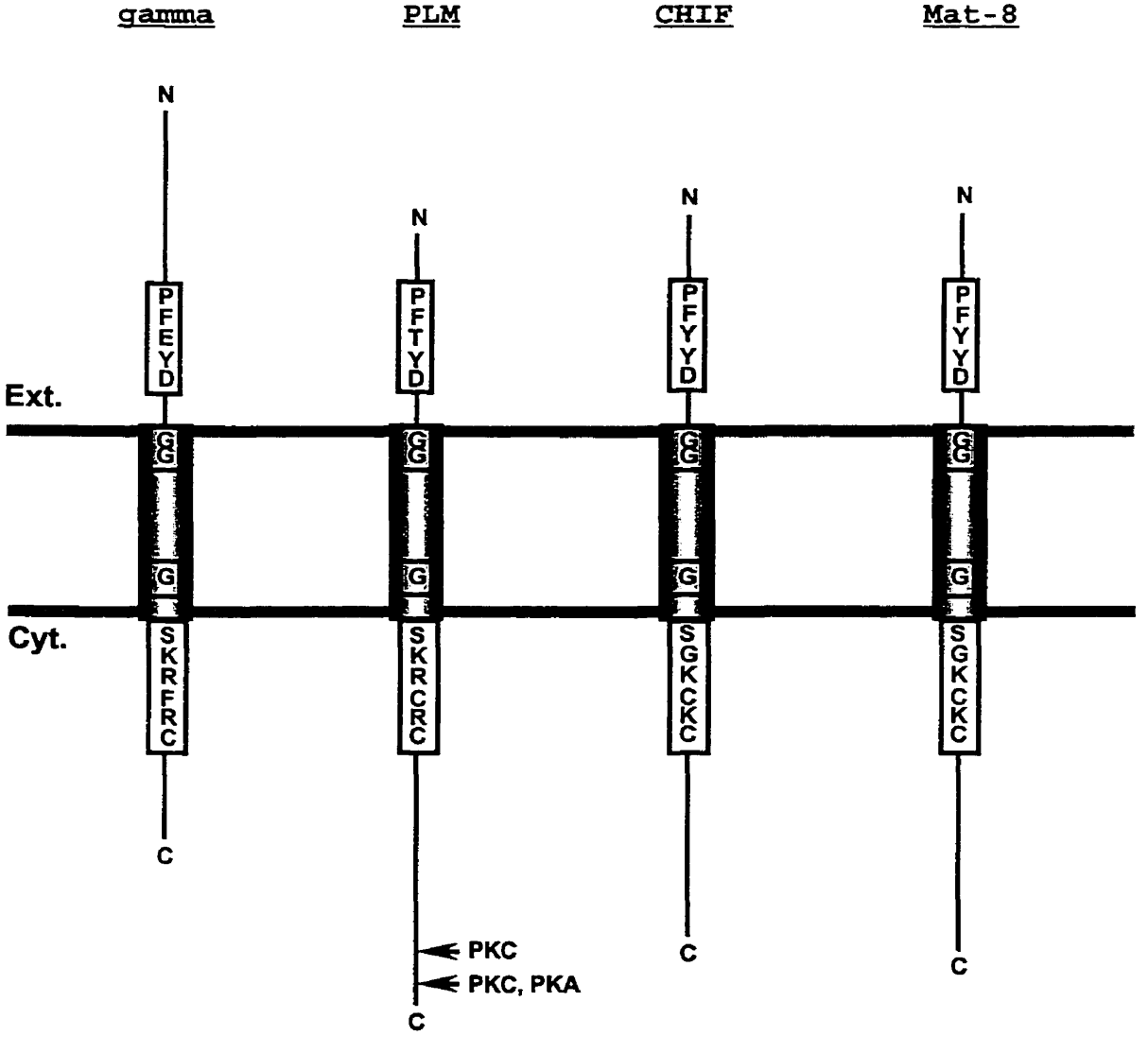
Fig. 1-3 **Comparison of the members of the γ subunit family of proteins.**

A) Amino acid sequences of rat γ subunit ("gamma"), rat phospholemman ("PLM"), rat channel inducing factor ("CHIF") and mouse 8 kDa mammary tumor-associated protein ("Mat-8"). For PLM, CHIF and Mat-8, the putative cleaved amino-terminal signal peptide is not shown (see text and ref. 381), while for gamma, the recently revised sequence is shown (see Chapter 5 and ref. 338). ▮, identical residues, ▩, conserved residues. B) Topology of gamma and putative topologies of PLM, CHIF and Mat-8. Conserved domains as well as PKC and PKA phosphorylation sites for PLM are indicated.

A


gamma MTELSANHGGSIKGTEN·IEIFIFIKIFIFI 40
PLM EIPQEPD·ITIHIIITAIIAI 30
CHIF NGPVDKGSIYIWISIQLIMISILCIA 31
Mat-8 NDPENKNDIYIWYIIVICIILCIC 31
gamma IFIFIGGSIHIQVNEIL 66
PLM ICIKFNQQQITGEPIEEGTFRSSIRRLSTRRR 72
CHIF IMIGICKIRRNITPSSLPEKVTPLITPGSAST 67
Mat-8 IMIGICKIKFRQIPSHRPGEGPPLITPGSAHNC 68

B


gamma
PLM
CHIF
Mat-8
N
PFEYD
PFTYD
PFYYD
PFYYD
Ext.
GG
G
Cyt.
SKRFRC
SKRCRC
SGKCKC
SGKCKC
C
PKC
PKC, PKA

Comparatively little is known about the last two members of the γ subunit family of proteins, CHIF and Mat-8. CHIF was discovered by screening a cDNA library of aldosterone-induced genes in rat colon (19). CHIF is also present in the kidney, but in this tissue, its expression is regulated by K^+ intake, and is aldosterone-independent (509). This finding indicates that CHIF may have a role in K^+ balance. Mat-8, on the other hand, was cloned from mouse mammary tumor cells as a neu- and ras-induced gene (347). Contrary to PLM, neither CHIF nor Mat-8 contain a known consensus sequence for PKA phosphorylation, and the cytoplasmic serines and threonines in these proteins, with the possible exception of the conserved S_{47} (using the numbering for the rat γ subunit), lack the nearby positive charges considered necessary for PKC phosphorylation (271).

As mentioned above, functional studies on these γ subunit-like proteins may yield valuable information on the role of γ in regulating cation transport. PLM, CHIF and Mat-8 have all been expressed in *Xenopus* oocytes and similar to results obtained with the γ subunit, have been found to induce ion channel activity in this system. Specifically, CHIF has been shown to induce K^+ fluxes (19), consistent with its putative role in K^+ homeostasis (509), Mat-8 induces chloride conductance (348) and phospholemman appears to have a broad substrate specificity as evidenced by its apparent permissiveness for cations, anions and zwitterions (283). Mutational studies on phospholemman have shown that residues in the transmembrane domain (346) and C-terminus (98) are important for the channel forming ability of this peptide. Overall, the available data indicate that members of the γ subunit family of proteins can induce or form ion channels in *Xenopus* oocytes and, in the case of phospholemman, in lipid bilayers (345). However, two recent observations have cast doubt on the physiological relevance of such channel-forming activity: (i) similar hyperpolarization-dependent Cl^- conductances were observed in *Xenopus* oocytes individually injected with the cRNA for a variety of structurally unrelated small membrane proteins including phospholemman, and (ii) hyperpolarizing pulses, albeit of greater magnitude, induced similar currents in uninjected oocytes (453). It may well be that the ion channel properties of small transmembrane proteins are non-specific, and that γ subunit-like proteins have other roles in regulating ion transport.

1.3.6 Circulating endogenous inhibitors

Although cardiac glycosides isolated from plants such as digoxin and ouabain, or amphibian skin (bufodienolides) have been used for several centuries to treat cardiac insufficiency, their molecular target, the Na,K-ATPase, has only been known for a few decades. Furthermore, the finding that endogenous cardiac glycosides (ECG) exist in animals, and, indeed, may have a physiological role, is even more recent. Very little is known about these substances since they seem to be present only at very low concentrations in the blood. Nevertheless, it may be a fair assumption that they function as endogenous sodium pump regulators (for reviews, see refs. 133, 134).

ECG have been detected in mammalian blood (211) and urine (199), and are believed to be synthesized by the adrenal gland (211, 289). Structurally, mammalian ECG are highly homologous to ouabain, consisting of a cholesterol core conjugated to either a lactone or pyrone ring and containing various combinations of hydroxyl, sulfate or carbohydrate groups (134). Several compounds have been identified as being ECG, including derivatives of bufodienolides (223), stereo- or regio-isomers of ouabain (539), and more recently, ouabain itself (440).

Being potent inhibitors of Na,K-ATPase activity, ECG are thought to act as natriuretic agents, that is to promote Na^+ excretion in the kidney. However, hypertension, a condition usually resulting from reduced Na^+ excretion, has been linked to increases in plasma ECG (424) and can result from long-term treatment with cardiac glycosides (532). The mechanism by which ECG mediate increased blood pressure is linked to the transmembrane equilibrium between Na^+ and Ca^{++} via the Na^+/Ca^{++} exchanger (reviewed recently in ref. 55). Thus, inhibition of the sodium pump in vascular smooth muscle cells and myocytes by ECG leads to an increase in the cytoplasmic Na^+ concentration, causing Ca^{++} to enter the cell and be sequestered in the sarcoplasmic reticulum. Increased Ca^{++} in the sarcoplasmic reticulum results in greater and sustained contractions of the vascular and heart muscle fibers, directly increasing blood pressure. Such a mechanism is also believed to be the basis for treatment of cardiac insufficiency with cardiac glycosides

(488). Presumably, the natriuretic effects of ouabain are not sufficient to counteract this phenomenon, possibly because the sodium pump of kidney, being mostly the α1 isoform, is more resistant to ouabain than the α2 isoform found in smooth muscle and heart (see previous section on isoforms).

1.3.7 Hormonal regulation

The Na,K-ATPase is subjected to both short- and long-term regulation by a variety of hormones. Short-term regulation involves either (i) direct effects on the kinetic behaviour of the enzyme, or (ii) translocation of sodium pumps to or from the plasma membrane from intracellular stores. Long-term regulatory mechanisms, on the other hand, are those that affect *de novo* Na,K-ATPase synthesis. Various hormones have been shown to alter activity, including catecholamines, insulin and steroid hormones. The regulatory role of many of these hormones, as well as the known cellular mechanisms by which this regulation is achieved, are described below.

1.3.7.a Catecholamines

Many catecholamines have been shown to regulate Na,K-ATPase activity. The two best-studied are norepinephrine and dopamine, which often act antagonistically as illustrated by their distinct roles in regulating salt-reabsorption in the kidney (for reviews, see refs. 11, 332).

Dopamine is a natriuretic factor synthesized in the kidney proximal tubule that acts in both paracrine and autocrine fashion (for reviews, see refs. 9, 297). Dopamine was first shown to be an inhibitor of Na,K-ATPase activity in the kidney proximal convoluted tubule (PCT; ref. 10), but similar effects have since been observed in other parts of the kidney, namely the medullary thick ascending limb (mTAL; ref. 12) and cortical collecting duct (CCD; ref. 433), as well as in cultured Maden-Darby canine kidney (MDCK) cells (446), neurons (43), arteries (413), retinal cells (458), aortic smooth muscle (412), small intestine (502) and lung (24). The overall consensus is that dopamine inhibits the Na,K-ATPase and that, in the kidney, this represents a physiologically

important mechanism for regulating salt reabsorption during high salt intake (see for examples refs. 21, 42, 361). Illustrating this point is the observation that the mechanisms of dopamine action are compromised in both old (260, 502) and hypertensive (96, 208, 233, 259, 361, 362) rats.

The cellular mechanisms of dopamine-dependent inhibition of Na,K-ATPase appear to be both age- and cell-specific (172). In the kidney, inhibition of sodium pumps in proximal segments of the nephron (for example, in the PCT) are mediated through both types of dopamine receptors, DA_1 and DA_2, and involve G-protein-linked, PKC-dependent pathways (10, 38, 40, 41, 175, 258, 432), while in distal segments (mTAL and CCD) and cultured cells of similar origin (MDCK), mainly DA_1 receptors and PKA pathways are involved (12, 432, 433, 484). However, this is probably an oversimplification, as PKA-mediated pathways appear to be necessary for modulation of the enzyme in the PCT (38), and PKC-mediated inhibition has been observed in MDCK cells (445, 446). A recent study has shed some light on the subject by showing that PKC-mediated pathways may be involved in short-term responses to dopamine inhibition, while PKA may have a role in long-term responses (397). Throughout the nephron, phospholipase A_2-activated elements, specifically arachidonic acid and its metabolites, also have a role in dopamine-mediated inhibition (233, 433, 435). The recent observation that dopamine inhibits the ouabain-sensitive component (α3 isoform), but not the relatively ouabain-resistant component (α1 isoform), of rat rod cells (458) has also raised the possibility that dopamine may act in an isoform-dependent fashion in some systems.

Many of the mechanistic details of regulation of the Na,K-ATPase by protein kinases will be discussed below, but two aspects particular to regulation by dopamine should be mentioned here. First, it was suggested by Baines *et al.* (21), and recently demonstrated by Chibalin *et al.* (101), that dopamine-activated PKC signaling pathways result in endocytosis of pumps, and that direct phosphorylation of the Na,K-ATPase at a specific serine residue (Ser^{18} of the rat enzyme, a putative PKC phosphorylation site) is involved (102). Second, the PKA-activated pathway of dopamine inhibition seems to involve phosphorylation of both the sodium pump and of so-called dopamine and cAMP-

regulated phosphoprotein (DARPP-32), the latter being an inhibitor of protein phosphatase 1 (PP1) (12, 171). In combination, the two mechanisms help keep the enzyme in an inactive phosphorylated state. Interestingly, Meister and co-workers (333) noted that DARPP-32 expression in the kidney seems to correlate with DA_1 receptor expression, which mediates cAMP-activated pathways (38, 484).

Despite the present consensus that dopamine is a specific inhibitor of the Na,K-ATPase, two recent reports have challenged this notion by showing that DA_2 agonists coupled to a pertussis toxin-sensitive G-protein can stimulate Na,K-ATPase activity through a decrease in cellular cAMP levels (232, 527).

In addition to dopamine, many other catecholamines have marked effects on Na,K-ATPase activity. In particular, adrenergic agents such as norepinephrine have been found to specifically stimulate sodium pump activity (for examples, see refs. 1, 15, 25, 94, 128, 209, 226, 256, 475, 510). The physiological role of catecholamines in stimulating Na,K-ATPase activity is probably tissue-specific. For example, epinephrine seems to be involved in stimulating K^+ uptake by skeletal muscle after exercise-induced hyperkalaemia (reviewed in refs. 109, 303), while norepinephrine, acting as a dopamine antagonist, appears to have a role in Na^+ reabsorption in the nephron (reviewed in refs. 11, 332). In addition, several catecholamines, including norepinephrine, act as neurotransmitters in the central nervous system, and their likely role in stimulating Na,K-ATPase in neural tissue is for reestablishing the electrochemical cation gradient across the membranes of neurons after electrical neural impulses (reviewed in ref. 221). The observation that norepinephrine stimulates Na,K-ATPase of glial cell, but not neurons (8, 220), indicates that glial cells may be the modulators of this K^+ reabsorption. In addition to these tissue-specific regulatory roles, adrenergic catecholamines may increase the susceptibility of the sodium pump to inhibition by ethanol (257, 411), although whether this has physiological relevance is unknown.

Adrenergic catecholamines modulate Na,K-ATPase activity through two general mechanisms. The first is non-receptor mediated, and involves direct effects on the plasma

membrane or chelation of inhibitory divalent metals (113, 420, 474). The physiological relevance of this mode of regulation is unknown, but the effects seem to occur only at very high concentrations of catecholamine (474). The second pathway, more likely to be physiologically important, is more complex, and involves stimulation via α-adrenergic or β-adrenergic receptors of both PKC and PKA pathways. The role of different protein kinases in catecholamine stimulation of Na,K-ATPase activity appears to be tissue-specific. Thus, catecholamine-dependent increases in cAMP levels, and therefore, stimulation of PKA, have been shown to activate Na,K-ATPase of brown adipose tissue (226), ventricular myocytes (179), kidney cortex (188), smooth muscle of the stomach (344) and arteries (512), skeletal muscle (301), macrophages (132), and lung (24), while PKC-mediated pathways appear to be responsible for sodium pump stimulation in hepatocytes (315), ventricular myocytes (510), and skeletal muscle (301). Regulation can be mediated through α-adrenergic receptors (15, 510), β adrenergic receptors (1, 256), or both (226, 475). Generally, β-adrenergic stimulation appears to be associated with activation of PKA pathways, while α-adrenergic agents stimulate PKC-dependent effects. Paradoxically, the β-adrenergic receptor agonist isoproterenol stimulates Na,K-ATPase activity in most tissues, but inhibits it in kidney medulla (188), brain (161) and COS-7 cells (99). These contradictory results have hindered a detailed understanding of adrenergic catecholamine regulation, but some progress has been made recently by Bertorello and co-workers (46) who showed that in lung alveolar cells, isoproterenol increases the number of sodium pumps at the plasma membrane through a PKA-mediated mechanism involving the cytoskeleton, but not direct phosphorylation of the pump. Isoproterenol has, however, been shown to mediate direct phosphorylation of the sodium pump, either at a PKA site of the rat enzyme transfected into COS cells (99), or at a PKC site of the brain enzyme (161). Unexpectedly, both effects appear to be mediated through PKA activation.

In the kidney proximal tubules, stimulation of the sodium pump by α-adrenergic agents has been shown to involve protein phosphatase 2B, a Ca^{++}- and calmodulin-dependent phosphatase also called calcineurin. For example, an inhibitor of calcineurin,

FK506, blocks oxymetazoline-dependent stimulation of the pump, while a calcium ionophore, A-23187, mimics it (15). Because norepinephrine's actions in the kidney appear to counter the inhibitory effects of dopamine, it has been suggested that the sodium pump is regulated in this organ by the antagonizing actions of calcineurin, which would serve to keep the pump in an active, dephosphorylated state, and protein kinases, which would keep the enzyme in an inactive, phosphorylated form (11, 14, 332).

While it is clear that catecholamines have highly specific effects on the Na,K-ATPase activity in most tissues and cells, there exists much controversy regarding the role of specific signalling pathways in catecholamine regulation of the sodium pump. Illustrating this point is the recent report showing that both adrenergic (α and β) as well as dopaminergic (DA_1) receptors transfected into COS-7 cells are linked to PKA-activated pathways (29). It is unclear how receptors that activate similar signalling mechanisms can mediate opposite effects.

1.3.7.b Insulin

Insulin is a major metabolic hormone that regulates glycolytic storage and plays an important role in K^+ homeostasis. In particular, increased uptake of K^+ by various tissues has long been a known effect of insulin, and this effect has been ascribed mainly to stimulation of the Na,K-ATPase (reviewed recently in ref. 479). The effects of insulin in the cell are mediated by its binding to the insulin receptor, and subsequent activation of a variety of intracellular signalling processes. The effectors of the short- and long-term effects of insulin on the Na,K-ATPase are numerous, and the main ones are highlighted below.

The earliest described short-term effect of insulin on the sodium pump involves translocation of sodium pumps to the cell surface from an intracellular storage compartment. This was first suggested and subsequently demonstrated in frog skeletal muscle (202, 370), and is thought to be the main mechanism of pump stimulation in skeletal muscle (reviewed in refs. 148, 479). Insulin-dependent increases in surface pump expression are independent of amiloride (184) and cycloheximide (202) and are thus not

secondary to changes in cytoplasmic Na^+ concentration or protein synthesis, respectively. Experiments on rat skeletal muscle have shown that the effect of insulin on cell-surface expression of pumps is specific to (i) the $\alpha 2$ and $\beta 1$ isoforms, with increases in $\alpha 1$ and $\beta 2$ not detected (231, 322), and (ii) oxidative slow-twitch muscles rather than glycolytic fast-twitch muscles (293).

Short-term insulin-dependent sodium pump stimulation can also involve increases in the cytoplasmic Na^+ concentration which, as described previously, can result in several-fold stimulation of the sodium pump under normal physiological conditions. For example, insulin mediates stimulation of the sodium pump secondary to an influx of Na^+ via stimulation of the $Na^+/K^+/Cl^-$ co-transporter or through Na^+ channels in adipocytes (81, 430), or the Na^+/H^+ exchanger in hepatocytes (314).

Another route by which insulin can upregulate Na,K-ATPase activity in the short term has been observed in the kidney. Experiments on the Na,K-ATPase of kidney cortical tubules have shown that insulin appears to increase the apparent affinity of the enzyme for Na^+ (152, 154). As with increases in Na^+ concentration, this can result in stimulation of the sodium pump in normally low Na^+ cells.

The molecular mechanisms by which insulin regulates Na,K-ATPase activity on a short-term basis are mostly unknown. It has been shown that PKC may have a role in the insulin-mediated activation of Na,K-ATPase in cultured rat skeletal muscle cells (428). More recently, Sweeney and Klip (479, 480) have shown that inhibition of specific kinases, namely (i) the phosphotidylinositol-3 kinase, (ii) a specific isoform of PKC (PKC-ζ) and (iii) p38MAP kinase, all abrogate the insulin effect on Na,K-ATPase activity in 3T3-L1 fibroblasts. In addition to their independent cellular roles, signalling cascades effected by these kinases converge on the phospholipase A_2 pathway, indicating that regulation of the Na,K-ATPase by insulin may involve arachidonic acid and its metabolites (see below).

In addition to the aforementioned short-term mechanisms of regulation, insulin also has long-term effects on the Na,K-ATPase. This type of regulation is especially

relevant to diabetes, where insulin function is impaired. Thus, isoform-specific alterations in the levels of Na,K-ATPase expression have been observed in streptozotocin-induced diabetic rats, and diabetic patients frequently suffer from hypertension due presumably to decreased sodium pump activities in their vasculature (reviewed in ref. 479). In addition, long-term exposure to insulin has been shown to upregulate de novo pump synthesis. Specifically, $\alpha 2$ but not $\alpha 1$ mRNA is upregulated by insulin in 3T3-L1 fibroblasts and vascular smooth muscle cells (425, 489). In addition, insulin-dependent increases in $\alpha 1$ protein have been shown in cultured rat astrocytes (329). The mechanisms for long-term upregulation of the sodium pump by insulin are unknown.

1.3.7.c Corticosteroids

Steroid hormones, in particular corticosteroids, have specific long- and short-term regulatory effects on the Na,K-ATPase. Long-term corticosteroid effects are generally mediated by changes in mRNA/protein synthesis induced by direct interactions of receptor/corticosteroid complexes with nuclear DNA. While many types of corticosteroids have been shown to mediate regulation of the Na,K-ATPase (reviewed in ref. 500), the most widely studied are the mineralocorticoid aldosterone and the glucocorticoid dexamethazone.

Aldosterone and dexamethazone are synthesized in and released by the adrenal cortex. Aldosterone in particular has long been known to have an important role in Na^+ and K^+ transport in epithelial tissues such as the kidney, and its physiological role is thought to be in long-term adaptation to decreases in Na^+ or increases in K^+ intake (reviewed in refs. 71, 372). It has been shown that the main effect of aldosterone and dexamethazone on the Na,K-ATPase involves increases in the long-term expression of sodium pumps. This effect is widespread and has been observed in toad bladder (186), and in many mammalian tissues including kidney (515) and kidney-derived cell-lines (447, 501, 516), colon (176), skeletal muscle (135), brain (201), heart (410), inner ear (398), cultured liver cells (48), vascular smooth muscle cells (366), and cultured cardiocytes (235). Experiments have shown that both steroid hormones can increase

mRNA expression of the α and β subunit genes: aldosterone increases sodium pump mRNA expression via mineralocorticoid (type I) receptors in toad bladder (185), and mammalian kidney (516) and hyppocampus (149), while dexamethazone, presumably bound to glucocorticoid (type II) receptors, has similar effects in colon (176, 511), skeletal muscle (135) and cultured liver cells (48). It has been shown that corticosteroid/receptor complexes mediate mRNA synthesis by interacting with regulatory elements 5' of both the α1 (366) and β1 (127) subunit genes. In addition, there is evidence that cAMP-inducible factors have a role in modulating aldosterone-dependent increases in both α and β subunit mRNA (4, 517). Corticosteroid-mediated increases in protein synthesis of sodium pumps may be dependent on changes in cytoplasmic Na^+ concentrations, as illustrated by abrogation of the effects in the presence of blockers of Na^+ transport (217, 235, 353), and may be facilitated by the thyroid hormone triiodothyronine (T3) in mammals (519), but not in amphibians (186). Interestingly, long-term stimulation of the sodium pump by aldosterone is abrogated by inhibitors of the protein phosphatase calcineurin in cultured *Xenopus laevis* kidney (A6) cells (422; see also Section 1.3.8.d on phosphatases below).

Recent experiments have shown that long-term upregulation of Na,K-ATPase by corticosteroids can be isoform-specific. Oguchi and co-workers first showed that the α1 isoform, but not the α2 and α3 isoforms, is upregulated by aldosterone in cultured vascular smooth muscle cells (366). However, α3 and α2 seem to be the prime targets for aldosterone-mediated regulation in the brain (149, 201) and heart (410), respectively.

While the classical effects of aldosterone on the Na,K-ATPase are on long-term expression of the enzyme as described above, this mineralocorticoid has also been shown to have specific short-term effects on Na,K-ATPase activity. These may be mediated by specific membrane-associated receptors, rather than the well-known soluble mineralocorticoid receptors (513). Specifically, two distinct types of aldosterone-mediated short-term effects have been described. The first type (reviewed in ref. 514) is dependent upon increases in cytoplasmic Na^+ concentration, since it is inhibited by amiloride (382, 395, 414, 447). The mechanism is thought to involve an increase in

membrane permeability to Na^+ leading to a higher cytoplasmic Na^+ concentration, a signal for translocation of pumps to the plasma membrane (69, 447). This mode of regulation does not involve synthesis of new protein, as it is not sensitive to either actinomycin D or cycloheximide, inhibitors of nucleic acid and protein synthesis, respectively (69, 447). A second type of short-term aldosterone-mediated upregulation of Na,K-ATPase has been observed in the rat cortical collecting tubule (23, 174) and A6 cells (36, 391). It is not inhibited by amiloride, nystatin, amphotericin B, or by incubation in the absence of extracellular Na^+, and thus is not dependent on increases in cytoplasmic Na^+ concentration. This type of modulation is sensitive to actinomycin D and cycloheximide and is partly stimulated by the hormone T3 (23, 35, 174, 391). The increase in activity may be secondary to changes in the number of plasma membrane sodium pumps (391), or to an increase in the intrinsic affinity of the enzyme for Na^+ (35). Recent evidence suggests that the Na^+-independent aldosterone-induced increase in Na,K-ATPase activity is isoform-specific since $\alpha 1$, but not $\alpha 2$ pumps transfected into A6 cells, were affected (396).

1.3.7.d Other hormones

In addition to those listed above, the Na,K-ATPase is known to be regulated by several other hormones and agents. These include: (i) thyroid hormone which appears to play a permissive role in the aldosterone-mediated stimulation of sodium pump synthesis (519); (ii) parathyroid hormone which inhibits Na,K-ATPase through PKC- and phospholipase A_2 (PLA_2) -mediated mechanisms (432); (iii) vasopressin which inhibits the sodium pump via cAMP/PKA pathways (170, 521); (iv) angiotensin II which stimulates sodium pump activity by inhibiting cAMP production (47); (v) atrial natriuretic peptide, which inhibits the pump via PKG-dependent mechanisms (32, 436); and (vi) the cytokine interleukin-1 (534), as well as (vii) endothelin (535), both of which appear to inhibit Na,K-ATPase activity through increased synthesis of prostaglandins.

1.3.8 Mechanisms of hormone action

Most of the hormones that regulate the Na,K-ATPase do so through signalling

mechanisms that modulate the activities of a group of protein kinases, phospholipases and phosphatases. The interplay between the main effectors of regulation of the sodium pump (protein kinases, protein phosphatases and PLA_2) and their effects on the Na,K-ATPase are shown in Fig. 1-4 and described below.

1.3.8.a PKA

cAMP-activated protein kinase, or PKA, is activated by the intracellular accumulation of cyclic adenosine monophosphate (cAMP). The enzymes that regulate cAMP levels in the cell are adenylate cyclase which synthesizes it, and cAMP phosphodiesterase which degrades it. Therefore, signals that activate or inhibit these two enzymes affect cAMP levels and therefore PKA activation. Increases in cAMP concentration can be effected either by incubation with various hormones (see previous sections), cAMP or cAMP analogs (such as Br-cAMP or dibutyryl-cAMP), stimulators of adenylate cyclase (such as forskolin) or inhibitors of phosphodiesterase (such as IBMX). Effects of cAMP levels on Na,K-ATPase activity has been observed in various tissues, and the nature of the effect varies in a tissue-specific manner as shown in Table 1-1. In addition to tissue-specific effects, there is evidence that PKA affects the Na,K-ATPase in a species-dependent manner. For example, sodium pump activity of salivary glands is stimulated in the dog (280), but unchanged in the rat (325), following incubation with cAMP.

The mechanisms by which PKA alters Na,K-ATPase activity are varied and complex, and have only recently begun to be investigated. The most straightforward effect of PKA is through direct phosphorylation of the sodium pump, which is suggested to be the mechanism of action of enzyme inhibition by β-adrenergic agents, such as isoproterenol (see for example ref. 99; see also section on catecholamine regulation). Bertorello *et al.* (45) first showed that the shark rectal gland and rat kidney enzymes are phosphorylated by PKA *in vitro*, with 1 mole of phosphate incorporated per mole of enzyme. Similar results were obtained with the enzymes of duck salt gland, *Bufo marinus* and *Xenopus laevis* (104). It was later shown that PKA phosphorylates the pump *in vivo*, and that the site of PKA phosphorylation is at Ser^{943} (Ser^{938} without counting the post-translationally cleaved N-terminus) in the enzyme of rat (160) and *Bufo marinus* (30).

Fig. 1-4 **Summary of the major mechanisms of hormonal regulation of the Na,K-ATPase.**

The main effectors of hormonal regulation of the sodium pump and their interactions are shown. The scheme is summarized from published reports of the various effects in different tissues, as described in the text. Activation is represented by ↓, and inhibition, by ⊥. Abbreviations are given in the text and on p. xiv.

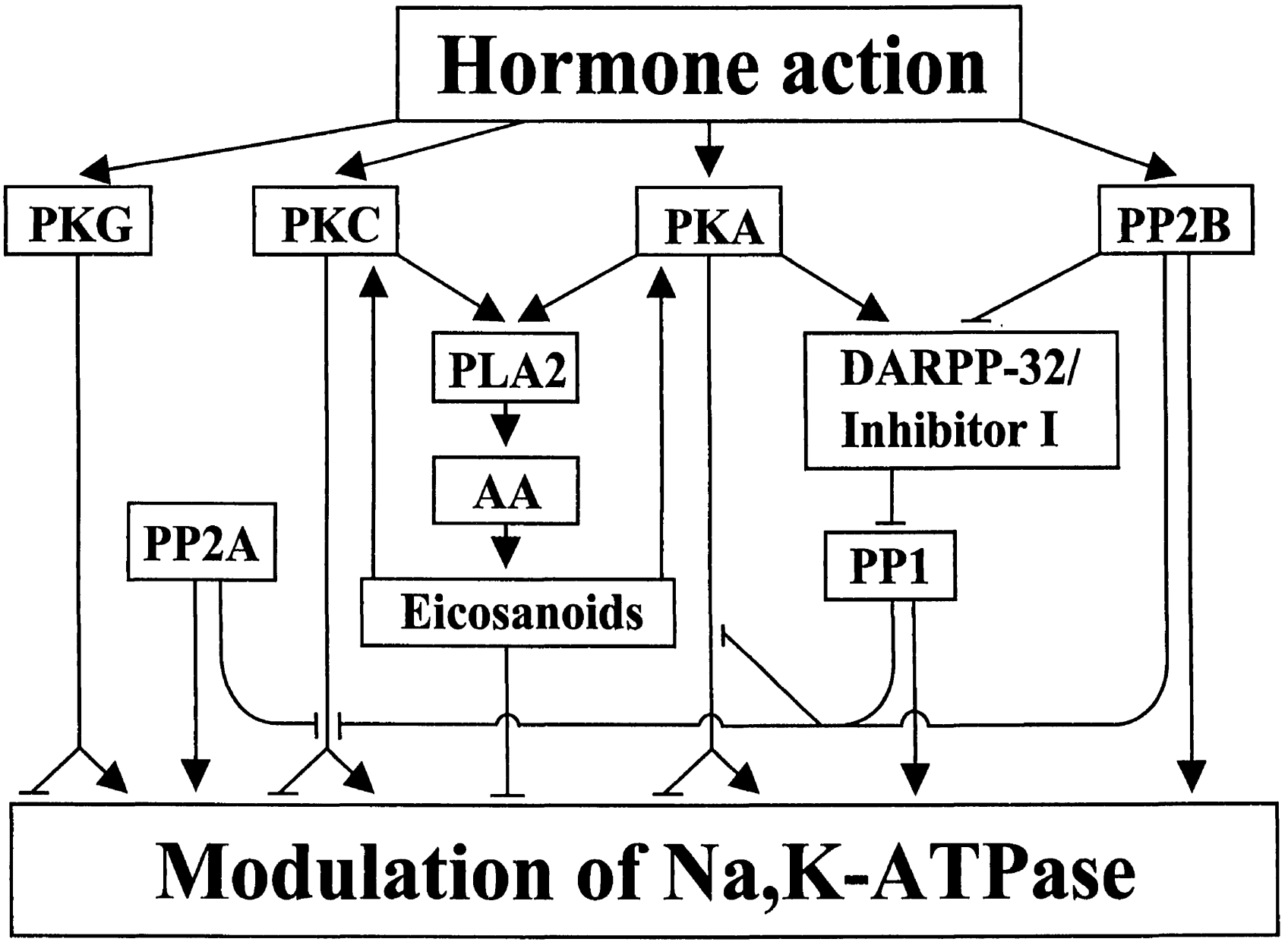

Hormone action
PKG
PKC
PKA
PP2B
PLA2
AA
Eicosanoids
DARPP-32/
Inhibitor I
PP1
PP2A
Modulation of Na,K-ATPase

Table 1-1

Summary of Na,K-ATPase regulation by PKA and PKC.

Reports showing effects of agonists or antagonists of protein kinases on the Na,K-ATPase in various tissues and cells are listed in chronological order. For effects of kinase inhibitors, results are shown in brackets. "Effect" indicates activation (↑) or inhibition (↓) of ^{3}H-ouabain binding, or strophanthidin/ouabain-sensitive ATPase activity, $^{86}Rb^{+}$ or $^{22}Na^{+}$ transport, PNPPase activity, current, or oxygen consumption. In cases where several studies have led to the same conclusion, only the first is cited. Abbreviations are given on p. xiii.

Tissue/cell	Effectors	Effect	Ref.
PKA			
brown adipose tissue (rat)	cAMP; adrenergic agents	↑	226
liver (rat)	(Chlorpropamide; phenformin)	(↑)	311
skin (frog)	cAMP; oxytocin	↑	2
reconstituted renal enzyme (hum.)	cAMP	↓	77
thyroid (guinea pig)	cAMP	↑	236
colon (rat)	cAMP; bisacodyl	↓	442
tail artery (rat and pig)	cAMP; isoproterenol	↑	512
Swiss 3T3 cells	Br-cAMP	↑	384
brain (rat)	cAMP; PKA	↓	304
sperm (hamster)	cAMP, PKA	↓	349
kidney CCT, cTAL (rabbit)	db-cAMP; isoproterenol; vasopressin	↓	521
kidney medulla (rat)	db-cAMP; forskolin; IBMX; isoproterenol	↓	188
kidney cortex (rat)		↑	
macrophage (mouse)	cAMP; IBMX	↑	132
hepatocytes (rats)	db-cAMP	↑	75
rectal gland (shark)	cAMP	↑	327
urethral smooth muscle (g. pig)	cAMP; PGE; forskolin; IBMX	↑	499
diaphragm muscle fibers (rat)	db-cAMP; theophylline; aminophylline	↑	125
retinal pigment epithelium (frog)	cAMP	↑	229
submandibular gland (dog)	db-cAMP	↑	280
pancreatic islets (rat)	db-cAMP; theophylline; caffeine	↓	493
rectal gland (shark)	purified PKA	↓	45
kidney cortex (rat)			
ciliary epithelium (rabbit)	db-cAMP	↓	123
rectal gland (shark)	db-cAMP; theophylline	↑	295
MDCK cells	db-cAMP; PGE	↑	487
kidney CCD (rat)	db-cAMP; dopamine, forskolin and others	↓	432
sciatic nerves (rat)	db-cAMP; cilostazol; iloprost	↓	455
sensory neurons (leech)	db-cAMP; forskolin; IBMX	↓	93
kidney PCT (rat)	db-cAMP; forskolin	↑	78
skeletal muscle (rat)	Br-cAMP; isoproterenol	↑	300
transfected COS cells (rat α1)	Forskolin; IBMX	↓	160
motor nerve (rat)	db-cAMP; aminophylline; PGE	↑	528

Tissue/cell	Effectors	Effect	Ref.
kidney PCT (rabbit)	db-cAMP	↑	27
kidney PCT (rat)	db-cAMP; Br-cAMP; forskolin; IBMX	↑	88
rectal gland (shark)	PKA	↑	117
transfected COD cells (rat α1)	isoproterenol; forskolin; IBMX	↓	99
transfected HeLa (rat α1,α2,α3)	forskolin; IBMX	↓	359
aortic smooth muscle cells	Br-cAMP; forskolin; IBMX; isoproterenol	↓	73
infected Sf-9 cells (rat α1, α2)	db-cAMP	↓	54
infected Sf-9 cells (rat α3)		↑	
RN22 Schwann cells	Br-cAMP; forskolin; cholera toxin	↑	473
skeletal muscle (squirrel)	cAMP	↑	317
<u>PKC</u>			
nerve (diabetic rat)	PMA; dioctanoylglycerol	↑	200
pancreatic acinar cells (guinea pig)	TPA	↑	225
erythrocytes (hypertensive human)	TPA	↑	407
brain (rat)	(ET-18-OCH3; BM 41.440)	(↓)	369
tracheal smooth muscle (rabbit)	DPB	↑	441
kidney PCT	OAG; PDB	↓	39
nerve (diabetic rabbit)	DOG; PMA	↑	290
oocytes (*Xenopus laevis*)	PMA	↓	497
ileal smooth muscle (guinea pig)	PDB, PDA, PMA	↑	431
aorta (rabbit)	PDB; endothelin	↑	207
rectal gland (shark)	purified PKC	↓	45
kidney cortex (rat)			
sciatic nerve (diabetic mouse)	(H7)	(↓)	219
L 1210 cells (mouse leukemia)	PMA	↑	272
kidney PCT (rat)	OAG (short-term)	↑	44
	OAG (long-term)	↓	
MDCK cells (dog)	OAG; DOG; PMA	↓	446
ciliary epithelium (rabbit)	PMA	↓	354
OK cells (opossum)	PDB	↓	336
MDCK (dog)	PMA; carbachol	↓	181
cultured ciliary epithelial (human)	PDB	↑	340
skeletal muscle cells (rat)	PMA; insulin	↑	428
brain (rat)	PDB; serotonin	↓	162

Tissue/cell	Effectors	Effect	Ref.
kidney PCT (rat)	PDB (+oxygen)	↑	151
	PDB (-oxygen)	↓	
vascular smooth muscle cells (rat)	PMA	↓	523
kidney PCT (rat)	PDB; DOG; dopamine; PTH	↓	371
cerebellar neurons (rat)	PMA	↓	320
arterial endothelial cells (cow)	(calphostin; staurosporine; H7)	(↑)	95
transfected oocytes (toad enzyme)	PMA	↓	34
transfected HeLa (rat α1,α2,α3)	PMA	↓	359
transfected OK cells (rat α1)	PMA	↑	388
transfected COS cells (rat α1)	20-HETE	↓	363
mucociliary cells (frog)	TPA; DOG	↓	187
aortic smooth muscle cells	PDB; PMA; AVP	↑	73
transf. *Xenopus* oocytes (rat α1)	PMA (endogenous PKC activation)	↓	496
	PKC (rat)	↓	
Xenopus oocytes (endogenous)	PMA (endogenous PKC activation)	↓	
	PKC (rat)	↑	
ciliate epithelial cells (rabbit)	PDB	↑	124
infected Sf-9 cells (rat α1, α2, α3)	PMA	↓	54
kidney cortex (rat)	PDB	↓	299
aorta (rat)	PDB	↑	288

Furthermore, Fisone and co-workers also showed that phosphorylation of Ser^{943} results in inhibition of enzyme activity, an effect abrogated by mutation of the serine to alanine. Similar experiments by Andersson *et al.* (7) showing that PKA-induced phosphorylation and inhibition of activity in rat α1-transfected COS-7 cells is not associated with internalization of the pumps have led these authors to suggest direct effects on the catalytic turnover of the enzyme. However, the role of direct phosphorylation in regulating sodium pump activity is not straightforward. Recent experiments have shown that phosphorylation of Ser^{943} plays a permissive role in allowing phosphorylation of the pump by PKC at Ser^{23} (ref. 100; see also Section 1.3.8.b). Consistent with a dependence of PKA-mediated phosphorylation on enzyme conformation, Feschenko and co-workers (156, 159), using rat enzyme and purified PKA, found that phosphorylation of Ser^{943} occurs mainly in the presence of stabilizers of the E_1 enzyme conformation.

Direct phosphorylation of the Na,K-ATPase may also be involved in the well-documented PKA-mediated stimulation of the sodium pump in renal proximal tubules, as evidenced by a correlation between enzyme phosphorylation and ouabain-sensitive $^{86}Rb^+$ uptake (88). In this tissue, however, activation appears to be secondary to an increase in plasma membrane pumps (89). Perhaps related to this are the observed PKA-induced increases in plasma membrane pumps of MDCK (487) and Schwann cells (473).

While direct phosphorylation of the Na,K-ATPase by PKA is an attractive simple mechanism for PKA-mediated regulation of the enzyme, and appears to apply to at least some systems, other more complex mechanisms have been observed. Lingham and Sen (304) were the first to suggest that PKA required the presence of an intermediate protein to mediate its effects on the sodium pump in rat brain. More recently, Satoh *et al.* (434) showed that PKA inhibits Na,K-ATPase activity in renal collecting duct by activating the PLA_2 pathway, specifically by increasing synthesis of eicosanoids which presumably downregulate Na,K-ATPase activity. In other systems, PKA appears to activate a protein phosphatase inhibitor, which in turn alters sodium pump activity (ref. 12; see also Section 1.3.8.d on protein phosphatases). In addition to the foregoing, the cytoskeletal protein

actin has been postulated to have a role in mediating PKA-regulation of the rat kidney sodium pump. Cantiello (83) showed that phosphorylation of monomeric actin by PKA prevented the actin-mediated stimulation of the sodium pump, while phosphorylation of polymeric actin promoted it. Finally, in some cases, PKA does not regulate the sodium pump directly, but rather alters the function of other Na^+ transporters, leading to changes in cytoplasmic Na^+ concentration, which in turn alter Na,K-ATPase activity (229, 473).

In recent years, isoform-specific effects of PKA have been observed. Nestor *et al.* (359) first showed that the PKA activators forskolin and IBMX effect a significant inhibition of the rat $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoforms in transfected HeLa cells. More recently, Blanco and co-workers (54), using Sf-9 cells infected with the individual rat isoforms, have shown that treatment with dibutyryl-cAMP (db-cAMP) inhibits $\alpha 1$ and $\alpha 2$ pumps, but activates $\alpha 3$ pumps, and results in direct phosphorylation of all three isoforms.

1.3.8.b PKC

The cascade that results in activation of calcium-dependent protein kinase (PKC) is usually initiated by activation of the membrane-bound phospholipase C which cleaves phospholipids into two components, phosphotidylinositol trisphosphate, which in turn increases cytosolic Ca^{++}, and diacyl glycerol (DAG). DAG allows the inactive, cytoplasmic form of PKC to bind to the membrane, and increases its affinity for Ca^{++} and phospholipids, its final activators. Activated PKC is a potent regulator of many enzymes, including the Na,K-ATPase. Experimentally, increases in PKC can be achieved in the cell by incubation in the presence of phorbol esters or DAG analogs (91). As is the case with cAMP/PKA-mediated regulation of the Na,K-ATPase, and as shown in Table 1-1, the effects of PKC activation on the enzyme are varied and tissue-specific. In particular, the Table highlights discrepancies in the effects of PKC on the Na,K-ATPase of renal proximal tubules (39, 44, 87, 151, 371) and OK cells (a cell line derived from proximal tubules of opossum kidney) (102, 103, 336, 388), where PKC has been shown to mediate either stimulation or activation of the enzyme, as discussed below.

The question of the mechanisms of PKC regulation of the Na,K-ATPase is

controversial. The aforementioned dichotomy regarding the enzyme of proximal convoluted tubules illustrates the many contradictions present in the literature regarding PKC-mediated pump regulation. PKC-dependent activation of the Na,K-ATPase in this tissue appears to be secondary to an increase in Na^+ influx, possibly via the Na^+/H^+ exchanger (44), and seems to be an oxygen-dependent process (151). Inhibition of proximal tubule enzyme by PKC, on the other hand, is mediated by one of two mechanisms. The first involves activation of the PLA_2 pathway (371), and is discussed in Section 1.3.8.e. The second involves direct phosphorylation of the sodium pump by PKC at Ser^{23} (18 with numbering starting after the first 5 post-translationally cleaved amino acids) of the α subunit, leading to endocytosis of pumps, as determined by Chibalin *et al.* using α1-transfected OK cells (102, 103). Endocytosis secondary to direct phosphorylation of the sodium pump is also the suggested mechanism of PKC-mediated inhibition of the Na,K-ATPase in *Xenopus* oocytes (496, 497). It has recently been shown that phosphorylation of the rat enzyme at Ser^{23} requires prior phosphorylation at the PKA site, Ser^{943} (100). Taken together with the fact that PKC-mediated internalization of sodium pumps is postulated to be the mechanism for dopamine-dependent inhibition of activity in proximal tubules (102), this observation may explain the requirement of both DA_1-activated PKA- and DA_2-activated PKC-mediated pathways for the full dopamine effect in this tissue (see Section 1.3.7.a on catecholamine regulation and refs. 38, 43). The observation that PKA mediates PKC phosphorylation in nerves (72) shows that this type of mechanism may not be restricted to proximal tubules.

As discussed above, direct phosphorylation of the Na,K-ATPase by PKC is one of the mechanisms by which sodium pump activity is regulated by this kinase. Such phosphorylation was first shown *in vitro* for the duck salt gland and dog kidney enzymes (308), and subsequently for the shark rectal gland, rat kidney (45), *Bufo marinus* kidney, and *Xenopus laevis* (104) enzymes. Middleton and co-workers (336) showed that phosphorylation of the sodium pump by PKC can occur *in vivo*. Their results showed that treatment of intact OK cells with the PKC activator phorbol dibutyrate results in phosphorylation of a protein that comigrates with the α subunit of the sodium pump on

Western blots, as well as inhibition of Na,K-ATPase activity. Similar treatment of the enzyme of LLC-PK cells was without effect. Identification of the PKC-phosphorylated residue has been hampered by the presence of several putative cytoplasmic PKC phosphorylation sites on the α subunit of the sodium pump (see for example ref. 158). Nevertheless, most researchers agree that PKC phosphorylation occurs primarily at the N-terminus of the catalytic subunit *in vivo*. For example, the *Bufo Marinus* enzyme is phosphorylated by PKC in intact transfected COS-7 cells mainly at Thr^{15} and Ser^{16} (Thr^{10} and Ser^{11}, numbering after the cleaved N-terminus) (30), while the mammalian enzyme is phosphorylated at low levels on Ser^{16}, and in the rat, at higher levels on serine Ser^{23} (158). Feschenko *et al.* (159) have recently examined two interesting aspects of phosphorylation of the Na,K-ATPase by PKC. Their group found that *in vitro* phosphorylation of the rat α1 enzyme by purified PKC is facilitated by agents that stabilize the E_2 conformation of the enzyme, and that the Na,K-ATPase itself can stimulate PKC autophosphorylation. The physiological consequences of these observations have yet to be determined. Although the absence of Ser^{23} in dog and pig enzyme shows that PKC may not have a major role in direct phosphorylation of the sodium pump *in vivo* in these species, as mentioned earlier, phosphorylation of Ser^{23} appears to be an important mechanism by which PKC modulates the rat kidney enzyme. Other experiments supporting this conclusion include recent studies showing that neither a Ser^{23} to alanine mutant transfected into COS cells (33), nor a deletion mutant lacking the first 31 amino acids transfected into OK cells (388), are modulated by PKC activators, even though the wild type enzyme is affected in both systems. Other experiments have shown that inhibition of the rat α1 enzyme by phosphorylation of Ser^{23} is due to a shift in the conformational equilibrium towards E_1, leading to a decreased apparent affinity for K^+ (307). In addition, direct phosphorylation of the sodium pump is the proposed mechanism of action of PKC in rat choroid plexus (162), aorta (288) and nerves (72).

The foregoing results notwithstanding, the physiological relevance of direct phosphorylation of the pump in regulating the Na,K-ATPase has recently been questioned. Thus, experiments have shown that a PKC-mediated decrease in plasma

membrane sodium pumps of A6 cells transfected with the *Bufo marinus* enzyme is not associated with phosphorylation of residues 15 and 16 (37). Consistent with this are the observations that (i) a deletion mutant of the rat α1 enzyme lacking the first 32 amino acids was recently shown to be inhibited by PKC activators to the same extent as the wild type enzyme (359), and (ii) phosphorylation of Ser^{18} by activators of PKC in a rat kidney cell line, NRK-52E, had no effect on either V_{max} or Na^+-affinity of the Na,K-ATPase (157). These experiments and others represent overwhelming evidence that direct phosphorylation of the sodium pump by PKC, at least at the N-terminus, cannot explain many of the PKC-mediated effects on the enzyme, and that other mechanisms must be involved, especially in species such as the dog and pig, where Ser^{23} is absent. Many PKC-dependent mechanisms of Na,K-ATPase regulation independent of pump phosphorylation have been observed. One involves stimulation of the sodium pump secondary to increases in cytoplasmic Na^+ via the Na^+/H^+ exchanger. Such a mechanism has been suggested to result in activation of the pump in cultured ciliary epithelial cells (340) as well as kidney proximal tubules (44). Another possible mechanism by which PKC regulates the Na,K-ATPase without directly phosphorylating it, involves stimulation by PKC of the PLA_2 pathway. As described in a later section, PLA_2 produces arachidonic acid, whose metabolites, the eicosanoids, can have highly specific effects on the sodium pump. PLA_2-mediated PKC regulation has been observed in kidney proximal tubules (371), as mentioned above, as well as in vascular smooth muscle cells (523) and pancreatic β-cells (379). In the latter case, however, both PLA_2-specific effects and PKC-mediated phosphorylation of the sodium pump were observed, suggesting that the two mechanisms may act in concert to inhibit Na,K-ATPase activity. Such a model is compatible with the observed dual mechanism of PKC-mediated pump inhibition in proximal tubules (for example, see ref. 371 and 102). Another mechanism of PKC-mediated regulation of the sodium pump has been recently described. Nemoto and co-workers (358) have shown that PKC-dependent mechanisms mediate the serum-induced increases in β1 subunit mRNA in vascular smooth muscle cells, which implies a role of PKC in long-term regulation of the sodium pump.

PKC can modulate Na,K-ATPase comprising various catalytic isoforms. Thus, PKC-dependent inhibition of the rat α1, α2 and α3 isoforms has been described in transfected HeLa cells (359), as well as Sf-9 cells (54). In the latter system, direct phosphorylation of the α isoforms was observed. Isoform-specific differences in the PKC effects were not observed, however. Conversely, PKC activation in frog mucociliary cells results in almost complete inhibition of the ouabain-sensitive isoform, without affecting the ouabain resistant isoform (187).

1.3.8.c PKG

cGMP-dependent protein kinase (PKG), a more recently discovered mediator of intracellular signalling, appears to have highly specific effects on the Na,K-ATPase. In a mechanism similar to the one involved in PKA-activation, PKG is activated by cyclic GMP (cGMP), whose cytoplasmic concentration is regulated via synthesis by guanylate cyclase and degradation by cGMP phopshodiesterase. Increases in cGMP have been shown to inhibit the Na,K-ATPase in colon (442), skeletal muscle (301), brain (402), cultured alveolar cells (205), and infected Sf-9 cells (54). Conversely, cGMP is involved in activation of the enzyme in duck salt gland (472), mammalian aorta and arteries (155), pulmonary arterial smooth muscle (486), ciliary epithelium (90), purkinje neurons (356) and NB-OK-1 cells (126). In the kidney, cGMP and PKG have been shown to inhibit (32, 482, 536) or stimulate (331, 436) the Na,K-ATPase. Although the basis for these conflicting results is unknown, the effects of cGMP/PKG appear to be antagonistic to those of cAMP/PKA, for example in ciliary epithelium (90), rat skeletal muscle (301) and hamster sperm (349). The mechanism of PKG-activation appears to involve activation of guanylate cyclase by nitric oxide (NO). For example, studies have shown that increases in NO via hormonal activation or incubation with NO donors, such as sodium nitroprusside, increase cGMP levels in cultured vascular smooth muscle cells (206), aorta and arteries (155), brain (402), renal proximal tubules (536), and alveolar cells (205). In intact cells, NO-stimulated cGMP synthesis is mediated by the neurotransmitters acetylcholine (331) and glutamate (356), as well as by atrial natriuretic peptide (32, 331, 436). Recently, isoform-specific effects of PKG on the Na,K-ATPase have been

observed. Thus, PKG modulates Na,K-ATPase activity of α3 but not α1 in Purkinje neurons (356), α1 but not α2 or α3 in brain endothelial cells (402), and α1 and α3 but not α2, in infected Sf-9 cells (54). Whether PKG regulates the pump through secondary modulators or by direct phosphorylation of the pump is unknown, although in some systems, cGMP is known to stimulate the sodium pump indirectly by increasing Na^+ influx via the Na/K/Cl co-transporter (365).

1.3.8.d Protein phosphatases

The many regulatory effects of protein kinases on the Na,K-ATPase can be reversed in many cases by protein phosphatases. Regulation of the sodium pump by the antagonistic actions of protein kinases and phosphatases has been studied extensively in the kidney and brain (reviewed in ref. 171; see also refs. 161, 299), and has also been observed in skeletal muscle (317) and ventricular myocytes (179). The major participants in protein phosphatase-dependent modulation of the Na,K-ATPase are protein phosphatases 1 and 2B (PP1 and PP2B).

The role of PP1 in countering the effects of protein kinases is believed to represent an important mechanism of pump inhibition by dopamine through the DA_1 receptor and isoproterenol via the β-adrenergic receptor. Such inhibition is mediated in part by the activation of PP1 inhibitors DARPP-32 and inhibitor-1 (I1) (see for example refs. 13, 161). Thus, it has been shown that the increase in cAMP levels mediated by dopamine or isoproterenol in kidney and brain, leads to phosphorylation of DARPP-32, which in turn becomes a potent inhibitor of PP1 (12, 161, 333). Therefore, the inhibition of Na,K-ATPase activity by stimulation of PKA in these two organs involves the synergistic effects of (i) direct phosphorylation of the enzyme by PKA and/or PKC, and (ii) inhibition of PP1, which normally counters PKA effects, by DARPP-32 and I1 (11, 161). Although DARPP-32 is involved in sodium pump regulation in most parts of the kidney and in brain, its low expression in renal PCT precludes such a role in this segment of the nephron (466). In addition to its role in regulating the kidney enzyme, inhibition of PP1 activity by okadaic acid, cyclosporin A or calyculin A has been shown to affect

Na,K-ATPase activity in ventricular myocytes (179) and both pump activity and phosphorylation level in the rat skeletal muscle cell line L6 (409).

The physiological role of PP2B, or calcineurin, in the kidney has recently been reviewed (492). It is a Ca^{++}- and calmodulin-dependent enzyme that, upon activation by norepinephrine and other α-adrenergic receptor agonists, activates the Na,K-ATPase of most segments of the nephron (294), although its main effects are on the enzyme of PCT (15). Other activators of calcineurin in the kidney include neuropeptide Y and the connecting peptide of pro-insulin, C-peptide (367). It has also been suggested that the role of calcineurin in the kidney is to counter dopamine-induced inhibition of the Na,K-ATPase, and that it does this by dephosphorylating targets of dopamine-stimulated protein kinases (11). It has been suggested that calcineurin mediates its stimulatory effects at least in part by increasing the apparent affinity of the sodium pump for Na^+ (15). In addition to its role in the kidney, calcineurin mediates ouabain-induced upregulation of surface expression of α1β1 pumps in cultured astrocytes (227), and has a role in sodium pump activation during glutamate toxicity in rat neurons (320) and in the long-term upregulation of the sodium pump by aldosterone in A6 cells (422).

Other protein phosphatases shown to be modulators of Na,K-ATPase activity include protein phosphatase 2A (PP2A), which increases pump plasma membrane expression in cortical collecting duct (68) and counters PKC-mediated inhibition of the Na,K-ATPase in Sf-9 infected cells (54), and inhibitors of which stimulate the pump in hepatocytes (314). In addition, tyrosine phosphatases also modulate Na,K-ATPase function, presumably by counteracting insulin- and epithelial growth factor-induced pump stimulation in PCT (153).

1.3.8.e Phospholipase A_2

As discussed above, the phospholipase A_2 (PLA_2) pathway of pump regulation can be activated by both PKA and PKC. Activated PLA_2 can cleave phospholipids in the membrane to generate lysophospholipids and arachidonic acid, which have both been shown to have specific effects on the Na,K-ATPase. In addition, arachidonic acid is

further metabolized in the cell by a variety of oxygenases to form eicosanoids, including prostaglandins (PG), thromboxanes (TX) and oxygenated compounds such as hydroxyeicosatetraenoic acids (HETE) and epoxyeicosatrienoic acids (EET), all of which are modulators of the Na,K-ATPase (434).

Effects of lysophospholipids and arachidonic acid as well as its metabolites on the Na,K-ATPase are generally inhibitory. Thus, addition of lysophosphatidylcholine to sarcolemmal membranes of mammalian heart caused a 50% inhibition of Na,K-ATPase activity (261). Similarly, arachidonic acid has been shown to be one of the mediators of dopamine-induced inhibition of the sodium pump in the kidney (432). Further studies using this system revealed that the effectors of arachidonic acid-mediated inhibition are its metabolites, specifically prostaglandin E (PGE) and the various products of P_{450}-dependent monooxygenase-mediated cleavage of arachidonic acid, including HETE and EET (434). In addition to effects on the renal enzyme, prostaglandins can alter Na,K-ATPase activity in other tissues (for examples, see refs. 124, 273, 385). Satoh and co-workers showed that PGE inhibits the pump by decreasing intracellular Na^+, while HETE and EET have direct effects on the sodium pump (434). The mechanisms whereby eicosanoids inhibit sodium pump activity are unknown, although their lipid nature suggests effects on membrane fluidity and/or thickness (see Section 1.3.3.a on effects of lipids) or perhaps direct interactions with hydrophobic domains of the pump.

In addition to acting directly on the sodium pump, eicosanoids have also been shown to stimulate protein kinases, resulting in modulation of the pump via mechanisms described in Sections 1.3.8.a and 1.3.8.b. For example, prostaglandins modulate cAMP levels thereby affecting sodium pump activity in several mammalian tissues and cells, including small intestine (452), smooth muscle (499), nerves (528), macrophages (76) and MDCK cells (487). Recently, a role of PKC in eicosanoid-mediated sodium pump regulation has also been observed in rat α1-transfected COS cells (363) and pancreatic β cells (379).

1.4 THESIS GOALS AND RATIONALE

The discovery of isoforms of the α and β subunits of the Na,K-ATPase has answered some of the questions regarding tissue-specific kinetic behaviour of the enzyme. Thus, the large tissue-specific difference in ouabain affinity between sodium pumps of brain and kidney (464) has been explained by the predominance in these two tissues of the α3 and α1 isoforms, respectively. However, as described above, the nature of catalytic isoforms cannot account for many of the discrepancies found in the literature regarding tissue-specific substrate-activation of the sodium pump. For example, the order of affinities for both Na^+ and K^+ appears to depend on the system used in various studies (for example, compare refs. 459, 476 with refs. 244, 351). Clearly, factors independent of the primary structure of α are important determinants of the kinetic behaviour of the Na,K-ATPase. The importance of understanding the mechanisms by which sodium pump activity is modulated is underscored in tissues where the sodium pump has a specialized role. Many hormones act on the enzyme of these tissues, but we are largely ignorant of the precise mechanisms by which these hormones modulate activity. In the case of long-term changes, increases or decreases in *de novo* pump synthesis are usually involved, but short-term regulation is often independent of changes in expression of sodium pumps. In such cases, alterations in the kinetic parameters that define Na,K-ATPase function, namely its affinity for its substrates or its catalytic turnover, are involved. While in some cases, direct phosphorylation of the enzyme appears to have a role in such modulations, other mechanisms must also be involved. The goal of this study has been to identify and characterize novel mechanisms of Na,K-ATPase modulation.

A first approach, described in Chapters 2 and 3, has been to identify functional differences between pumps of different tissues comprising identical α isoforms. By taking advantage of the large difference in ouabain sensitivities between α1 and the other isoforms, I was able to study the cation activation kinetics of the α1 and α3 isoforms of various tissues and cells, and show that there exist tissue-specific, isoform-independent differences in the affinities of the sodium pump for Na^+ and K^+. The ubiquitous nature of

the α1 isoforms has allowed me to further characterize these differences for this isoform, and to carry out a characterization of the mechanistic and structural bases for distinct tissue-specific cation activation kinetics, specifically at cytoplasmic (Na^+) activation sites.

The second approach used in this study (Chapters 4 and 5) stems from attempts at identifying the molecular nature of the modulator responsible for observed tissue-specific differences in cation activation kinetics. Using an antiserum specific for the so-called γ subunit of the Na,K-ATPase, I discovered a tissue-specific pattern of expression for the peptide. Although its tissue distribution did not correlate with previous kinetic studies, I became interested in the possible role of γ in modulating Na,K-ATPase behaviour. Inspired by the classical functional studies on the L_p antigen of low-K^+ sheep red blood cells, I first looked at effects of our γ-specific antiserum on the kinetic behaviour of the sodium pump. Combined with studies using transiently-transfected HEK cells, I have completed a thorough investigation of the modulatory role of the γ subunit.

CHAPTER 2

TISSUE-SPECIFIC VERSUS ISOFORM SPECIFIC DIFFERENCES IN CATION ACTIVATION KINETICS OF THE NA,K-ATPASE

2.1 PREFACE

This manuscript has been published in the Journal of Biological Chemistry (Therien, A.G., N.B. Nestor, W.J. Ball and R. Blostein. *J. Biol. Chem.* 271: 7104-7112, 1996.) and describes experiments aimed at reconciling the many controversies present in the literature regarding isoform-specific kinetic behaviour of the Na,K-ATPase.

The co-authors have contributed in the following manner. Dr. William J. Ball provided the isoform-specific antisera and his unpublished results. Nestor B. Nestor helped develop the immunoprecipitation protocol.

2.2 ABSTRACT

The experiments described in this paper reconcile some of the apparent differences in isoform-specific kinetics of the Na,K-ATPase reported in earlier studies. Thus, tissue-specific differences in Na^+ and K^+ activation kinetics of Na,K-ATPase activity of the same species (rat) were observed when the same isoform was assayed in different tissues or cells. In the case of $\alpha 1$, $\alpha 1$-transfected HeLa cell, rat kidney and axolemma membranes were compared. For $\alpha 3$, the ouabain-insensitive $\alpha 3$*-transfected HeLa cell (c.f. Jewell, E.A. and Lingrel, J.B. (1991) *J. Biol. Chem.* 266, 16925-16930), pineal gland and axolemma (mainly $\alpha 3$) membranes were compared. The order of apparent affinities for Na^+ of $\alpha 1$ pumps was axolemma $\approx$ rat $\alpha 1$-transfected HeLa > kidney, and for K^+, kidney $\approx$ $\alpha 1$-transfected HeLa > axolemma. For $\alpha 3$, the order of apparent affinities for Na^+ was pineal gland $\approx$ axolemma > $\alpha 3$*-transfected HeLa, and for K^+, $\alpha 3$*-transfected HeLa > axolemma $\approx$ pineal gland. In addition, the differences in apparent affinities for Na^+ of either kidney $\alpha 1$ or HeLa $\alpha 3$* as compared to the same isoform in other tissues were even greater when the K^+ concentration was increased. A kinetic analysis of the apparent affinities for Na^+ as a function of K^+ concentration indicates that isoform-specific as well as tissue-specific differences are related to the apparent affinities for both Na^+ and K^+, the latter acting as a competitive inhibitor at cytoplasmic Na^+ activation sites. Although the nature of the tissue-specific modulation of K^+/Na^+ antagonism remains unknown, an analysis of the nature of the β isoform associated with $\alpha 1$ or $\alpha 3$ using isoform-specific immunoprecipitation indicates that the presence of distinct β subunits does not account for differences of $\alpha 1$ of kidney, axolemma and HeLa, and of $\alpha 3$ of axolemma and HeLa; in both instances $\beta 1$ is the predominant β isoform present or associated with either $\alpha 1$ or $\alpha 3$. However, a kinetic difference in K^+/Na^+ antagonism due to distinct β's may apply to $\alpha 3$ of axolemma ($\alpha 3\beta 1$) and pineal gland ($\alpha 3\beta 2$).

2.3 INTRODUCTION

The sodium potassium adenosine triphosphatase (Na,K-ATPase) or sodium pump is responsible for maintaining the electrochemical gradient of Na^+ and K^+ across the plasma membrane of animal cells. It normally couples the hydrolysis of one molecule of ATP to the transport of three Na^+ ions out and 2 K^+ ions into the cell (for reviews, see refs. 7, 16, 42, 45). This cation pump is a heterodimer comprised of a catalytic α subunit ($\approx$105 kDa) and a highly glycosylated β subunit (45-55 kDa), and may (4, 33) or may not (28, 36) form larger oligomers. The α subunit contains the binding sites for Na^+, K^+, ATP and the highly specific cardiac glycoside inhibitors such as ouabain, as well as the site of phosphorylation (45). The function of the β subunit is not completely understood; it appears to be essential for the normal delivery and correct insertion of α into the plasma membrane (15) and to have some influence on the catalytic activity of α (5, 6, 17, 18, 37). A third peptide subunit known as the γ subunit (6.5 kDa) appears to exist in association with α and β, at least in certain tissues, although its role is yet to be determined (29).

In mammals, three isoforms of the α subunit (α1, α2 and α3) and two of the β subunit (β1 and β2) are known to exist (42). Isoforms of the α subunit are expressed in a tissue-specific manner: α1 is present ubiquitously, α2 is detected mainly in skeletal muscle, heart and certain neuronal cells (neurons and astrocytes), and α3, mainly in neurons (42, 43).

Earlier studies of cation activation of the Na,K-ATPase by Sweadner using rat kidney and axolemma (40) and later studies by Shyjan *et al.* using kidney, brain and pineal gland (38) indicated a higher affinity for Na^+ in preparations now known to be predominantly α3. Thus, the order of apparent affinities for Na^+ in the former study was axolemma (predominantly α3) > kidney (α1 only) and in the latter, pineal gland (predominantly α3) $\approx$ brain (a mixture of α1, α2 and α3) > kidney. In contrast, Jewell and Lingrel, using membranes isolated from HeLa cells transfected with the individual α

isoforms, reported that the order of apparent affinities for Na^+ is $\alpha 1 \approx \alpha 2^* > \alpha 3^*$, and for K+, $\alpha 3^* > \alpha 2^* \approx \alpha 1$, where $\alpha 2^*$ and $\alpha 3^*$ denote ouabain-resistant mutants of $\alpha 2$ and $\alpha 3$, respectively (19). Moreover, studies of pump-mediated K^+ (Rb^+) influx into these individual isoform-transfected cells confirmed the general conclusions drawn from the aforementioned work, except that considerably larger kinetic differences among the isoforms were observed (32). Interestingly, in experiments carried out with kidney and axolemma microsomal membranes delivered by membrane fusion into red cells, the order of apparent affinities for cytoplasmic Na^+ and K^+ resembled those of $\alpha 1$- and $\alpha 3^*$-transfected HeLa cells, respectively (32).

The aim of the experiments described in this paper was to reconcile the discordant results obtained in the foregoing studies, as well as numerous earlier reports, regarding the order of apparent affinities for Na^+ and K^+ of different tissues and/or isoforms (for review, see 42). In particular, the question of isoform-specific versus tissue-specific properties of the rat Na,K-ATPase has been addressed by studying the same isoform, either $\alpha 1$ or $\alpha 3$, in the membranes of various cells. Thus, the properties of the $\alpha 1$ isoform were examined in kidney, axolemma and rat $\alpha 1$-transfected HeLa cells, and those of the $\alpha 3$ isoform, in axolemma, pineal gland and $\alpha 3^*$-transfected HeLa cells. The results provide evidence for isoform-independent, tissue-specific modulation of the kinetic behavior of the Na,K-ATPase, the most striking being the differences in the effects of intracellular K^+ as a competitive inhibitor of Na^+ at cytoplasmic Na^+ activation sites.

2.4 EXPERIMENTAL PROCEDURES

Antibodies. Antibodies used include M7-PB-E9, a rat α3-specific monoclonal antibody, and polyclonal antisera 757 and 50946 which recognize rat β1 and β2, respectively (1, 39), and 754 which was raised against the NH_2-terminal (amino acids 1-13) sequence of lamb α1. A polyclonal antiserum specific for rat α3 isoform and monoclonal antibodies specific for α1 (6H) were generous gifts from Dr. Michael Caplan, Yale University. Goat anti-mouse antibodies used for immunoprecipitations were purchased from Tago Immunologicals, and horseradish peroxidase-labeled secondary antibodies (donkey anti-rabbit), from Bio/Can Scientific.

Cell culture, membrane preparations. Rat kidney microsomes were prepared as described by Jørgensen (21) and stored in a sucrose-histidine-EDTA buffer (SHE buffer: 0.25 M sucrose, 0.03 M histidine, 1.0 mM tris-EDTA, pH 7.5) at -70°C. Rat axolemma membranes were prepared as described by Sweadner (41) and stored at -70°C in a solution comprising 0.315 M Sucrose, 10 mM Tris, and 1mM EDTA, at pH 7.4. Rat pineal gland membranes were prepared as described by Ceña *et al.* (9), with the following modifications. After sonication (Braun-Sonic 1510 sonicator) four times at low setting for 3 seconds in SHE buffer, the protein was collected by centrifugation at 100,000 x g for 30 minutes at 4 °C using a TLA100 rotor in a Beckmann TL-100 centrifuge, resuspended in SHE buffer (≈500 µl / 10 mg of original tissue), and stored at -70 °C. Membranes were isolated from rat α1- and α3*-transfected HeLa cells as described elsewhere (19, 25) and stored at -70 °C. Protein concentrations of the tissue preparations were determined using the Lowry assay as modified by Markwell *et al.* (27). Specific activities are indicated in the legends to Figs. 2-1 and 2-2.

Enzyme assays. Membranes were permeabilized as described by Forbush (12). Briefly, they were diluted to 0.06-0.5 mg/ml and treated for 10 minutes at 22°C with 1% BSA, 0.65 mg/ml SDS and 25 mM imidazole, after which they were diluted 6-fold with 0.3% BSA, 25 mM imidazole. ATP hydrolysis was measured as described previously (46), in a final volume of 100 µl containing 30 mM Tris-HCl (pH 7.4), 1 mM EDTA, 3

mM $MgCl_2$, and, unless indicated otherwise, concentrations of NaCl varying from 0.5 to 100 mM with KCl kept constant at 10 mM, or KCl concentrations varying from 0.2 to 50 mM with NaCl kept constant at 100 mM, with choline chloride added so that ([NaCl] + [KCl] + [ChCl]) was constant at 150 mM. Prior to the assay, membranes were pre-incubated in the reaction medium without or with 10 μM or 5 mM ouabain for 10 minutes at 37 C. The reaction was initiated by adding [γ-^{32}P] ATP (final concentration of 1 mM) and NaCl, KCl and choline chloride to the concentrations listed above.

Immunoprecipitation and Immunoblotting. Axolemma membranes (0.4 mg/ml) were solubilized for 20 minutes at room temperature in solubilizing buffer comprising 1% Triton X-100 or 1% CHAPS, 0.32% BSA, 5 mM EDTA, dissolved in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4). The insoluble material was removed by centrifugation for 1 minute at 500 x g and the supernatant was then incubated for 1 hour at 4°C with monoclonal mouse antibodies specific for α1 or α3 (6 μg per 150 μl solubilized axolemma). Protein G covalently linked to agarose beads (Pharmacia), pretreated for 3 hours at 4°C with goat anti-mouse antibody (100 μl antibody added to 125 μl dry beads), were added to the antibody-treated solubilized axolemma (25 μl of the original dry beads added to 150 μl solubilized axolemma) and incubated overnight at 4°C. After several washes of the beads (suspension in 200 μl of solubilizing buffer, centrifugation for 1 minute at 500 x g), the protein was eluted with 60 μl sample buffer (0.06 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.00125% bromophenol blue), incubated at 37°C for 5 minutes and then separated on a 12% SDS polyacrylamide gel using a Bio-Rad mini gel apparatus as described by Laemmli (24). Proteins were transferred to a polyvinylidinedifluoride (PVDF) membrane (Millipore) which was then blocked for 1 hour at 37°C in blocking buffer (PBS containing 5% milk powder and 0.1% Tween 20) and probed overnight at 4°C with polyclonal rabbit anti-α1, -α3, -β1 or -β2 antisera diluted in blocking buffer. After several washes with 0.1% Tween 20 in PBS, the membranes were probed (1 hour, 37°C) with horseradish peroxidase-labeled donkey anti-rabbit antibody (diluted 1:5000 in blocking buffer) and exposed for 1 minute to the

Enhanced ChemiLuminescence (ECL) reagents obtained from Amersham. Densitometry was carried out on several exposures of the Kodak Imaging Film using a SciScan 5000 Scanner and software (United States Biochemical Corp.).

Analysis of kinetic data. Results are expressed as percentages of V_{max} and were analyzed using the Kaleidagraph computer program with either (i) the equation for the non-cooperative, three-site model described by Garay and Garrahan (14):

$$v = V_{max}/(1+K'_{Na}/[Na])^3, \qquad \text{(Equation1)}$$

where K'_{Na} is the apparent affinity for Na^+, and [Na] is the Na+ concentration; or (ii) the cooperative, n-site model described by the following form of the Hill equation (cc. ref. 34):

$$v = V_{max}[cat]^n/(K+ [cat]^n), \qquad \text{(Equation 2)}$$

where "cat" represents the cation (K^+ or Na^+). Equation 2 was fitted to the experimental points and the values of V_{max}, K and n obtained. $K'_{0.5(cat)}$ representing $K'_{0.5(Na)}$ or $K'_{0.5(K)}$ was obtained from equation 2 through the relationship $K'_{0.5(cat)} = (K)^{1/n}$.

2.5 RESULTS

Na^+ and K^+ activation profiles of distinct α isoforms. To gain insight into the basis for the discrepancies in apparent cation affinities, a series of experiments were carried out in which the cation activation profiles of pumps of the same α isoform but from different tissues were compared. This comparison was confined to α1 and α3 of the rat. It was technically not feasible to include α2 in the analysis since there are virtually no suitable tissues with predominantly this isoform. (Although α2 may predominate in adult skeletal muscle, a high background Mg-ATPase activity precludes meaningful kinetic analysis of Na,K-ATPase). For α1, the tissues compared were kidney, α1-transfected HeLa cells and axolemma. The activity of α1 in axolemma was determined by taking advantage of the low sensitivity of the rodent α1 isoform to cardiac glycosides. Thus, axolemma α1 was assayed in the presence of 10 μM ouabain which effectively inhibits α2 and α3 (26). The α3-rich tissues compared were pineal gland, α3*-transfected HeLa cells, and axolemma. The difference in activity observed in the absence and presence of 10 μM ouabain was ascribed mainly to α3 since the proportion of α2 in axolemma is relatively low (32). In the case of the pineal gland, we have confirmed the report by Shyjan *et al.* (38) showing that the predominant α isoform detected in immunoblots of the adult rat pineal gland is α3 (results not shown). In addition, α1 is also detected, but the immunoblots do not provide information regarding the relative activities of the two isoforms. Therefore, assays to quantify ATPase activity sensitive to low (10 μM) versus high (5 mM) ouabain concentrations were carried out, and the results indicated that the activity of α1 is less than 5% that of α3 in pineal gland (experiment not shown).

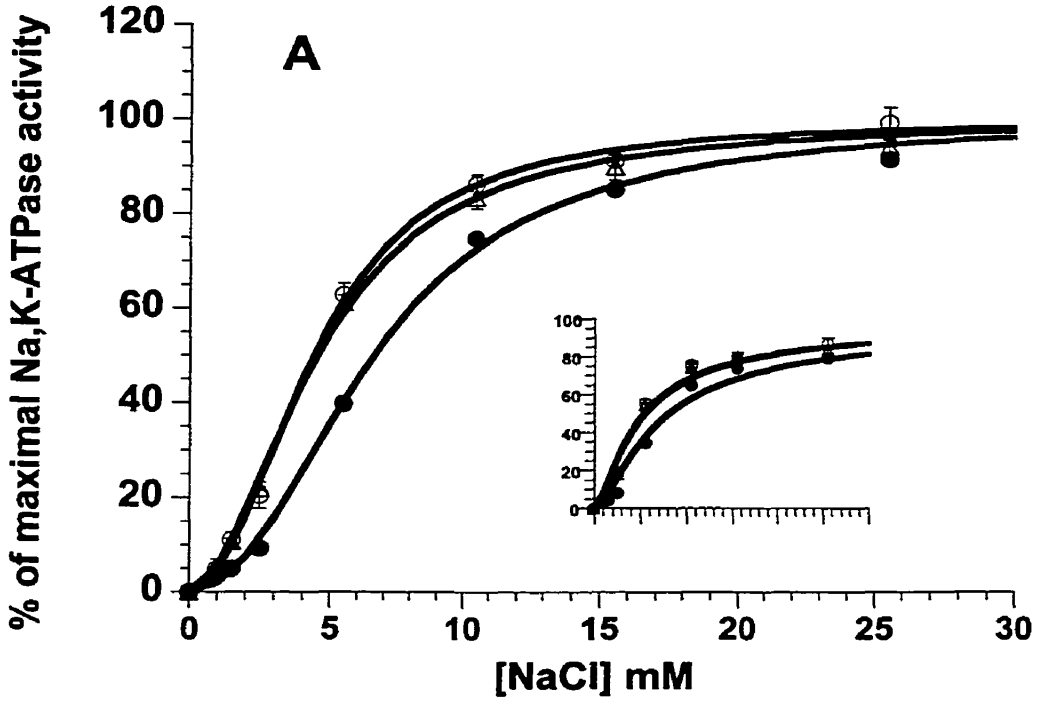
The results of kinetic experiments carried out with α1-containing membranes isolated from rat kidney, axolemma, and rat α1-transfected HeLa cells are shown in Fig. 2-1, and the results for membranes rich in α3, in Fig. 2-2. The data are expressed as percentages of V_{max} and the curves are best-fits to equation 2. The inserts in Figs. 2-1A and 2-2A represent the same data fitted to equation 1.

As shown in Fig. 2-1A, the apparent Na^+ affinity of $\alpha 1$ from kidney, with the K^+ concentration held constant at 10 mM, appears somewhat lower than that of $\alpha 1$ from either axolemma or HeLa; $K'_{0.5(Na)}$ values were 6.6 ± 0.6, 4.7 ± 0.9 and 5.0 ± 0.3 mM for the three tissues, respectively. At a higher K^+ concentration (20 mM; c.f. ref. 18), the difference in the $K'_{0.5(Na)}$ value for $\alpha 1$ of kidney became greater as indicated below (see Fig. 2-4). In the case of K^+ activation (Fig. 2-1B), the order of apparent affinities (assayed at 100 mM Na^+) are as follows: axolemma < kidney ≈ HeLa, with $K'_{0.5(K)}$ values of 2.4 ± 0.5, 0.9 ± 0.1 and 1.1 ± 0.3 mM, respectively. For the $\alpha 3$ isoform of the enzyme, Fig. 2-2A shows that the apparent affinity for Na^+ of $\alpha 3^*$ from HeLa cells is markedly lower than that of $\alpha 3$ from either pineal gland or axolemma; $K'_{0.5(Na)}$'s were 11.1 ± 1.5, 4.9 ± 0.8 and 5.7 ± 0.9 mM, respectively. The K^+-activation profiles of $\alpha 3$ pumps (Fig. 2-2B) show that the apparent affinity for K^+ of HeLa $\alpha 3^*$ pumps is higher than that of other tissues ($K'_{0.5(K)}$'s were 0.7 ± 0.1, 1.6 ± 0.1 and 1.4 ± 0.4 mM, respectively). The kinetic constants and Hill coefficients (n) are shown in Table 2-1.

In other experiments (not shown), the possibility that the results from axolemma membranes were flawed by an incomplete distinction of $\alpha 1$ from $\alpha 3$ was tested. Thus, since axolemma $\alpha 1$ activity is measured as the difference in ATPase activity in the presence of low and high ouabain concentrations, and $\alpha 3$, as the difference in activity in the absence and presence of low ouabain, a spuriously lower-than-true apparent affinity of axolemma $\alpha 1$ and higher-than-true apparent affinity of $\alpha 3$ for K^+ may have resulted from the well documented K^+-mediated decrease in ouabain binding (2). In other words, incomplete inhibition of $\alpha 3$ at high K^+ concentrations would effect an apparent affinity decrease in the former and affinity increase in the latter curves relating activity to K^+ concentration. In order to rule out this possibility, even though this K^+/ouabain antagonism is minimal in rat brain preparations (2), an experiment was carried out in which the K^+ concentration was varied in the presence of different concentrations of ouabain (5, 10 and 20 μM). It was found that $K'_{0.5(K)}$ for the $\alpha 1$ enzyme remained

Fig. 2-1 **Activation by Na^+ or K^+ of rat α1 Na,K-ATPase from kidney, axolemma and transfected HeLa cells.**

Membranes were prepared and assayed as described under "Experimental Procedures". Ouabain-sensitive activities are the difference between hydrolysis measured in the presence of 10 μM and 5 mM ouabain, and are the means ± S.D. (triplicate determinations) expressed as percentages of V_{max}. The curves were fitted to equation 2. Representative experiments are shown and values of $K_{0.5}$ and n for replicate experiments are shown in Table 2-1. A) Activation by Na^+ at 10 mM KCl (V_{max}'s are 3.72 ± 0.07, 0.260 ± 0.004 and 0.107 ± 0.003 μmol/(mg·min) for kidney, axolemma and HeLa cells, respectively), B) Activation by K^+ at 100 mM NaCl (V_{max}'s are 4.32 ± 0.14, 0.330 ± 0.030 and 0.108 ± 0.003 μmol/(mg·min) for kidney, axolemma and HeLa cells respectively). ●, kidney; ○, axolemma; △, transfected HeLa cells. In the insets the data were fitted to equation 1.


A
% of maximal Na,K-ATPase activity
120
100
80
60
40
20
0
0
5
10
15
20
25
30
[NaCl] mM

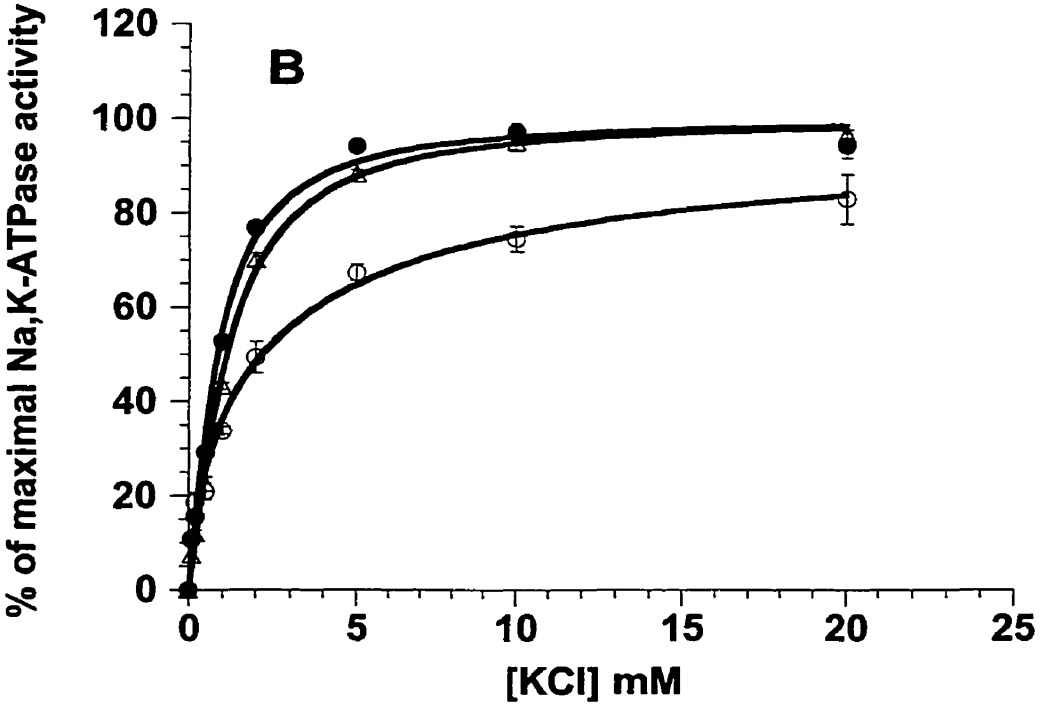
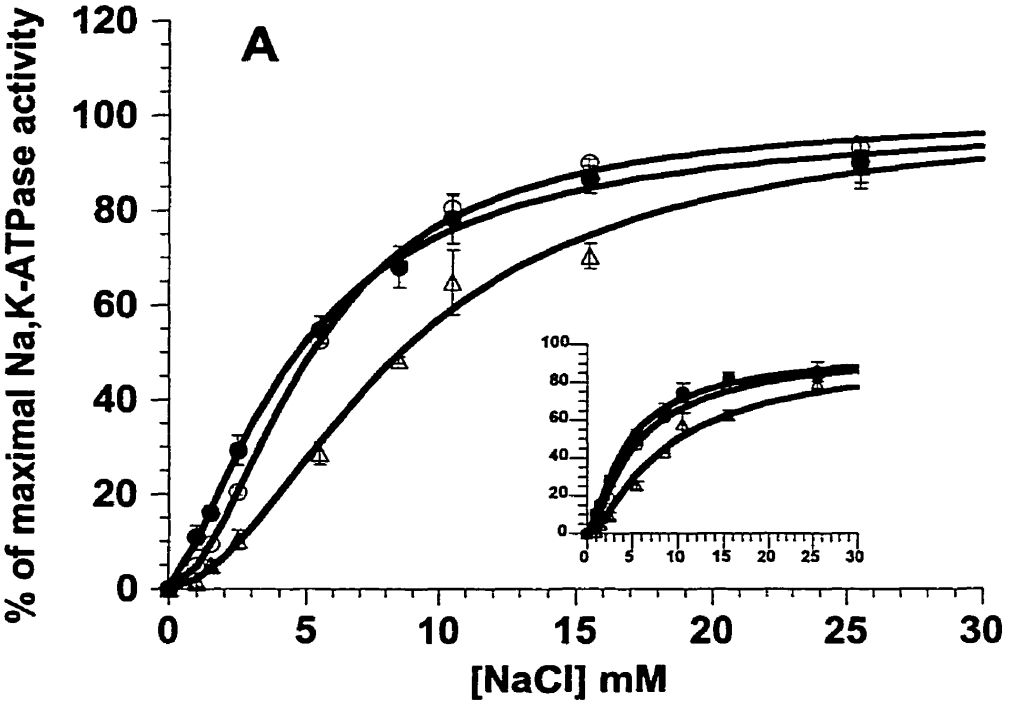

B
% of maximal Na,K-ATPase activity
120
100
80
60
40
20
0
0
5
10
15
20
25
[KCl] mM

Fig. 2-2 **Activation by Na^+ and K^+ of rat α3 Na,K-ATPase from pineal glands, axolemma and transfected HeLa cells.**

Assays were carried out and analyzed as described in Fig. 2-1, except that ouabain-sensitive activities attributed mainly to α3 are the differences between hydrolysis measured in the absence and presence of 5 mM ouabain (pineal gland) or the absence and presence of 10 μM ouabain (axolemma) or in the presence of 10 μM and 5 mM ouabain (α3*-transfected HeLa cells). A) Activation by Na^+ at 10 mM KCl (V_{max}'s are 0.353 ± 0.011, 3.34 ± 0.07 and 0.078 ± 0.002 μmol/(mg·min) for pineal gland, axolemma and HeLa cells, respectively), B) Activation by K^+ at 100 mM NaCl (V_{max}'s are 0.465 ± 0.008, 5.19 ± 0.07 and 0.075 ± 0.003 μmol/(mg·min) for pineal gland, axolemma and HeLa cells, respectively). ●, pineal gland; ○, axolemma; △, transfected HeLa cells. In the insets the data were fitted to equation 1.


A
% of maximal Na,K-ATPase activity
120
100
80
60
40
20
0
0
5
10
15
20
25
30
[NaCl] mM

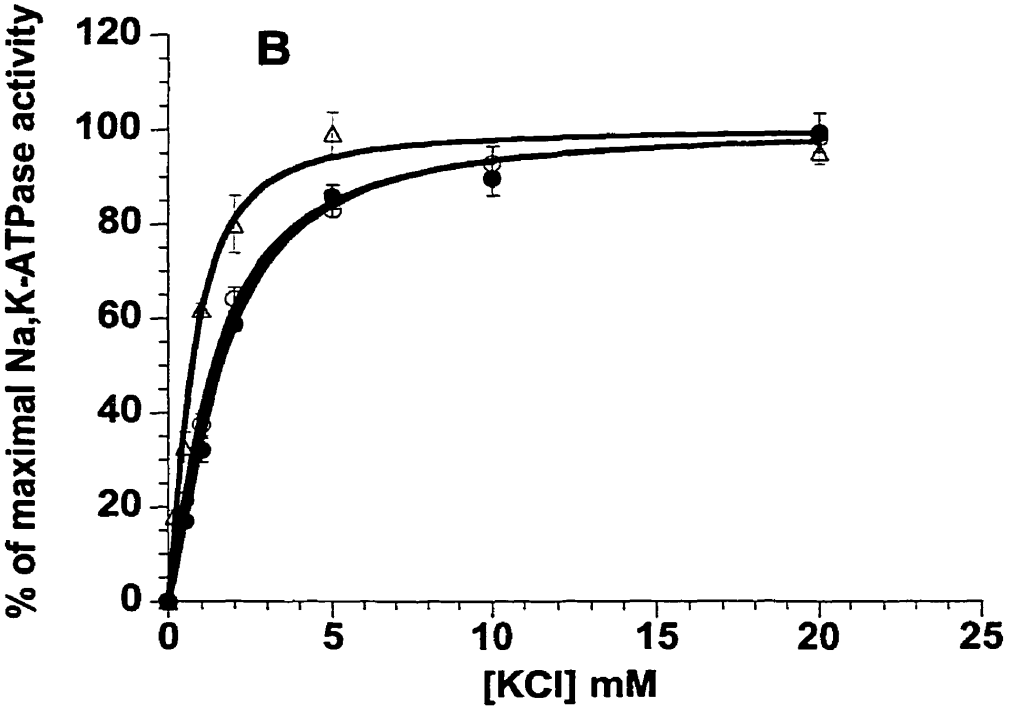

B
% of maximal Na,K-ATPase activity
120
100
80
60
40
20
0
0
5
10
15
20
25
[KCl] mM

Table 2-1

Apparent affinities for Na^+ and K^+ of Na,K-ATPase of various tissues

ATP hydrolysis was assayed with NaCl concentration varied and KCl kept constant at 10 mM, or KCl concentration varied and NaCl kept constant at 100 mM, as described in Experimental Procedures. Values for $K'_{0.5}$ and the Hill coefficient, n, are averages ± S.D. of the number of experiments shown in parentheses, and were calculated from curves fitted to equation 2.

Tissue/cell	Na^+		K^+	
	$K'_{0.5}$	n	$K'_{0.5}$	n
	mM		mM	
α1				
Kidney	6.57 ± 0.58 (7)[a]	2.02 ± 0.26	0.87 ± 0.09 (6)	1.56 ± 0.35
Axolemma	4.67 ± 0.92 (9)	1.98 ± 0.46	2.40 ± 0.54 (5)[c]	1.01 ± 0.31
HeLa	4.95 ± 0.28 (7)	1.73 ± 0.28	1.09 ± 0.28 (6)	1.47 ± 0.24
α3				
Pineal gland	4.89 ± 0.80 (6)	1.51 ± 0.30	1.57 ± 0.14 (4)	1.47 ± 0.12
Axolemma	5.74 ± 0.94 (8)	1.53 ± 0.18	1.37 ± 0.39 (7)	1.34 ± 0.23
HeLa	11.10 ± 1.46 (6)[b]	1.69 ± 0.10	0.70 ± 0.09 (5)[d]	1.37 ± 0.27

[a]Different from α1 of axolemma and HeLa ($p < 0.01$ for both)
[b]Different from α3 of axolemma and pineal gland ($p < 0.01$ for both)
[c]Different from α1 of kidney and HeLa ($p < 0.01$ for both)
[d]Different from α3 of pineal gland and axolemma ($p < 0.01$ for both)

constant at all three ouabain concentrations. Moreover, it should be noted that the enzyme was preincubated with ouabain in the absence of K^+ (see Experimental Procedures), and that the enzyme activity measured thereafter remained constant as a function of time.

K^+ interactions at cytoplasmic Na^+ binding sites. One of the inherent problems in kinetic studies of Na,K-ATPase in membrane fragments is the lack of control of the composition of cations at the cytoplasmic versus extracellular milieu. Specifically, it has been shown that K^+ binding and inhibition at the cytoplasmic Na^+ activation sites alters the enzyme's apparent affinity for Na^+ (14). To determine whether Na^+/K^+ interactions are, indeed, distinct for the sodium pumps of different tissues, a series of activity measurements were carried out at varying K^+ concentration and Na^+ maintained constant at a low 5 mM rather than 100 mM concentration. The results shown in Figs. 2-3A & 2-3B indicate that the extent of K^+-inhibition at the presumably cytoplasmic Na^+ binding site is at least partly affected by the nature of the tissue. As shown in Fig. 2-3A, the kidney $\alpha 1$ enzyme is significantly more sensitive to K^+-inhibition than $\alpha 1$ from either HeLa cells or axolemma; at 20 mM KCl, a concentration at which the $[K^+]/[Na^+]$ ratio of 4 is still lower than the normal physiological value (>10), the activity of kidney $\alpha 1$ is reduced by 40% whereas that of the other tissues is minimally affected. The results for $\alpha 3$ (Fig. 2-3B) show that the transfected HeLa enzyme is much more sensitive to inhibition by K^+ than either the axolemma or pineal gland enzymes.

It has been observed that the antagonistic effect of vanadate, a potent inhibitor of the sodium pump, is facilitated by the presence of K^+ ions (8, 22). To ensure that the K^+-mediated inhibition observed in this study is not the result of vanadate present in the kidney preparation, two control experiments were performed: (i) in one, assays were carried out in the presence of 2.5 mM norepinephrine, which reverses the effect of vanadate (22), and (ii) in the other, the assay time was reduced 10-fold and the amount of kidney microsome sample increased 10-fold; if vanadate were present in the microsome suspension, such an increase in endogenous vanadate concentration should result in greater inhibition of activity. Neither of these conditions altered the K^+-inhibition

profiles, which argues against an apparent K^+-inhibition secondary to the presence of vanadate in the kidney preparation.

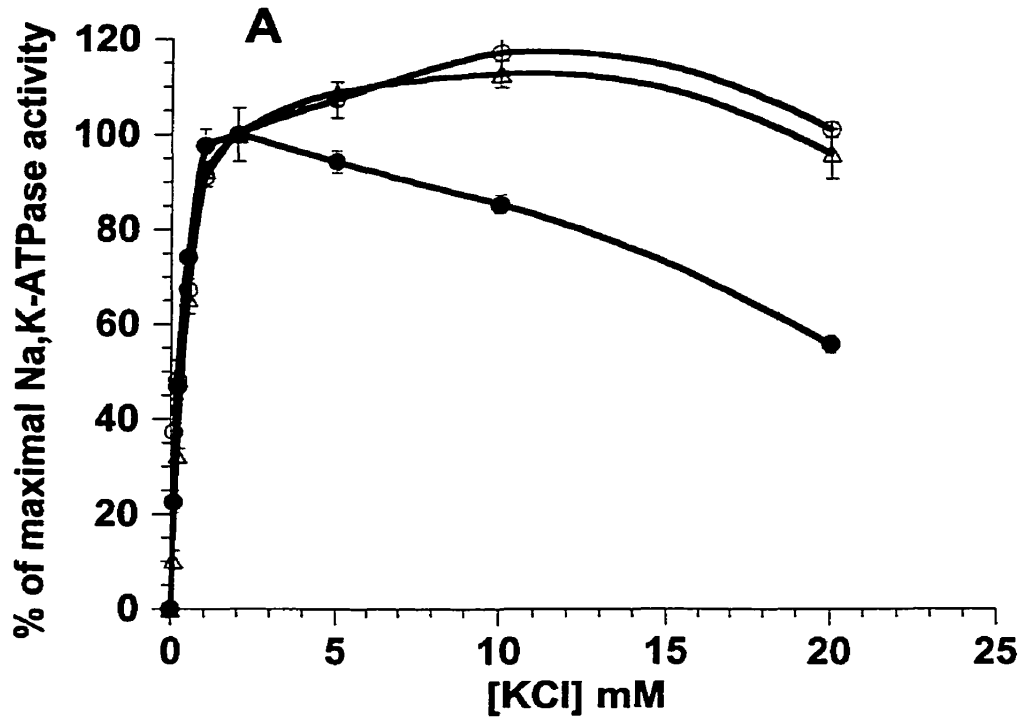
Based on the Albers-Post model of the Na,K-ATPase reaction mechanism and, more specifically, on the model which assumes random binding of Na^+ and K^+ to (the same) three equivalent sites on the cytoplasmic side of the enzyme, Garay and Garrahan, in their studies on Na^+ efflux in red cells (14), and Sachs, in studies of ouabain-sensitive ATPase activity in broken red cell ghosts (35), showed that activity adhered closely to the following relationship:

$$v = \frac{V_{max}}{\left(1 + \frac{K'_{Na}}{[Na]_{in}}\left(1 + \frac{[K]_{in}}{K_K}\right)\right)^3} \quad \text{(Equation3)}$$

where $[Na]_{in}$ and $[K]_{in}$ are the cytoplasmic concentrations of Na^+ and K^+, respectively. In accordance with this model, the plot of K'_{Na}, the apparent affinity for sodium, as a function of K^+ concentration yields the linear relationship $K'_{Na} = K_{Na}(1+[K]_{in}/K_K)$ (equation 4; see ref. 35). From this plot, K_{Na}, the apparent affinity for Na^+ when the K^+ concentration is zero, as well as K_K, the apparent affinity for K^+ at the cytoplasmic Na^+ binding site, are readily obtained (c.f. ref. 35). In order to apply this analysis to our system, a series of experiments were carried out in which K'_{Na} was determined at various K^+ concentrations for each of the tissues studied in Figs. 2-1 and 2-2. It should be noted that the plots of K'_{Na} shown in Figs. 2-4A & 2-4B are best-fits to the model described by equation 1 (see inserts of Figs. 2-1A and 2-2A), which adheres to the non-cooperative assumptions of Garay and Garrahan, while the data of Figs. 2-1 & 2-2 were best-fits to equation 2. The values of K_{Na} and K_K, representing the cytoplasmic binding constants for Na^+ and K^+, respectively, and calculated from the Fig. 2-4 plots, are shown in Table 2-2.

Fig. 2-3 **Potassium inhibition of Na,K-ATPase of kidney, axolemma, pineal gland and transfected HeLa cells at low Na^+ concentration.**

Membranes were prepared and ATPase activity assays performed as described in Figs. 2-1 and 2-2, but in the presence of 5 mM NaCl. The representative experiments show the mean ± S.D. of triplicate determinations expressed as percentages of the activity measured at 2 mM KCl. A) α1 pumps: ●, kidney; ○, axolemma; △, transfected HeLa cells. B) α3 pumps: ●, pineal gland; ○, axolemma; △, transfected HeLa cells.


A
% of maximal Na,K-ATPase activity
120
100
80
60
40
20
0
0
5
10
15
20
25
[KCl] mM

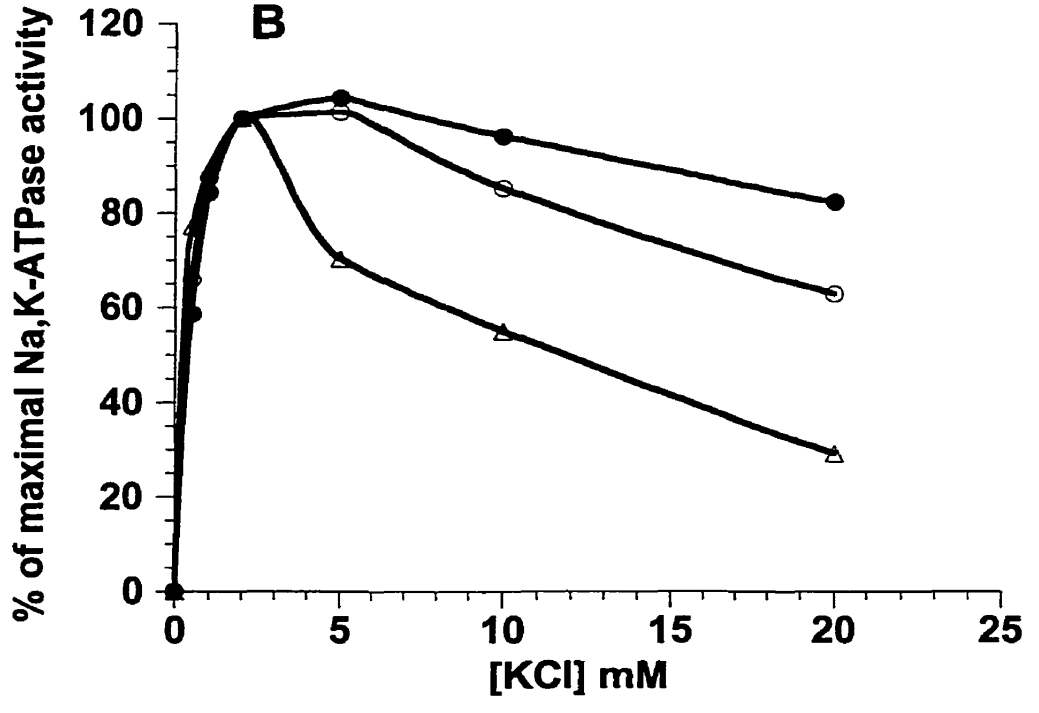
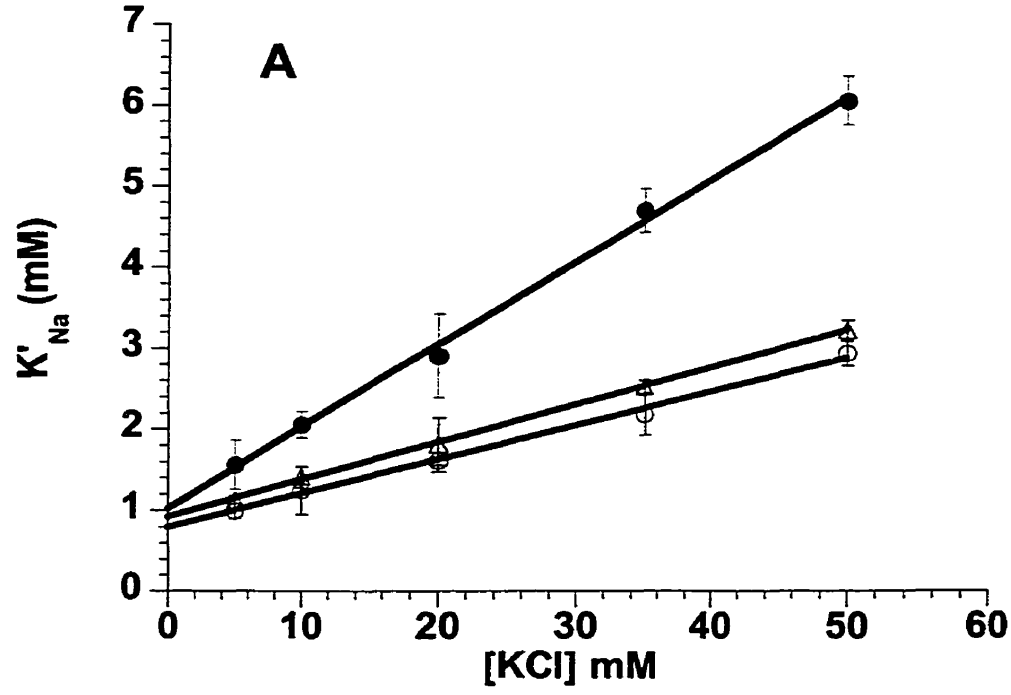

B
% of maximal Na,K-ATPase activity
120
100
80
60
40
20
0
0
5
10
15
20
25
[KCl] mM

Fig. 2-4 **Dependence of K'_{Na} on K^+ concentration for rat pumps from kidney, axolemma, pineal gland and transfected HeLa cells.**

Assays were carried out as described in Fig. 2-1, but at varying concentrations of KCl (5, 10, 20, 35 and 50 mM). K'_{Na} were first determined by fitting the data obtained for each Na^+-activation curve to equation 1, and were then plotted as a function of KCl concentration. Each point represents an average ± S.D. of at least three separate experiments, and the values of K_{Na} and K_K obtained are shown in Table 2-2. A) α1 pumps: ●, kidney; ○, axolemma; △, transfected HeLa cells. B) α3 pumps: ●, pineal gland; ○, axolemma; △, transfected HeLa cells.


A
K'Na (mM)
[KCl] mM
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1
2
3
4
5
6
7
0
10
20
30
40
50
60

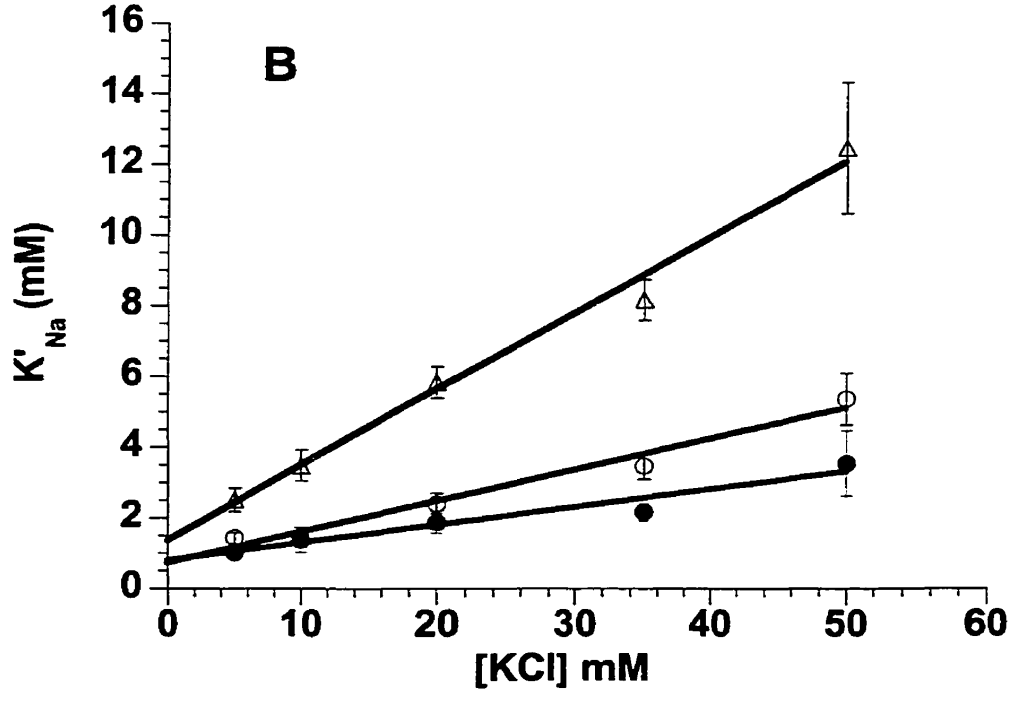
B
K'Na (mM)
[KCl] mM
0
2
4
6
8
10
12
14
16
0
10
20
30
40
50
60

Table 2-2

Affinities for Na^+ and K^+ binding to Na^+-activation sites of the Na,K-ATPase of various tissues

K_{Na} and K_K values were calculated from the plots shown in Fig. 2-4 using the relationship $K'_{Na} = K_{Na}(1+[K]_{in}/K_K)$, where K'_{Na} is the observed apparent affinity for Na^+ in the presence of K^+. K_{Na} is the apparent affinity for Na^+ as an activator in the absence of potassium, and was obtained from the y-intercept. K_K is the apparent affinity for K^+ as a competitive inhibitor of Na^+ at the cytoplasmic binding site, and is the negative of the x-intercept.

	Tissue/cells	K_{Na}	K_K	K_{Na}/K_K
α1	Kidney	1.02	10.0	0.102
	Axolemma	0.78	18.7	0.042
	HeLa	0.92	19.9	0.046
α3	Pineal gland	0.79	15.5	0.051
	Axolemma	0.72	8.20	0.088
	HeLa	1.35	6.30	0.214

In comparing these values, it is evident that the main difference between the α1 pumps of kidney and those of HeLa and axolemma is the higher apparent affinity for K^+ as a competitive inhibitor of Na^+. In the case of the α3 isoforms, however, the lower apparent affinity for Na^+ characteristic of transfected HeLa cells (Fig. 2-2A) is a function of both a lower affinity for Na^+ as an activator as well as a higher affinity for K^+ as a competitive inhibitor, as compared to either the pineal gland or axolemma α3 enzymes. Thus, the ratio K_{Na}/K_K reflects the ability of K^+ to compete with Na^+ for the cytosolic cation binding site and the larger the ratio, the more susceptible the enzyme is to competitive inhibition by K^+. A large K_{Na}/K_K ratio explains the greater K^+-inhibition, at low Na^+ concentration, observed in the case of kidney pumps and HeLa α3* pumps, as depicted in Figs. 2-3A and 2-3B. As well, since K^+/Na^+ antagonism should decrease as the Na^+ concentration is increased, the curves of enzyme activity versus Na^+ concentration shift to the right resulting in the lower apparent affinities for Na^+, as noted in Figs. 2-1A and 2-2A.

Are the tissue-specific kinetic differences the result of α associations with different β isoforms? The question as to whether differences in cation activation are due, at least to some extent, to differences in the β isoform which associates with α was approached by carrying out a series of experiments involving immunoprecipitation of α1 and α3 from axolemma, followed by immunoblotting with α and β isoform-specific antibodies to determine the nature of the associated β subunits. The results of these experiments are shown in Fig. 2-5 and summarized below. This question is relevant only to axolemma membranes since only β1 is present in kidney and only β2, in the adult pineal gland. In addition, HeLa cells contain β1 message (23, 30) and β1 protein has been detected by Western blotting[1]; neither β2 message nor protein were detected by

[1] W. J. Ball, unpublished results

polymerase chain reaction, Northern analysis, or Western blotting[2] .

When a mouse monoclonal antibody specific for α1 was used to immunoprecipitate the enzyme of Triton X-100-solubilized axolemma membranes, the only subunit isoforms detected on Western blots using rabbit polyclonal antisera specific for α1, α3, β1 and β2 as primary antibodies, were α1 and β1 (Fig. 2-5, lanes 3). Further, when a mouse monoclonal antibody specific for α3 was used, α3 and β1 were detected along with a barely visible band corresponding to β2 (Fig. 2-5, lanes 4). Control experiments carried out omitting the precipitating antibody showed minimal amounts of non-specific binding of the axolemma Na,K-ATPase subunits to the protein G-linked agarose beads (Fig. 2-5, lanes 5). The fact that the appearance of two bands, one of slightly higher mobility than α1 and α3 (Figs. 2-5A and 2-5B, lane 3), the other at ≈50 KDa (lanes 3-5 of Fig. 2-5C), reflect non-specific reactions was evidenced in the following controls (not shown). (i) The first band was present even when the primary detecting antibody was omitted, thus indicating that it is the result of non-specific binding of the secondary blotting antibody to the primary immunoprecipitating antibodies. (ii) The second non-specific band appeared even when the primary antibody (mouse anti-α1 or -α3) was omitted (Fig. 2-5C) or when the procedure was carried out in the absence of solubilized axolemma (not shown), indicating that it probably represents a non-specific reaction involving the primary blotting antibody to β1 and goat anti-mouse antibodies.

α–β stoichiometries in axolemma. The stoichiometry of the α–β associations was then evaluated in order to assess whether these associations may have been disrupted as a result of the membrane solubilization procedure. These analyses were done utilizing different exposures of the Western blots from several replicate experiments which were quantified as described in Experimental Procedures. In this work, two assumptions were made; first, that the α1:β1 subunit stoichiometry of the kidney enzyme, as based on

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[2] R. Levenson, personal communication

studies of the purified enzyme, is 1:1 (for example, see ref. 20) and second, that heterodimers comprising β1 are not preferentially immunoprecipitated compared to those comprising β2.

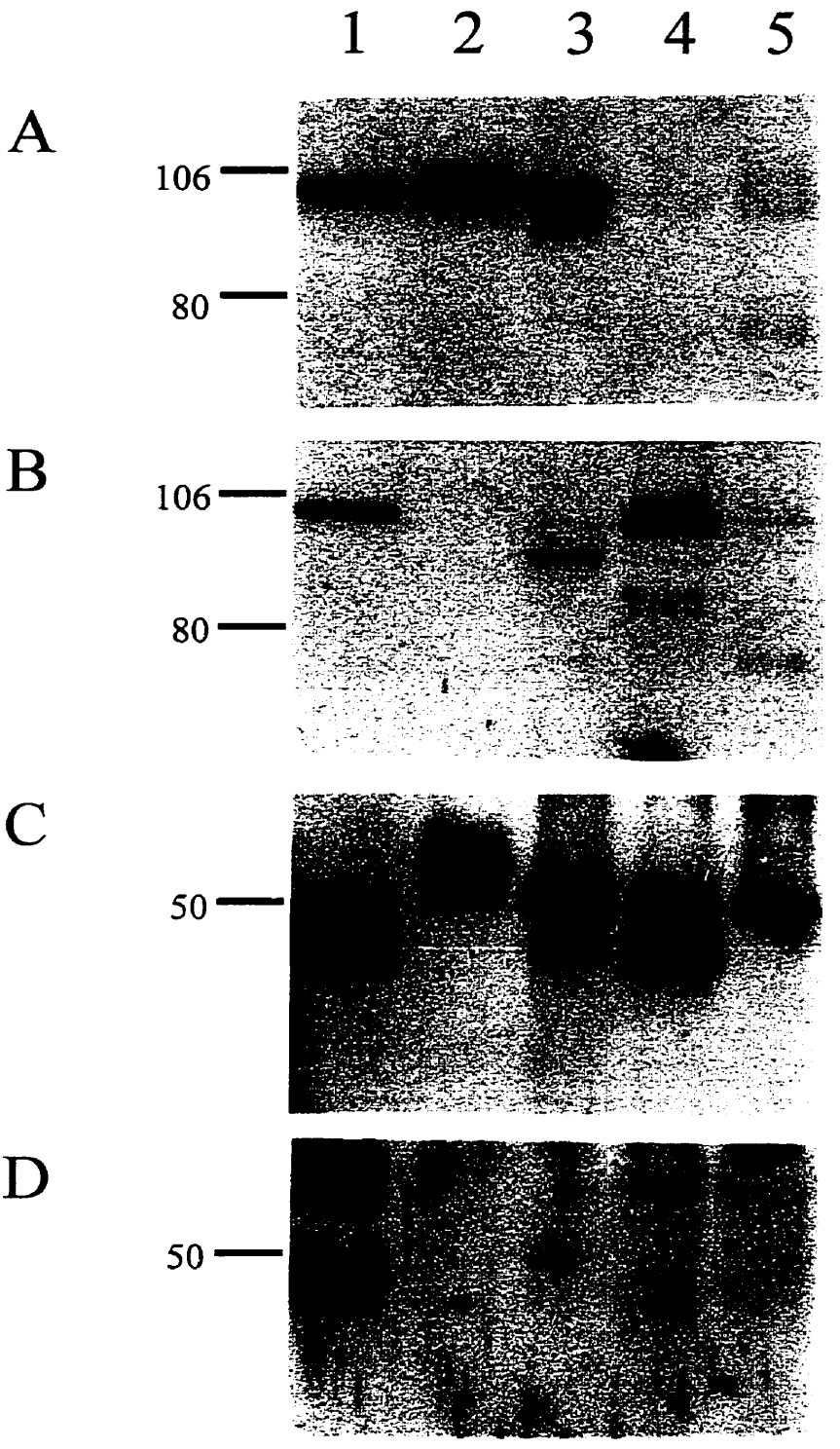
The estimate of α1:β1 stoichiometry in axolemma was based on a comparison of the densities of the α and β bands of axolemma immunoprecipitated with anti-α1 monoclonal antibody with those of unprecipitated kidney microsomes, following exposures to anti-α1 and anti-β1 antisera as shown in Figs. 2-5A and 2-5C (lanes 2 and 3). The ratio of α1 to β1 in precipitated axolemma was found to be 1.08 ± 0.17 (S.E.M. for five independent experiments).

Because there is no tissue in which α3 and β1 have been shown to be expressed in a 1:1 ratio, the question of possible α3–β1 versus α3–β2 associations was evaluated as follows. The α3:β1 ratio as detected in anti-α3-immunoprecipitated axolemma samples was compared to that found in unprecipitated axolemma membranes, after correcting the ratio for the proportion of β1 presumed to associate with α1. The ratio in the immunoprecipitate of axolemma sample was found to be reasonably close to the "corrected" ratio observed in the unprecipitated membranes. Thus, if the corrected α3:β1 ratio of unprecipitated axolemma is normalized at 1.00, the ratio of the precipitate is 1.08 ± 0.09 (S.E.M. for four independent experiments).

It should also be mentioned that in other experiments (not shown) aimed to determine whether the detergent Triton X-100 interfered with subunit interactions, immunoprecipitations were also carried out with a 4-fold lower concentration of Triton X-100 (0.25%) and with 1% CHAPS. Under both conditions, the α:β ratios obtained were not significantly different from those observed with 1% Triton X-100 (data not shown).

Fig. 2-5 **Co-immunoprecipitation of the β subunit with α1 and α3 from rat axolemma.**

Membranes were prepared, solubilized in 1% Triton X-100 and immunoprecipitated with either an α1- or α3-specific monoclonal antibody as described in "Experimental Procedures". Following SDS-PAGE the proteins were analyzed by Western blotting using polyclonal antisera specific for: A) α1, B) α3, C) β1, and D) β2. Lanes are: 1- Axolemma, 2- Kidney; 3- Immunoprecipitate from axolemma using α1-specific monoclonal antibody 6H; 4- Immunoprecipitate from axolemma using α3-specific monoclonal antibody M7-PB-E9; and 5- Control: immunoprecipitation performed in the absence of the primary antibody. Molecular weights are given in KDa.


1 2 3 4 5
A
106
80
B
106
80
C
50
D
50

2.6 DISCUSSION

In this paper we show that the divergent results regarding the relative affinities of the Na,K-ATPase of the different rat isoforms as reported in different laboratories are not simply accounted for by differences in the experimental conditions used. Thus, we have reproduced the relative cation affinities for α1 versus α3 as reported by Jewell and Lingrel and Munzer *et al.* on the one hand (19, 32), and those of Sweadner (40) and Shyjan *et al.* (38), on the other. To gain insight into the basis for this dichotomy, we have assessed the apparent cation affinities of pumps of the same catalytic isoform, either α1 or α3, but from different tissues and, therefore, membrane environments. Marked differences in the apparent affinities for both Na^+ and K^+ were observed in pumps of the same α isoforms isolated from different cellular sources. These data and previous work in lamb (39) and dog (3) tissues are consistent with the conclusion that factors other than the type of catalytic isoform influence interactions of the pump with Na^+ and K^+.

The most obvious tissue-specific protein component which interacts with the pump is the β subunit. In fact, effects of different β subunits on both K^+- (5, 6, 17) and Na^+- (5, 6) affinities have been described. Accordingly, one question is whether the kidney enzyme's lower apparent affinity for Na^+ and higher apparent affinity for K^+ as compared to other α1 pumps are the result of interactions of α1 with different β subunits. To address this question, particularly in axolemma, in which both β1 and β2 have been identified, the nature of the β subunit which co-immunoprecipitates with the distinct α subunits was assessed. The results of these experiments indicate that β1 associates with α1 in axolemma and that the stoichiometry of the association is close to 1.0. The determination of α/β1 stoichiometries imply that little, if any, β2 associates with either α1 or α3. Whether β2 associates preferentially with α2 in axolemma remains to be determined.

Although HeLa cells contain human β1, while kidney cells contain rat β1, these subunits are 95% identical (30). A difference in both type and amount of glycosylation has been observed between kidney and brain β1 (44) and is presumably the basis for the

differences in mobilities in immunoblots of kidney and axolemma as shown in Fig. 2-5C. The possibility remains that these differences are at least partly responsible for the distinct kinetics, even though there is evidence that the oligosaccharides are not essential for primary function (reviewed in ref. 10).

It is unlikely that the presence of distinct β's account for differences of α1 of kidney, axolemma and HeLa, and of α3 of axolemma and HeLa; in both instances, β1 is the predominant β isoform present or associated with α1 or α3. However, tissue-specific differences in cation affinities, despite similar αβ pairing, does not imply that the β subunit has no effect on function. In fact, a kinetic difference due to the distinct β subunits is observed in the case of α3. Thus, as shown in Table 2-2, the ratio K_{Na}/K_K is 1.7-fold lower in pineal gland compared to axolemma, reflecting the 1.9-fold difference in K_K. That this difference is a result of β2 association with α3 in the pineal gland and of β1 with α3 in axolemma is supported by a recent report showing a 1.6-fold higher apparent affinity for Na^+ of α3β2 compared to α3β1 in Sf-9 cells transfected with these isoform pairs (6). In that study, the Na^+ activation kinetics from which the kinetic constants were obtained were carried out in the presence of 30 mM K^+ so that the difference in apparent affinity for Na^+ may also reflect a difference in K_K. Other kinetic differences were not detected. Taken together, these results are consistent with a role for the distinct β's in modulating K^+ interactions at cytoplasmic Na^+ sites.

An important observation regarding the co-immunoprecipitation studies presented here is the isoform specificity of the reactions as evidenced in the co-immunoprecipitation of α with β, but not of α1 with α3. This lack of co-immunoprecipitation between the different α isoforms is in contradiction with reports that pumps co-immunoprecipitate as α heterodimers in rat brain and in bacculovirus infected Sf-9 cells (4). Although it is possible that the detergent (1% CHAPS) used in that study (4) did not fully solubilize the membranes, or that the Triton X-100 used in this study disrupted α–α interactions, these are unlikely explanations, since we confirmed our results using 1% CHAPS.

It can be argued that certain methodological procedures may be responsible for some of the differences observed for the α3 enzyme of axolemma as compared to that of other tissues. As described above, the activity ascribed to α3 in axolemma is that which is sensitive to 10 μM ouabain. Unfortunately, the ouabain-affinities of α2 and α3, both present in axolemma, are quite similar (42), and it was technically difficult to distinguish the two on that basis. However, the amount of α2 in axolemma is relatively low (≈25%; ref. 20), so that its effect on the kinetic behavior cannot account for the magnitude of the differences in the observed kinetic constants as discussed below. As well, the similar fold difference in apparent affinity for external K^+ ascribed to α1 versus α3 in two separate systems (transfected HeLa cells compared to axolemma- and kidney-fused red blood cells; see ref. 32) argues against a substantial contribution of α2 to the behavior of the ouabain-sensitive pumps of axolemma. For the same reasons, it is unlikely that the mutation of α3 to render it ouabain resistant in HeLa cells alters its behavior.

There has been some evidence that detergents, such as SDS used here to increase the permeability of membrane vesicles to substrates, can have an effect on cation activation kinetics of the Na,K-pump (13). Although such an effect was not observed in the case of the Na^+-activation profile of axolemma enzyme, or in the case of the Na^+- and K^+- activation profiles of transfected HeLa cells (data not shown), it is entirely possible that other pumps might react differently to SDS-treatment. However, the SDS concentration and the SDS:protein concentration ratio were identical in all of our experiments. Therefore, if SDS affects the different enzymes to varying extents, it should be the result of differential interactions with the surrounding environment, which would be consistent with the notion that the catalytic behavior of the pump does not depend solely on the isoform of the α subunit.

The most intriguing results of this study concern tissue-distinct K^+/Na^+ antagonism. Differences in K_K, the apparent affinity for K^+ at (cytoplasmic) Na^+ activation sites, underlie tissue-specific differences in the sodium-activation profiles noted in both the present and earlier studies (19, 32, 38, 40). There is evidence also of

differences in apparent Na^+-affinity, independent of K^+ concentration, between pumps of the same catalytic isoform, although this difference was slight in the case of the kidney enzyme compared to other α1 pumps (see Table 2-2). In general, the ability of K^+ to act as a competitive inhibitor of Na^+ binding is reflected in the ratio of K_{Na} to K_K, the cytosolic binding constants for Na^+ and K^+, respectively. It is apparent from Table 2-2 that, of the membrane systems examined, α1 in the kidney and α3 transfected into the HeLa cell have the largest K_{Na}/K_K ratios when compared to other pumps with the same α isoforms. It is these two enzyme preparations which exhibit the greatest sensitivity to inhibition by K^+ as depicted in Fig. 2-3. Specifically, it seems that in the case of kidney α1, this inhibition is due to its relatively lower K_K compared to the other α1 pumps, whereas with HeLa α3 pumps, it is due mainly to a higher K_{Na} (Table 2-2). Whether these characteristics are intrinsic to the enzyme, for example due to tissue-specific co- or postranslational modification(s), or rather, the result of modulation of the enzyme by another associated protein remains unresolved.

The kinetic analyses of the sigmoid activation kinetics described in this and previous studies (19, 32, 38, 40) are based on conventional cooperative or non-cooperative models. Using the non-cooperative model, the apparent affinities of α1 and α3 for intracellular Na^+ and K^+ of the same tissue (HeLa) derived in the present study can account for the low affinities of α3 compared to α1 observed in studies of Munzer *et al.* using intact cells (32), with the following provisos. Those authors pointed out that their data points for Na^+ activation of α3 pumps could be obtained only in the region of the curve well below saturation due to the technical difficulty of raising intracellular Na^+ above 45 mM. This precluded a reliable estimate of the kinetic constants for α3 when using the non-cooperative model. However, the data fitted well to a cooperative model (equation 2 in ref. 32) giving $K'_{0.5}$ values for intracellular Na^+ of 17.6 mM for α1 and 63.5 for α3. In the present study, the K_{Na} and K_K values for rat α1 and α3 in HeLa membranes (Table 2-2) were used to obtain the observed apparent affinity, K'_{Na}, at 135 mM intracellular K^+, a concentration approximating that of the intact cells used by

Munzer *et al.* (32). Using these values of K'_{Na} (7.1 mM for α1 and 30.3 mM for α3) we derived curves of pump activation as a function of varying intracellular Na^+ using the cooperative model (Fig. 2-6). The curves (solid lines) and the values of $K'_{0.5}$ thus obtained (19.2 mM for α1 and 75.2 mM for α3) are similar to those derived from the data of previous studies (32) with intact cells (dashed lines, Fig. 2-6). Therefore, these results indicate that the physiologically significant extremely low apparent affinity of α3 reflects its much greater sensitivity to inhibition by intracellular K^+.

Modulation of pump behavior by the membrane environment is most likely the explanation for the discrepancies among previous reports concerned with isoform-specific behavior. A slightly higher Na^+ affinity in axolemma compared to kidney was reported first by Sweadner (40), and later by others including Shyjan *et al.* (38) who compared brain and kidney. This observation was replicated in the present study of kidney and axolemma Na,K-ATPase, with values very close to those reported by Sweadner, i.e. K_{Na} values of 0.72 mM and 1.02 mM, respectively (Table 2-2) corresponding to $K'_{0.5}$ values of 4.0 mM and 4.3 mM for axolemma and kidney, respectively (not shown).

Tissue-specific as well as isoform-specific behavior is evident not only in apparent affinities for cytoplasmic Na^+ and K^+ but also for K^+ at extracellular sites (Table 2-1). Thus, it was only in the same (red cell or HeLa cell) environment, that a higher affinity for extracellular K^+ of α3 or axolemma compared to kidney (α1) was observed (19, 32). It may be relevant that exogenous kidney pumps fused into red cells and endogenous red cell pumps behave identically with respect to the apparent affinity for extracellular K^+, $K_{K(ext)}$ (31). (The greater difference between α1 and α3 observed in studies with intact cells may reflect the limitation of kinetic studies with unsided preparations).

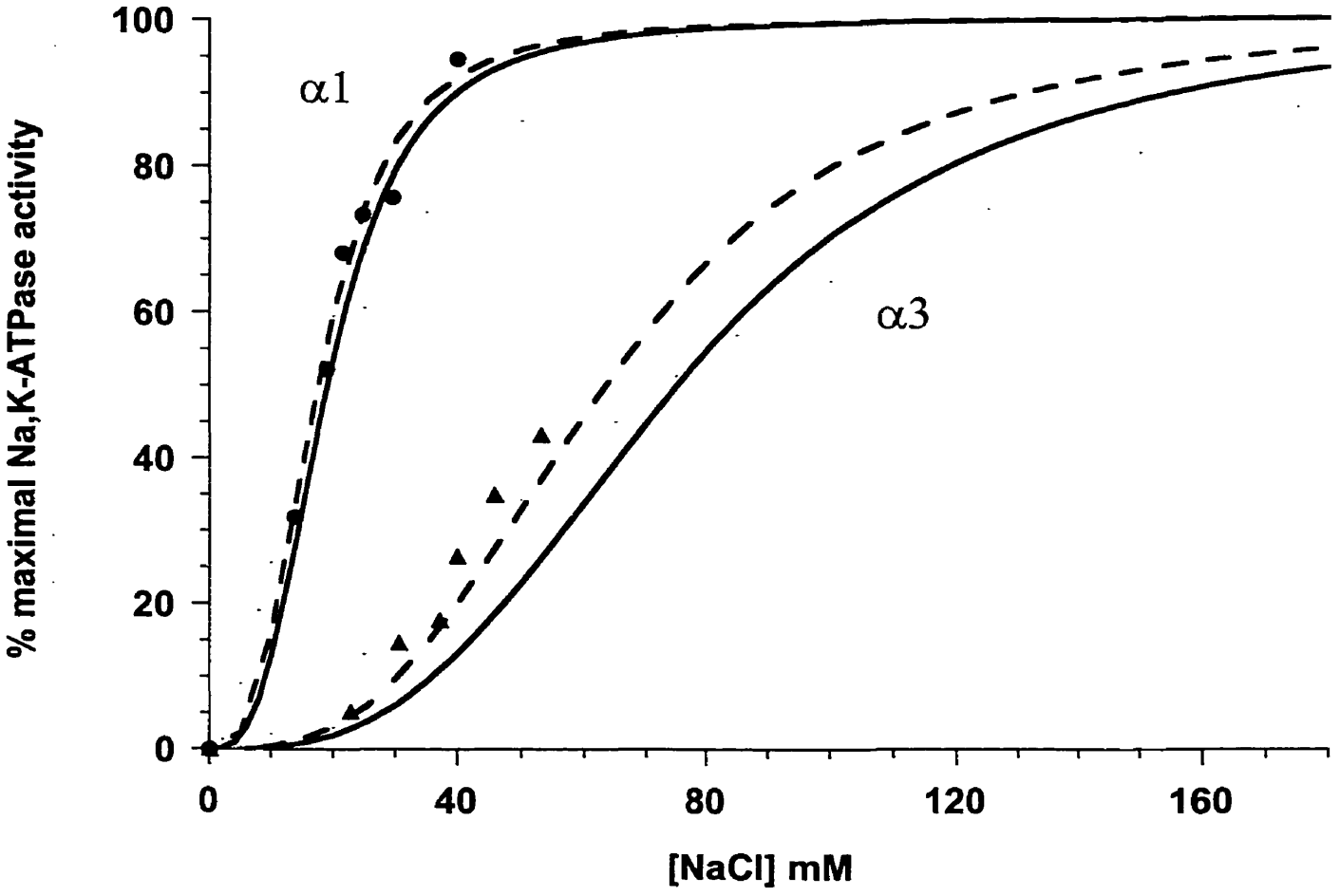
The foregoing considerations argue in favor of the conclusion that the primary structure of the α isoform is not the sole determinant of the magnitude of cation affinity/selectivity. Our observations are consistent with the existence of some pump

modulator, for example one which interacts and effects a greater sensitivity of $\alpha 1$ to K^+ inhibition in the kidney compared, for example, to $\alpha 1$ of axolemma; in the microsome-fused red cell system, association of the α subunit with the putative regulator may be interrupted following its association with other components of the new (red cell) environment. This kind of regulation is reminiscent of the effects of an intrinsic red cell membrane (blood group) antigen, L_p, found in genetically low potassium (LK) sheep red blood cells. This protein or glycoprotein interacts with the pump and effects K^+-inhibition (for review, see 11). Interestingly, when kidney pumps are delivered from microsomes into LK sheep red cells, the L_p antigen effects susceptibility to K^+ inhibition (47), which supports the idea that fusion into red blood cells confers a new membrane environment for the pump.

The study described in this paper provides evidence in support of the conclusion that factors in addition to the primary structure of the α isoforms dictate the kinetic behavior of the Na,K-ATPase. Likely candidates include other membrane-bound components or modulation by co- or post-translational modifications of either subunit.

Fig. 2-6 **Comparison of Na^+-activation curves derived from kinetic constants in Table 2-2 and those obtained in transport studies with intact cells.**

Using equation 4, K_{Na} and K_K values from Table 2-2 were used to obtain K'_{Na} for $\alpha 1$ and $\alpha 3$ at 135 mM K^+ (c.f. ref. 20). The K'_{Na} values thus obtained (7.1 mM for $\alpha 1$ and 30.3 mM for $\alpha 3$) were used to derive curves (solid lines) of percent of V_{max} as a function of Na^+ concentration using the cooperative model (equation 2) with $n = 3.0$ as in ref. 32. Data points for $\alpha 1$ (●) and $\alpha 3$ (▲) were taken from Fig. 6 of ref. 32, and are also expressed as percent of V_{max}. The dashed curves were derived from the $K'_{0.5}$ values for intracellular Na^+ shown in Table II of ref. 32.


100
80
60
40
20
0
% maximal Na,K-ATPase activity
α1
α3
0
40
80
120
160
[NaCl] mM

2.7 ACKNOWLEDGMENTS

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CHAPTER 3

K^+/NA^+ ANTAGONISM AT CYTOPLASMIC CATION ACTIVATION SITES IS A TISSUE-SPECIFIC MECHANISM OF NA,K-ATPASE REGULATION

3.1 PREFACE

This manuscript has been accepted for publication in the American Journal of Physiology (Cell Physiology) (Therien, A.G. and R. Blostein. *Am. J. Physiol.* (in press), 1999). Its aim is to further characterize the tissue-specific differences in K^+/Na^+ antagonism at cytoplasmic cation activation sites described in the previous chapter, and to gain insight into the functional and structural basis for such differences.

3.2 ABSTRACT

Tissue-distinct interactions of the Na,K-ATPase with Na^+ and K^+, independent of isoform-specific properties, were reported previously (Therien *et al.*, *J. Biol. Chem.* 271: 7104-7112, 1996). In this paper, we describe a detailed analysis of tissue-specific kinetics particularly relevant to regulation of pump activity by intracellular K^+, namely K^+ inhibition at cytoplasmic Na^+ sites. Our results show that the order of susceptibilities of α1 pumps of various rat tissues to K^+/Na^+ antagonism, represented by the ratio K_{Na}/K_K, is red blood cell < axolemma ≈ rat α1-transfected HeLa < small intestine < kidney < heart. In addition, we have carried out an extensive analysis of the kinetics of K^+ binding and occlusion to the cytoplasmic cation binding site and find that for most tissues, there is a relationship between the rate of K^+ binding/occlusion and the apparent affinity for K^+ as a competitive inhibitor of Na^+ activation, the order for both parameters being heart ≥ kidney > small intestine ≈ rat α1-transfected HeLa cells. The notion that modulations in cytoplasmic K^+/Na^+ antagonism are a potential mode of pump regulation is underscored by evidence of its reversibility. Thus, the relatively high K^+/Na^+ antagonism characteristic of kidney pumps was reduced when rat kidney microsomal membranes were fused into the dog red blood cell.

Keywords: sodium pump; α1 isoform; heart; kidney

3.3 INTRODUCTION

The Na,K-ATPase, or sodium pump, is a ubiquitous membrane protein complex which maintains the high electrochemical gradient of Na^+ and K^+ ions across the plasma membrane of animal cells (for reviews, see refs. 13, 20, 29). It comprises two essential subunits, α and β. The catalytic α subunit encompasses the sites of nucleotide and cation binding and undergoes conformational transitions associated with the coupling of ATP hydrolysis to the translocation of Na^+ and K^+. The β subunit is required for proper insertion and stability of the enzyme in the plasma membrane and also has a role in modulating cation affinity (reviewed in ref. 4). Multiple isoforms of both the α (α1, α2, α3, α4) and β (β1, β2, β3) subunits are expressed in a tissue- and development-specific manner (3, 19).

Although the basic function of the Na,K-ATPase is the maintenance of cation homeostasis, modification of its behaviour in certain tissues may be critical to specialized functions such as Na^+ reabsorption across epithelia, plasma K^+ clearance by skeletal muscle, adjustment of the set-point for Na^+/Ca^{++} exchange in the heart and restoration of the electrochemical cation gradient following propagation of the nerve impulse. Relevant to such diversity of function in various tissues is an increasing body of evidence suggesting that the Na,K-ATPase is subject to complex short- and long-term regulation. In intact cells, sodium pump activity may be modulated by alterations in (i) intrinsic kinetic behaviour, (ii) cell surface expression and (iii) *de novo* pump synthesis (for reviews, see refs. 2, 8). Furthermore, the distinct properties of the different isoforms of the catalytic subunit as well as their putative distinct susceptibilities to regulatory processes comprise a diverse and elaborate set of modulatory mechanisms.

Although the nature of the α subunit isoform may be the primary determinant of the intrinsic kinetic properties of the enzyme, other cell-specific components may interact with, and modulate kinetic behaviour. In an earlier study we described tissue-specific differences in the interactions of the enzyme with Na^+ and K^+ (28). A particular intriguing finding, albeit rudimentary, was a notable difference in the effects of K^+ as a competitive

inhibitor at cytoplasmic Na^+-activation sites of pumps comprising either $\alpha 1\beta 1$ or $\alpha 3\beta 1$. In this report, we describe a more extensive analysis of this tissue-specific K^+/Na^+ antagonism and its mechanistic basis as it pertains to $\alpha 1\beta 1$ pumps. Using the technique of fusing $\alpha 1\beta 1$ pumps from one tissue (kidney medulla) into another (red cell), we show that the tissue-specific effects are, in at least one instance, subject to modification.

3.4 MATERIALS AND METHODS

Cell Culture and Membrane Preparations. Rat α1-transfected HeLa cells were grown and maintained in culture as described elsewhere (27). Membranes from kidney, axolemma, heart, α1-transfected HeLa cells and red blood cells were prepared as described previously (27, 28). Epithelial cells from small intestine were isolated by a method adapted from ref. (18). Briefly, rat small intestines were sliced longitudinally and washed with ice-cold 340 mM NaCl, and epithelial cells were detached from the intestine by incubation in 240 mM NaCl containing 2.5 mM EDTA for 1 hour at 4°C. Following removal of the remaining intestine, the detached cells and cellular debris were pelleted by centrifugation for 15 min at 39,000 x g. Membranes were prepared from the pellet by the method used for kidney medulla. All membrane preparations were stored at –70°C in a solution containing 1 mM EDTA.

Enzyme assays. Prior to all experiments, membranes were permeabilized by preincubation for 10 min at room temperature in 15 mM Tris-Cl (pH 7.4) containing 1% BSA and 0.65 mg/ml SDS, followed by dilution with 15 mM Tris-Cl (pH 7.4) containing 0.3% BSA, essentially as described by Forbush (11). Such treatment was previously shown to yield maximally permeabilized membranes, at least for kidney (not shown). Assays of Na,K-ATPase activity were carried out essentially as described previously (28), except that 5 μM ouabain was included to inhibit the activity of isoforms other than the rat α1. Accordingly, α1-specific activities were determined as the difference in ATP hydrolysis measured in the presence of 5 μM ouabain and either 5 mM ouabain or 100 mM KCl in the absence of NaCl, with no detectable differences between these two baselines. Average of activities of membrane preparations of rat kidney medulla, axolemma, heart, α1-transfected HeLa cell, red blood cell, and small intestine membranes (μmoles Pi/mg protein/minute) were: 2.8, 0.23, 0.14, 0.15, 0.05, and 0.5, respectively. For membrane preparations of mouse kidney (whole), axolemma and heart, the activities were: 0.84, 0.15, and 0.10, respectively. Assays of the K^+-dependence of K^+-occlusion ($E_1 + K^+ \leftrightarrow E_2(K)$) and the rate of deocclusion ($E_2(K) \rightarrow\rightarrow E_1 + K^+$) were also carried out as

described elsewhere (6), with the following modifications: (i) the final assay volume was 100 µl and contained 5 µM ouabain, (ii) ionic strength was kept constant at 4 mM with choline chloride during the pre-incubations with K^+, and (iii) for the deocclusion assays, enzyme was pre-equilibrated with 4 mM K^+ which was found to be sufficient to form maximal $E_2(K)$ for all tissues used.

PEG-mediated fusion of kidney microsomes into dog red blood cells. Fusion of rat kidney microsomes into dog red blood cells was carried out essentially as described by Munzer *et al.* (22) with minor modifications. Dog blood was collected into 1/10 volume of 0.1 M EDTA. The red blood cells were isolated by centrifugation (2 min at 500 x g) and washed four times at 4°C with 10 volumes of Wash Buffer (140 mM NaCl, 10 mM KCl, 5 mM glucose, 68 mM sucrose and 20 mM Tris-PO_4, pH 7.4). The cells were then suspended and incubated in Wash Buffer for 1 hour at 37°C, centrifuged and washed once at 4°C with Wash Buffer containing 1 mM adenosine and 0.5 mM adenine. One hundred and fifty µl of SHE solution (0.25 M sucrose, 0.03 M histidine, 1.0 mM EDTA-Tris (pH 7.4)), or SHE containing 0.20 to 0.45 mg rat kidney microsomes was added to 1 ml of the packed red blood cells, and the cells were then incubated 15 min at room temperature, followed by dropwise addition of 70% polyethylene glycol to a final concentration of 45%. The suspension was further incubated with gentle mixing for 45 sec at room temperature, then 90 sec at 37°C, followed by successive dilutions with 6, 14 and 26 ml Repletion Buffer (Wash Buffer containing 1 mM adenosine, 0.5 mM adenine, and 2 mM $MgCl_2$), with 30-sec incubations at room temperature prior to each dilution. Cells were then incubated further for 1 hour at 37°C, centrifuged for 5 min at 100 x g, and washed 4 times at 4°C with 10 vol Wash Buffer containing 2 mM $MgCl_2$, by resuspension and centrifugation at 100 x g. These last steps resulted in the isolation of cells free of unfused microsomes. Membranes from fused and mock-fused red blood cells were prepared by the same method as that used for rat red blood cells.

Analysis of kinetic data. Data for Na^+-activation of Na,K-ATPase activity and for K^+-dependence of K^+-occlusion were expressed as percentages of maximal activity or maximal occlusion, as determined by extrapolation of the curves, using the Kaleidagraph

computer program (Synergy software) with the non-interactive model of cation binding described by Garay and Garrahan (12):

$$m = M / (1 + K'_{cat} / [cat])^n \qquad \text{(Equation 1)}$$

where m represents either the rate of the reaction, or the level of K^+-occluded enzyme [$E_2(K)$]; M represents either maximal Na,K-ATPase activity or maximal K^+-occlusion; K'_{cat} represents either K'_{Na}, the apparent affinity for Na^+, or K_{occ}, the apparent affinity for K^+-occlusion; [cat] represents the concentration of cation, either Na^+ or K^+; and n represent the number of binding sites, either 3 in the case of the Na^+-activation experiments, or 2 in the case of the K^+-occlusion experiments. Variances are given as standard deviations, except for Table 1 in which case the standard errors obtained from least square fits are shown. In the case of the estimations of k_o ($k_o=k_d/K_{occ}$; Table 2), the standard deviations were obtained from a Monte Carlo simulation of a joint probability distribution.

3.5 RESULTS

Previous studies of the Na^+- and K^+-activation kinetics of the Na,K-ATPase have shown both isoform- and tissue-specific differences in apparent affinities for Na^+ and K^+ at activating cytoplasmic and extracellular sites (17, 24, 28). Our earlier analysis provided rudimentary evidence of tissue-specific interactions of Na^+ and K^+ at cytoplasmic sites that are particularly relevant to the behaviour of the pump *in vivo* under the normal or resting steady-state condition of high K^+ and low Na^+ concentrations in the cytosolic milieu (28). Thus, the kidney $\alpha1\beta1$ enzyme is notably more sensitive to K^+-inhibition at cytoplasmic Na^+-activation sites than pumps of the same enzyme (rat $\alpha1\beta1$) of either rat $\alpha1$-transfected HeLa cells or axolemma, the latter two assayed in the presence of low ouabain to inhibit activity due to ouabain-sensitive forms. The comparative behaviour of pumps of these and of other tissues is shown in Fig. 3-1A. The plots depict the activities of rat pumps as a function of Na^+ concentration under conditions of relatively high (50 mM) K^+ concentration. The behaviour suggests that pumps of heart, kidney and intestine have lower apparent affinities for Na^+ compared to pumps of $\alpha1$-transfected HeLa cells, axolemma and red blood cells. Fig. 3-1B shows a similar pattern, albeit with somewhat smaller differences, for pumps of another species (mouse), namely heart, kidney and axolemma.

To gain insight into the mechanistic basis for these tissue-specific differences, we carried out a series of analyses of the kinetic behaviour of $\alpha1$ pumps of each tissue, in which Na^+-activation profiles were determined as a function of K^+ concentration. As in our previous study and based on the Albers-Post model with the assumption that Na^+ and K^+ ions bind randomly at three equivalent cytoplasmic sites, the data were analyzed by the relationship described by Garay and Garrahan (12), i.e.

$$m = \frac{M}{\left(1 + \frac{K_{Na}}{[Na]_{in}}\left(1 + \frac{[K]_{in}}{K_K}\right)\right)^3} \qquad \text{(Equation. 2)}$$

where $[Na]_{in}$ and $[K]_{in}$ are the cytoplasmic concentrations of Na^+ and K^+ respectively, and m and M represent v and V_{max}, respectively. This model predicts a linear relationship between the apparent affinity constant for cytoplasmic Na^+, K'_{Na}, and $[K]_{in}$ according to the following relationship:

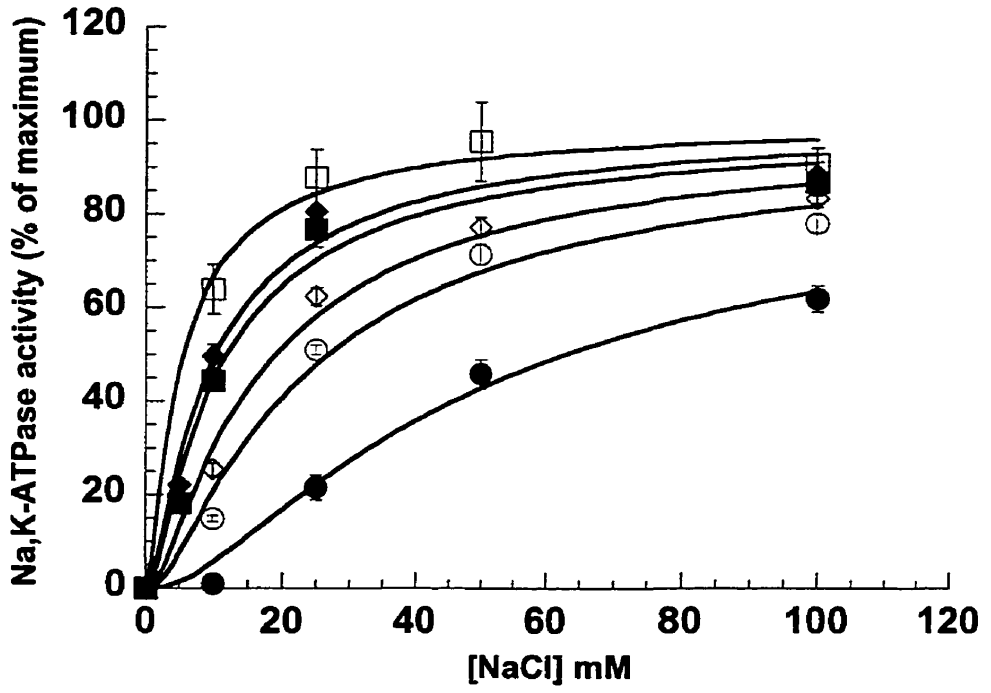
$$K'_{Na} = K_{Na}(1 + [K]_{in} / K_K) \qquad \text{(Equation 3)}$$

The good fits of the data to equation 3 (Fig. 3-2) indicate that this non-interactive model provides a valid basis for analyzing and quantifying K^+/Na^+ antagonism and deriving values for the apparent affinity constants for (i) Na^+ binding at cytoplasmic activation sites in the absence of K^+ (K_{Na}), and (ii) K^+ acting as a competitive inhibitor of Na^+ binding at cytoplasmic sites (K_K). As shown by the data summarized in Table 1, the order of susceptibility to competitive inhibition by K^+, expressed as the ratio K_{Na}/K_K, is heart > kidney > small intestine > axolemma α1 $\approx$ α1-transfected HeLa > red blood cells (K_{Na}/K_K = 0.239 ± 0.025, 0.102 ± 0.003, 0.070 ± 0.005, 0.042 ± 0.002, 0.046 ± 0.001, 0.027 ± 0.005, respectively). For heart and kidney, the high K^+ inhibition can be attributed to higher affinities for K^+ (lower values of K_K) at the cytoplasmic Na^+ binding sites (K_K = 3.96 ± 3.05 and 10.0 ± 0.9 mM , respectively) compared to axolemma, α1-transfected HeLa cell and small intestine (K_K = 18.7 ± 1.8, 19.9 ± 0.7, 20.8 ± 3.6, respectively). With pumps of small intestine, however, the relatively high K_{Na}/K_K value is associated with a lower affinity for Na^+ (K_{Na}= 1.46 ± 0.16 mM) than those in kidney, axolemma α1, and α1-transfected HeLa cell pumps (K_{Na} = 1.02 ± 0.09, 0.78 ± 0.05 , 0.91 ± 0.02 mM, respectively), while the relatively low K^+/Na^+ antagonism characteristic of red blood cells may be due primarily to a high intrinsic affinity for Na^+ (K_{Na} = 0.51 ± 0.16).

Fig. 3-1 **Na^+-activation of α1 Na,K-ATPase from various tissues.**

Membranes were prepared and assayed at various NaCl concentrations and 50 mM KCl, as described under "Materials and Methods". The data were fitted to equation 1 (n=3). Representative experiments are shown, and the data points are the means of triplicate determinations ± S.D., expressed as % of V_{max}. A, Na^+-activation of rat α1 pumps of kidney (○), red blood cells (□), small intestine (◇), heart (●), α1-transfected HeLa cells (■) and axolemma (◆). B, Na^+-activation of mouse α1 pumps of kidney (○), axolemma (◆) and heart (●).

A


Na,K-ATPase activity (% of maximum)
120
100
80
60
40
20
0
0
20
40
60
80
100
120
[NaCl] mM

B

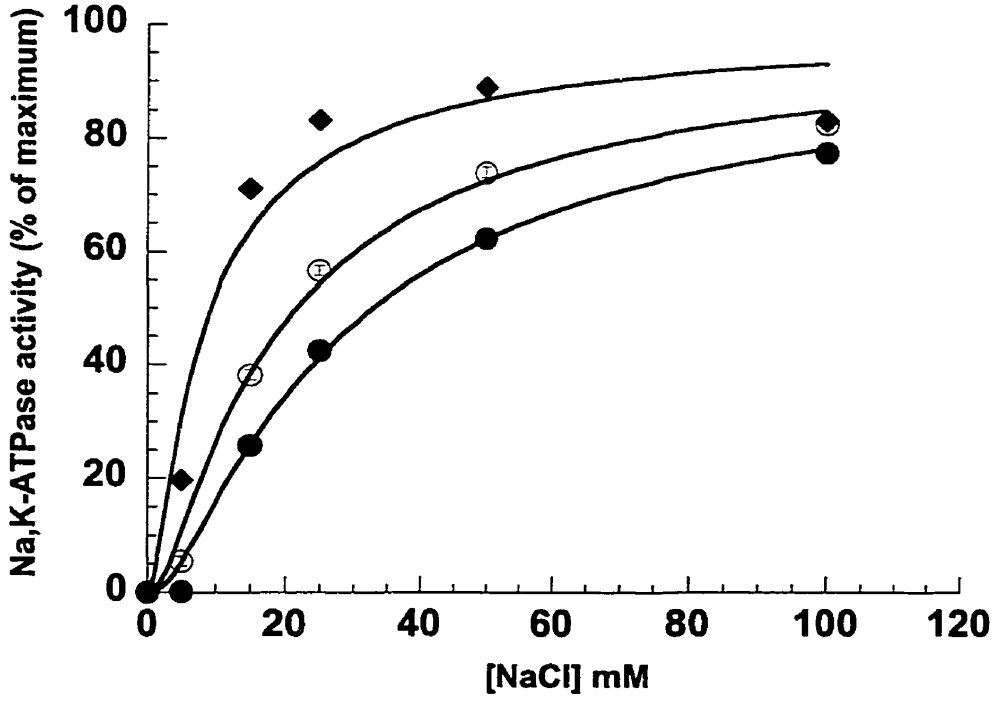

Na,K-ATPase activity (% of maximum)
100
80
60
40
20
0
0
20
40
60
80
100
120
[NaCl] mM

Fig. 3-2 **Dependence of K'_{Na} on K^+ concentration for rat α1 Na,K-ATPase from various tissues.**

Na^+-activation of Na,K-ATPase was determined as described in Fig. 1. K'_{Na} obtained from plots of activity versus Na^+ concentration (equation 1 with n=3) were plotted as a function of KCl concentration. Data points shown are the means ± S.D. of at least three separate experiments, and the values obtained for K_{Na}, K_K and K_{Na}/K_K are shown in Table 1. Dashed lines are the curves obtained for kidney, axolemma and α1-transfected HeLa cells, taken from Fig. 4 of Therien *et al.* (28). Symbols are as described in the legend to Fig. 1.

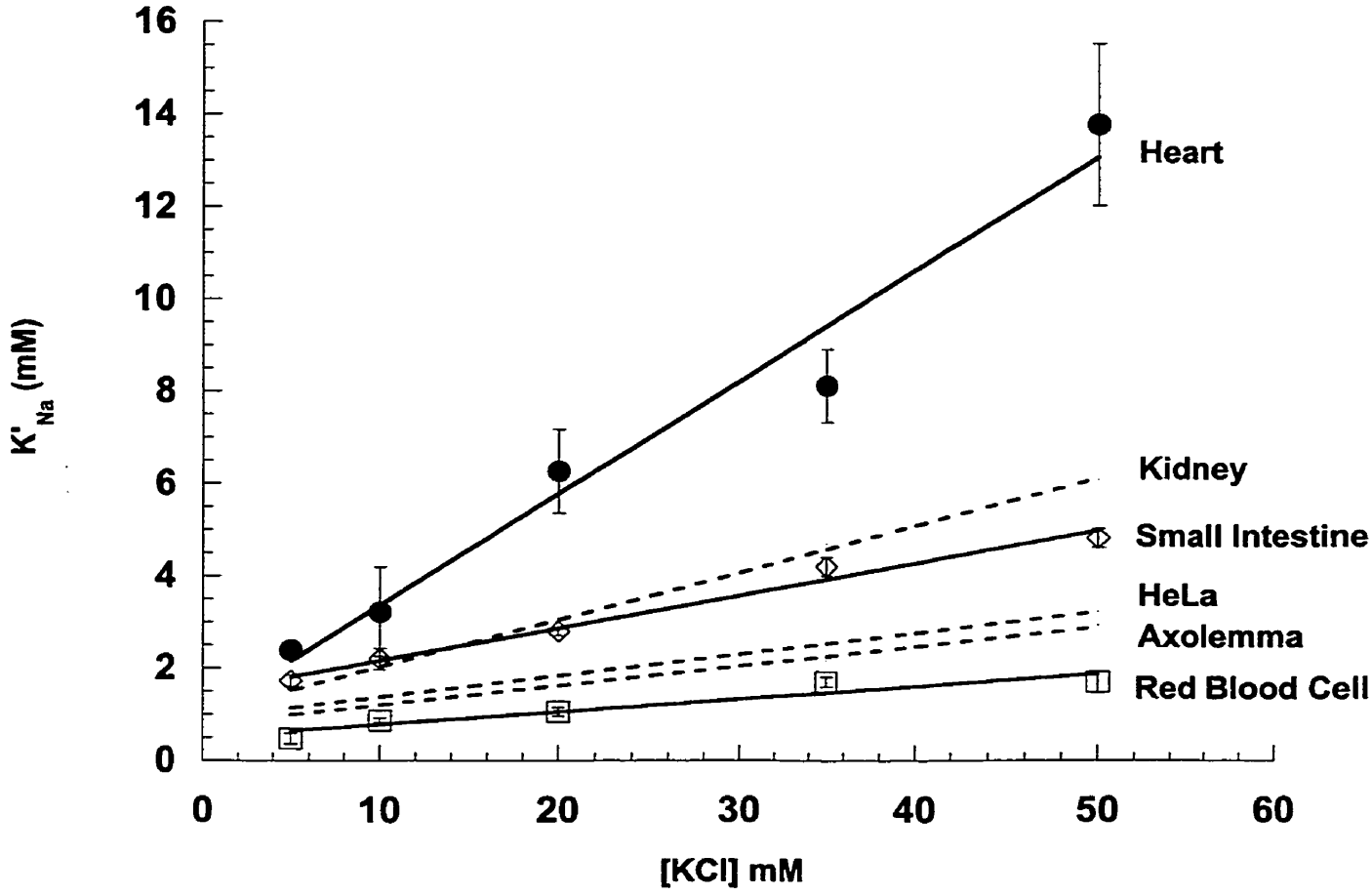

16
14
12
10
8
6
4
2
0
K'Na (mM)
0
10
20
30
40
50
60
[KCl] mM
Heart
Kidney
Small Intestine
HeLa
Axolemma
Red Blood Cell

Table 3-1

Affinities for Na^+ and K^+ binding to Na^+-activation sites of $\alpha 1$ pumps of various tissues

Tissue/cell	K_{Na}[1] (mM)	K_K[1] (mM)	K_{Na}/K_K[1]
red blood cell	0.51 ± 0.16[3]	18.6 ± 9.1	0.027 ± 0.005[7]
axolemma[2]	0.78 ± 0.05	18.7 ± 1.8	0.042 ± 0.002
HeLa[2]	0.91 ± 0.02	19.9 ± 0.7	0.046 ± 0.001
small intestine	1.46 ± 0.16[4]	20.8 ± 3.6	0.070 ± 0.005[7]
kidney[2]	1.02 ± 0.09	10.0 ± 1.2[5]	0.102 ± 0.003[7]
heart	0.95 ± 0.71	3.96 ± 3.32[6]	0.239 ± 0.025[7]

[1] K_{Na} is the affinity constant for Na^+ in the absence of K^+, and K_K, the affinity constant for K^+ as a competitive inhibitor. Values of K_{Na}, K_K and K_{Na}/K_K were determined from plots in Fig. 3-2 using equation 3. Because of the interdependence of K_{Na}, K_K and K_{Na}/K_K, standard errors were obtained by fitting the data to equation 3 with (i) K_{Na} and the ratio K_{Na}/K_K treated as two variables, and (ii) K_{Na} and K_K treated as two variables.

[2] Values taken from Table II of Therien *et al.* (ref. 28).

[3] Different from HeLa, small intestine, kidney ($p < 0.02$).

[4] Different from red blood cell, axolemma, HeLa and kidney ($p < 0.02$)

[5] Different from axolemma, HeLa, small intestine ($p < 0.01$) and may be different from heart ($p < 0.1$).

[6] Different from axolemma, HeLa, small intestine ($p < 0.01$) and may be different from kidney ($p < 0.1$).

[7] Different from all other tissues shown ($p < 0.01$).

Analysis of K^+ interactions: K^+-occlusion and the rate of the $E_2(K) \rightarrow \rightarrow E_1 + K^+$ reaction. In order to gain more insight into the interactions of K^+ ions with cytoplasmic binding site(s), a detailed analysis of the binding and occlusion of K^+ was carried out according to the following simplified equilibrium relationship, which does not distinguish the individual steps of sequential binding/occlusion of the two K^+ ions:

$$E_1 + K^+ \underset{k_d}{\overset{k_o}{\leftrightarrow}} E_1 \cdot K \leftrightarrow E_2(K) \qquad \text{(Scheme 1)}$$

The experimental approach involved indirect assays of K^+-occlusion and deocclusion as described previously (6). Na,K-ATPase-containing membranes were equilibrated with various amounts of K^+, under which condition K^+ binds to E_1 (presumably at the cytoplasmic binding site) and becomes occluded such that an equilibrium between E_1 and $E_2(K)$ is established, as shown in Scheme 1 (10, 14). Taking advantage of the high rate of Na^+-dependent phosphorylation of the K^+-free enzyme, E_1, and low rate of K^+-deocclusion from $E_2(K)$ at 0°C, the enzyme is then phosphorylated at this temperature in the presence of γ-^{32}P-ATP. The K^+-dependent reduction in amount of E_1 susceptible to phosphorylation provides an estimate of K^+-occluded enzyme as described previously (6). Accordingly, Fig. 3-3A shows both the percentage phosphoenzyme (right axis) as measured directly and percentage K^+-occluded enzyme (left axis), as estimated from the difference between maximal phosphoenzyme measured in the absence of K^+, and phosphoenzyme formed following equilibration with K^+ (see relationship given at top of Fig. 3-3A). For this series of experiments, we compared α1 pumps of heart, kidney, small intestine and α1-transfected HeLa cells. The results indicate notable tissue-specific differences in the apparent affinity constant of the enzyme for K^+ occlusion (K_{occ}). Thus, the order of apparent affinities for K^+ occlusion is heart ≈ kidney > HeLa > small intestine (K_{occ} = 0.014 ± 0.001, 0.017 ± 0.003, 0.058 ± 0.014, and 0.158 ± 0.041 mM respectively). A further series of experiments was carried out to

determine whether and to what extent these differences are secondary to distinct rates of deocclusion. Accordingly, the rate of E_1 formation from $E_2(K)$ was estimated at 10°C as described previously (6). The results shown (Fig. 3-3B) indicate dramatic tissue-specific differences in the rates of deocclusion, represented in Scheme 1 by the deocclusion rate constant, k_d. The occlusion rate constant, k_o, was then obtained according to the relationship $K_{occ} = k_d/k_o$ (see Scheme 1). Values of K_{occ}, k_d and k_o for each tissue are given in Table 2. It is interesting, albeit possibly fortuitous, that a comparison of k_o, obtained from experiments done at equilibrium, with the K_K values obtained from the steady-state Na,K-ATPase reaction using the Garay-Garrahan model for K^+/Na^+ antagonism (see Table 1) shows a notable inverse correlation between the two. Thus, the order of k_o values, is heart > kidney > HeLa ≈ small intestine, and the order of K_K calculated from Fig. 3-1 is heart ≤ kidney < HeLa ≈ small intestine.

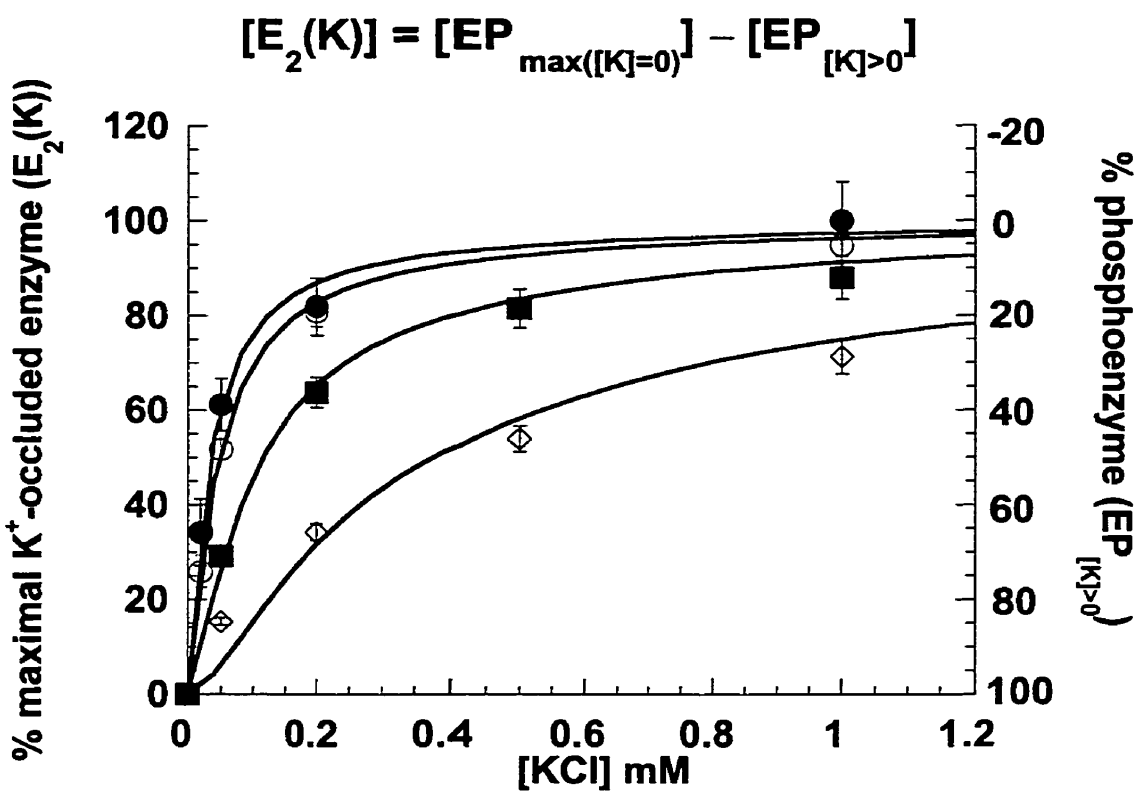
Can tissue-specific differences in K^+/Na^+ antagonism be attributed to distinct membrane environments? To test whether susceptibility to high K^+/Na^+ antagonism is reversible and related to the membrane environment of the Na,K-ATPase, pumps were transferred from the kidney into the red blood cell membrane by the fusion procedure described earlier (21, 22). In those studies, we showed that kidney microsomes can be functionally inserted into mature red blood cells; those of the dog have the advantage of not containing significant levels of endogenous Na,K-ATPase. In this study, we compared the Na^+-activation profiles of fused and non-fused rat kidney pumps. We first carried out fusions in the presence or absence of kidney microsomes. Membranes were prepared and three fusion conditions were used for subsequent functional assays: (i) kidney microsome-fused red blood cells, (ii) kidney microsome/mock-fused red blood cells (kidney microsomes added following a mock fusion) and (iii) mock fused red blood cells. Membranes from mock-fused red blood cells (condition iii) contained no ouabain-sensitive ATPase activity (not shown). Fig. 3-4 shows that kidney microsome/mock fused red blood cells have a typically high K'_{Na} in the presence of 100 mM KCl (K'_{Na} = 12.2 ± 2.4 mM, similar to values obtained with kidney pumps alone; K'_{Na} = 13.1 ± 1.7 (average of 12 experiments), not shown). However, membranes of kidney microsome-fused red

blood cells showed a 1.9-fold decrease in K'_{Na} (6.3 ± 1.1 mM) towards that of α1 pumps of rat red blood cells, axolemma and α1-transfected HeLa.

Fig. 3-3 **Occlusion/deocclusion of K^+ for Na,K-ATPase of various tissues.**

Representative experiments are shown. A, Formation of phosphoenzyme (EP) at 0°C following preincubation in the presence of various concentrations of KCl was carried out as described in "Materials and Methods". Values shown are the means ± S.D. of triplicate determinations of occluded enzyme ($E_2(K)$), which is defined as the difference between (i) maximal EP formed in the absence of KCl ($EP_{max([K]=0)}$) and (ii) EP formed in the presence of various KCl concentrations ($EP_{[K]>0}$). The values are expressed as % of maximal occluded enzyme on the left axis, as determined by fitting the curves to equation 1 (n=2). % phosphoenzyme is indicated on the right axis. Values for K_{occ}, the apparent affinity constant for K^+ occlusion, are shown in Table 2. B, Formation of EP at 10°C was measured at the indicated times as described in "Materials and Methods". Points are the means ± S.D. of triplicate determinations of occluded enzyme, defined as in A, and are expressed as % of maximal occluded enzyme, defined as the difference between EP formed in the absence of KCl and EP formed in the presence of 4 mM KCl at 0°C. Measured values of k_d, the rate constant for K^+ deocclusion, are shown in Table 2. ◇, small intestine; ●, heart; ■, α1-transfected HeLa cells; ○, kidney.

A


$[E_2(K)] = [EP_{max([K]=0)}] - [EP_{[K]>0}]$
% maximal K+-occluded enzyme ($E_2(K)$)
% phosphoenzyme ($EP_{[K]>0}$)
120
100
80
60
40
20
0
-20
0
20
40
60
80
100
0
0.2
0.4
0.6
0.8
1
1.2
[KCl] mM

B

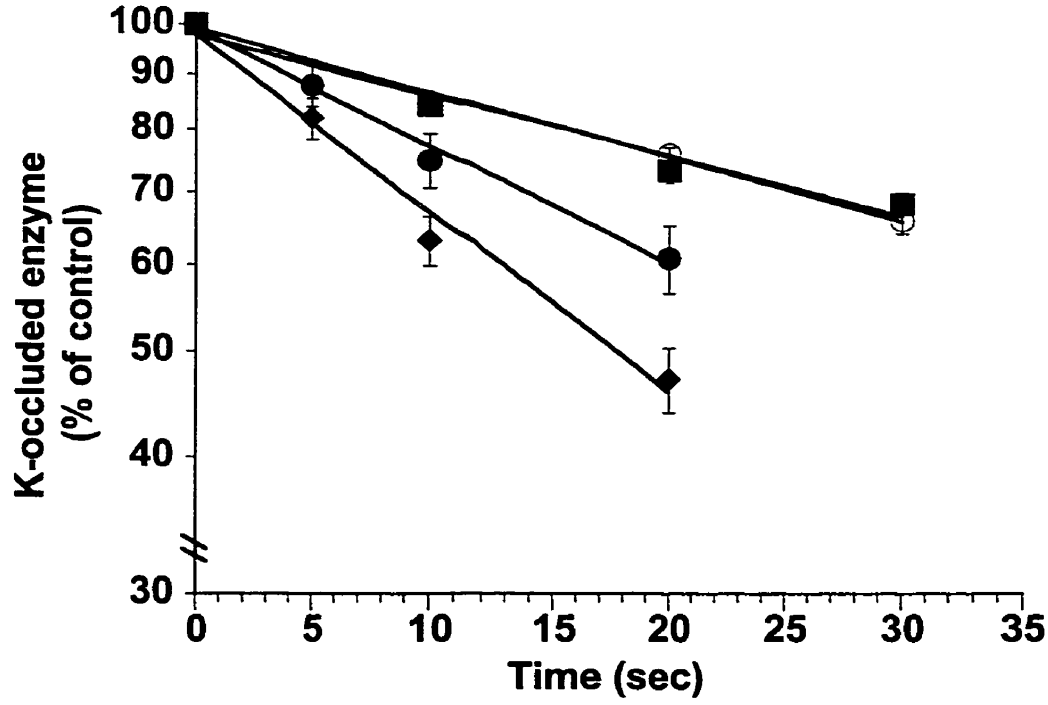

K-occluded enzyme (% of control)
100
90
80
70
60
50
40
30
0
5
10
15
20
25
30
35
Time (sec)

Fig. 3-4 **Na^+-activation of membranes of kidney pumps fused or mock fused into dog red blood cells.**

Pumps of kidney microsome-fused red blood cells and kidney microsome/mock fused red blood cells (see text for definitions) were assayed as in Fig. 1, but in the presence of 100 mM KCl. Points are the means of triplicate determinations ± S.D. and expressed as % of V_{max}, and curves were fitted to equation 1 (n=3). Representative experiments are shown. ●, kidney microsome-fused red blood cells; ■, kidney microsome/mock fused red blood cells.

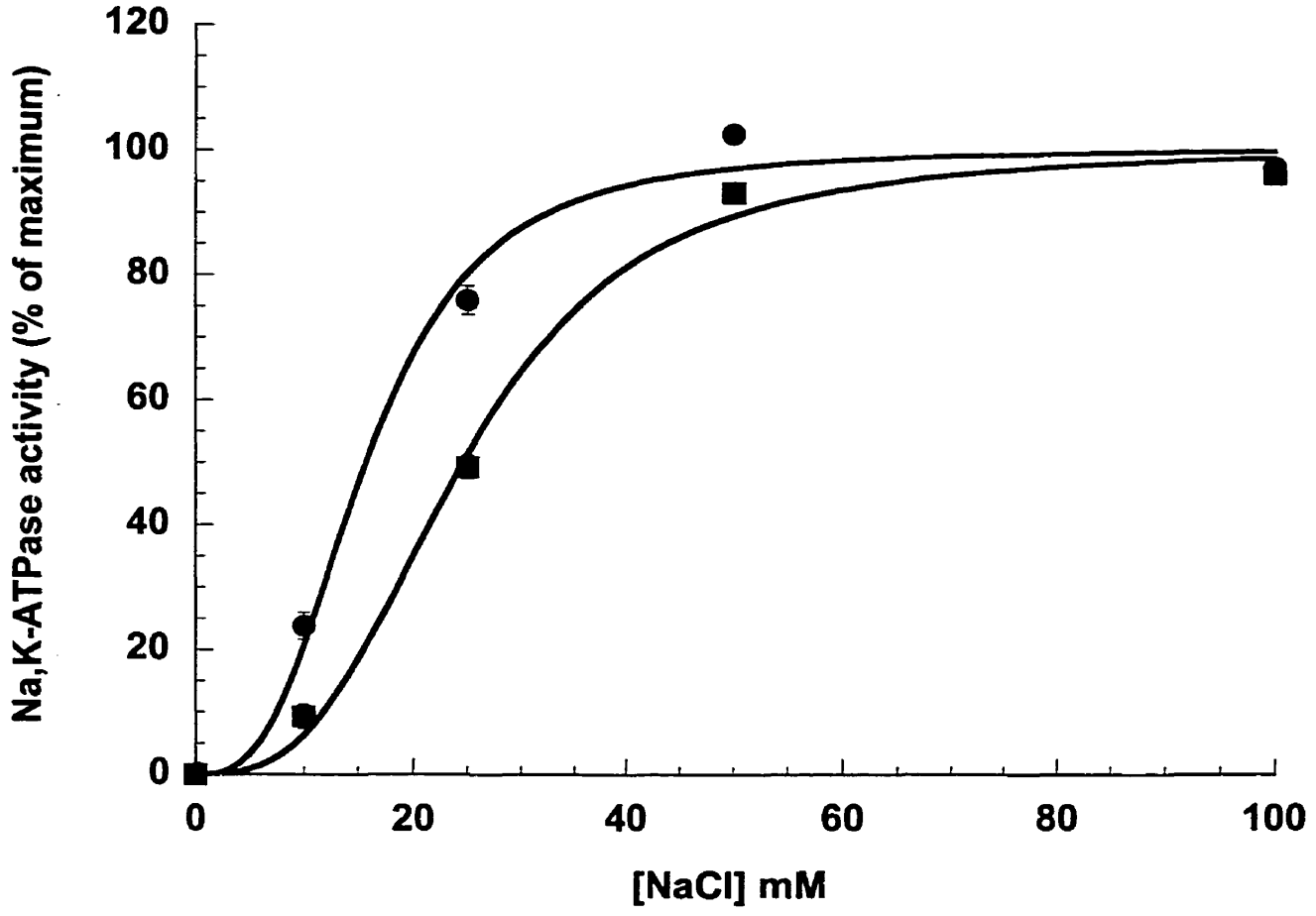

120
100
80
60
40
20
0
Na,K-ATPase activity (% of maximum)
0
20
40
60
80
100
[NaCl] mM

Table 3-2

Comparison of kinetic parameters K_{occ}, k_d and k_o

Tissue/cell	K_{occ}[1] (mM)	deocclusion rate constant (k_d[2]) (sec^{-1})	occlusion rate constant (k_o[3]) ($sec^{-1} \cdot mM^{-1}$)
	a	*b*	*b/a*
kidney	0.017 ± 0.003	0.013 ± 0.002	0.81 ± 0.19[4]
heart	0.014 ± 0.001	0.024 ± 0.001[5]	1.70 ± 0.16[4]
HeLa	0.058 ± 0.014[4]	0.011 ± 0.001	0.20 ± 0.06
small intestine	0.158 ± 0.041[4]	0.033 ± 0.005[5]	0.21 ± 0.07

[1] K_{occ} values are the affinity constants for the reaction sequence depicted in Scheme I, and were determined from plots in Fig. 3-3A.

[2] k_d values are the rate constants for the $E_2(K) \rightarrow E_1 + K^+$ process, and were determined from plots in Fig. 3-3B.

[3] k_o values are the rate constants for the $E_1 + K^+ \rightarrow E_2(K)$ and were calculated from the relationship $k_o = k_d/K_{occ}$.

[4] Different from all other tissues shown ($p < 0.02$).

[5] Different from all other tissues shown ($p < 0.05$).

3.6 DISCUSSION

Several studies (17, 21) have clarified apparent anomalies noted in earlier analyses of the Na,K-ATPase kinetics of tissues comprising different pump isoforms. In essence, it is now clear that the distinct membrane environments of diverse tissues are an important determinant of kinetic behaviour. Notable cases in point are the comparisons of α1 pumps of kidney with α3 pumps of either the pineal gland (24) or axolemma (26). Whereas α3 pumps appeared to have a higher apparent affinity for Na^+ at cytoplasmic activation sites compared to α1 pumps of kidney, the order of apparent affinities was reversed for α3 and α1 pumps transfected into HeLa cells (17) or delivered from either rat axolemma or kidney into the dog red blood cell (21).

A more detailed evaluation of the tissue- versus isoform-specific behaviour of pumps further underscored the importance of the membrane environment as a determinant of pump behaviour (28). In that study, it became apparent also that there are notable tissue-specific differences in the extent to which K^+ behaves as a competitive inhibitor at cytoplasmic Na^+-activation sites of pumps comprising predominantly α1 or α3 catalytic isoforms. In fact, the relative concentrations of Na^+ and K^+ under which K^+/Na^+ antagonism is marked are those which prevail under steady-state physiological concentrations and, as discussed below, this behaviour may have important ramifications vis-à-vis the regulation of intracellular Na^+.

In this study, the interaction of the enzyme with cytoplasmic K^+ was analyzed in terms of the well-documented formation of K^+-occluded enzyme by the direct binding of K^+ to the E_1 conformation of the enzyme (reviewed in refs. 10, 14). Based on the premise that the avidity of K^+ for cytoplasmic cation binding sites should be evidenced in the rate of formation of the K^+-occluded enzyme [$E_1 + K^+ \rightarrow E_1{\cdot}K \rightarrow E_2(K)$; see Scheme 1], the present analysis of the K^+-occlusion pathway of several tissues which vary in K^+/Na^+ antagonism (heart, kidney, small intestine, rat α1-transfected HeLa) shows an intriguing inverse relationship between the rate constant for K^+-occlusion (k_o), calculated from determinations of K_{occ} and k_d as defined by the simple equilibrium relationship in Scheme

1, and the affinity constant for K^+ acting as a competitive inhibitor of Na^+ binding (K_K) measured under steady-state conditions. Thus, our data show a high rate of K^+-occlusion in the tissue (heart) having a low K_K (high affinity for K^+ as a competitive inhibitor of Na^+ binding), an intermediate rate in the tissue (kidney) with an intermediate K_K, and lower rates in tissues (HeLa, small intestine) with the highest K_K values. This analysis was not extended to either axolemma or red blood cells, since (i) in axolemma, conditions for measuring binding/occlusion and deocclusion of K^+ could be confounded by the high proportion of the other (mainly α3) isoforms, and (ii) in the red cell, the relatively high background activity and low specific activity of the enzyme precludes accurate measurements of these parameters.

The correlation between the affinity constants (K_K) for K^+ as a competitive inhibitor of Na^+ binding (as determined by plotting K'_{Na} of various tissues versus $[K^+]$; Table 1), and the apparent rate constants for binding and occlusion of K^+ ions (k_o; Table 2) in pumps of kidney, heart, α1-transfected HeLa cell and small intestine membranes, supports a model whereby K^+/Na^+ antagonism is related to the competing reactions represented by (i) binding/occlusion of Na^+ to form $E_2P(Na)$ in the forward direction, and (ii) binding/occlusion of K^+ to form $E_2(K)$ in the backward reaction. This result also supports a model of ion exchange whereby the K^+-release site and the Na^+-binding site is the same, consistent with several studies showing that mutational or biochemical alterations of residues deemed important for cation binding and occlusion can affect both Na^+ and K^+ interactions (23, 30).

Although Western blots of the various tissues showed a correlation between α1-antibody reactivity and amount of α1 activity analyzed in each lane (not shown), there remains the possibility that our analysis is confounded by the presence of other ouabain-sensitive ATPases, such as the putative "non-gastric" H,K-ATPases, namely the 'colonic' H,K-ATPase found in the colon, kidney and uterus of mammals (5), the H,K-ATPase found in amphibian bladder (16), and ATP1AL1 first cloned from human skin (15). We consider this possibility unlikely, since in the rat, mRNA for the colonic ATPase was not detected in brain or small intestine, and only trace amounts were

detected in kidney and heart by Northern blot analysis, while ATP1AL1 message is absent in all of these tissues (5).

In order to better understand the structural basis for the observed differences in K^+/Na^+ antagonism, we compared the Na^+-activation profiles (at 100 mM K^+) of kidney pumps fused into dog red blood cells, to that of unfused kidney pumps in the presence of mock-fused dog red cell membranes. Kidney membranes and dog red blood cells were used since kidney microsomal membrane preparations are predominantly right-side out and thus are conducive to efficient fusion, and dog red blood cells are devoid of endogenous Na pumps (see ref. 22). Our results show a notable decrease in K'_{Na} of kidney pumps fused into red blood cells, consistent with the conclusion that either components of the red cell membrane are interacting with the exogenous pumps, or that components of the kidney membrane are dissociating from these pumps, resulting in a decrease in their susceptibility to inhibition by K^+. This effect requires intimate interactions between the pumps and the membrane environment since the mere presence of mock-fused red blood cell membranes in the assay medium had no effect on activity. Although our results suggest an effect of the membrane environment, we cannot rule out another possibility: since fusion requires a one-hour incubation of the fused cells at 37°C to insure integration of the exogenous pumps, it is possible that the pumps are somehow altered during this period by a cytosolic factor present in the red blood cell, for example through phosphorylation/dephosphorylation. In either case, these results show clearly that the high affinity for K^+ acting as a competitive inhibitor of Na^+ binding, which is characteristic of kidney pumps, is reversible and can be regulated by some cellular component(s). This type of reversible modulation of exogenous pumps by components of the red blood cell membrane is reminiscent of the observed regulation of pumps by the so-called L_p-antigen of low-K^+ sheep red blood cells. Using the same fusion strategy, we showed that interaction of L_p antigen with exogenous kidney pumps conferred the distinctive K^+-inhibition of pumps of genetically low K^+ sheep red blood cells (31).

It could be suggested that the membrane component that modulates susceptibility of the renal enzyme to K^+/Na^+ antagonism is the so-called γ subunit since this peptide has

been detected in this, but not in other tissues (27). We consider this possibility unlikely for the following reasons: (i) γ subunit protein was not detected in heart (27) even though pumps of this tissue are even more susceptible to K^+/Na^+ antagonism than kidney pumps, and (ii) the fusion experiments would indicate that the γ subunit dissociates from kidney pumps upon fusion into red blood cells, yet fused and unfused kidney pumps were inhibited by anti-γ antiserum (c.f. ref. 27) to a similar extent (experiments not shown).

Association of α1 pumps with distinct β isoforms is also unlikely to be the basis for tissue-specific differences in K^+/Na^+ antagonism. We have already shown in a previous report (28) that the α1 isoform associates only with the β1 isoform in kidney, HeLa and axolemma, and we have since determined that the β1, but not the β2, isoform is detected in Western blots of heart and small intestine (not shown). In addition, we consider it unlikely that the β subunit could dissociate from α upon fusion of pumps into the red blood cell. We cannot rule out, however, that distinct β isoforms are the basis for the high sodium-affinity (low K_{Na}; see Table 1) of pumps of red blood cells, since message for the β2 and β3, but not the β1 isoforms was detected recently in human reticulocytes (25).

In previous studies of Na^+-dependent Rb^+ transport (21), we showed that kidney pumps have a higher affinity for cytoplasmic Na^+ than axolemma pumps when fused into dog red blood cells. However, in a more recent paper (28), we showed that native kidney pumps have a lower affinity for Na^+ than axolemma pumps in Na^+-activated ATPase assays. We postulated that this discrepancy may be related to a lower K^+/Na^+ antagonism after fusion of the pumps. Here we show that this hypothesis is at least partly correct. Thus, the high K^+-inhibition characteristic of kidney pumps is reversible and related to the membrane environment of the pump.

Since the concentration of K^+ in cells is generally high compared to that of Na^+ (roughly 10-fold higher), modulation of K^+/Na^+ antagonism could be a physiologically important mechanism of pump regulation, especially in tissues where the role of the pump is specialized, such as the heart, the kidney and the small intestine. Although the

sodium pump has a fundamental role as a 'housekeeping' transporter responsible for maintaining Na^+ and K^+ homeostasis, in the kidney and small intestine, it is also responsible for the absorption or reabsorption of Na^+ and other solutes that are transported across the apical membrane by Na^+-dependent transporters (for reviews, see 7 and 9). In the heart, it is an indirect regulator of cardiac muscle contraction, as originally formulated by Baker *et al.* (1). Thus variations in K^+/Na^+ antagonism of heart enzyme could alter intracellular Ca^{++} via Na^+/Ca^{++} exchange secondary to alterations in cytoplasmic Na^+ concentration. It is likely that such a regulatory mechanism extends to species other than the rat since similar results were obtained with the murine enzyme. The reversibility of K^+/Na^+ antagonism as evidenced in the experiments with kidney pumps fused into red blood cells underscores the potential importance of intracellular K^+ as a regulator of pump activity.

3.7 ACKNOWLEDGMENTS

We thank Drs. E. A. Jewell-Motz and J. B. Lingrel, University of Cincinnati, for their generous gift of the rat α1-transfected HeLa cells, and Dr. Lawrence Joseph, McGill University, for help in the statistical analysis of the data. The assistance of Ania Wilczynska and Richard Daneman is gratefully acknowledged.

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CHAPTER 4

TISSUE-SPECIFIC DISTRIBUTION AND MODULATORY ROLE OF THE γ SUBUNIT OF THE NA,K-ATPASE

4.1 PREFACE

This manuscript has been published in the Journal of Biological Chemistry (Therien, A.G., R. Goldshleger, S.J.D. Karlish, and R. Blostein. *J. Biol. Chem.* 272: 32628-32634, 1997). My work on the γ subunit stems from searching for a structural basis for the tissue-specific differences in cytoplasmic cation binding described in chapters 2 and 3. Although there was no correlation between γ subunit expression and K^+/Na^+ antagonism, the interesting finding that γ is expressed only in kidney tubules led me to carry out structural and functional analysis of the γ subunit, as described in the next two chapters. This particular manuscript describes initial studies on the functional relevance of the γ subunit using antiserum directed towards the C-terminus of the peptide.

Dr. Steven J.D. Karlish and Rivka Goldshleger of the Weizmann Institute of Science in Rehovot, Israel, carried out the experiments on topology and Rb^+-protection of trypsin digestion, as well as provided the anti-γ antiserum used in this study.

4.2 ABSTRACT

The Na,K-ATPase comprises a catalytic α subunit and a glycosylated β subunit. Another membrane polypeptide, γ, first described by Forbush *et al.* (Biochemistry, 17: 3667-3676, 1978) associates with α and β in purified kidney enzyme preparations. In this study, we have used a polyclonal anti-γ antiserum to define the tissue specificity and topology of γ, and to address the question of whether γ has a functional role. The trypsin sensitivity of the N-terminus of the γ subunit in intact right-side out pig kidney microsomes has confirmed that it is a type I membrane protein with an extracellular N-terminus. Western blot analysis shows that γ subunit protein is present only in membranes from kidney tubules (rat, dog, pig), and not those from axolemma, heart, red blood cells, kidney glomeruli, cultured glomerullar cells, α1-transfected HeLa cells, all derived from the same (rat) species, nor from three cultured cell lines derived from tubules of the kidney, namely NRK-52E (rat), LLC-PK (pig) or MDCK (dog). To gain insight into γ function, the effects of the anti-γ serum on the kinetic behavior of rat kidney sodium pumps was examined. The following evidence suggests that γ stabilizes E_1 conformation(s) of the enzyme and that anti-γ counteracts this effect: (i) anti-γ inhibits Na,K-ATPase and the inhibition increases at acidic pH under which condition the $E_2(K) \rightarrow E_1$ phase of the reaction sequence becomes more rate limiting, (ii) the oligomycin-stimulated increase in the level of phosphoenzyme was greater in the presence of anti-γ indicating that the antibody shifts the $E_1 \leftrightarrow \leftrightarrow E_2P$ equilibria towards E_2P and (iii) when the Na^+-ATPase reaction is assayed with the Na^+ concentration reduced to levels (≈ 2 mM) which limit the rate of the $E_1 \rightarrow \rightarrow E_2P$ transition, anti-γ is stimulatory. These observations taken together with evidence that the pig γ subunit, which migrates as a doublet on polyacrylamide gels, is sensitive to digestion by trypsin, and that Rb^+ ions partially protect it against this effect, indicate that the γ subunit is a tissue-specific regulator which shifts the steady-state equilibria towards E_1. Accordingly, binding of anti-γ disrupts αβ-γ interactions and counteracts these modulatory effects of the γ subunit.

4.3 INTRODUCTION

The Na,K-ATPase is a ubiquitous membrane protein complex that couples the exchange of three cytoplasmic sodium ions for two extracellular potassium ions to the hydrolysis of one molecule of ATP. The minimal functional enzyme consists of two subunits, α and β. Catalytic functions of the sodium pump including Na^+- plus K^+-activated ATP hydrolysis and the binding and occlusion of cations have been ascribed to the α subunit, whereas the role of β is mainly structural (for recent reviews, see refs. 8, 20, 21, 38). A third subunit, γ, was discovered nearly 20 years ago when a $\approx$12 kDa peptide was specifically labeled by a photoactivatable derivative of ouabain, a cardiac glycoside which specifically binds to and inhibits the sodium pump (18). The γ subunit has since been found to co-immunoprecipitate with both the α and β subunits, and the γ subunit of rat, mouse, cow and sheep (27), and more recently, human (24) and *Xenopus laevis* (3) have been cloned. Although the γ subunit of the rat contains 58 residues with a predicted molecular weight of 6.5 kDa (27), its mobility on SDS-PAGE corresponds to molecular weights as high as 12 kDa.

To-date, little is known about the tissue distribution of the γ subunit, or whether, and in what way, γ has a role in Na,K-ATPase function. Hardwicke and Freytag (22) reported that detergent-mediated dissociation of the γ subunit did not affect ATPase activity, and Scheiner-Bobis and Farley (33) saw no difference in enzymatic or transport activities in yeast cells that had been transfected with all three subunits compared to only the α and β subunits. More recently, however, Béguin *et al.* (3) saw a small change in the apparent affinity for external cations in *Xenopus* oocytes transfected with the γ subunits of rat and *Xenopus*.

Although the γ subunit is not essential for function, several observations suggest that it may have an important role in sodium pump function. The high degree of similarity between γ subunits of different species (93%; see ref. 27), the observation that γ subunit RNA is expressed in a tissue-specific fashion (3, 24, 27), and the recent reports of sequences homologous to γ (2, 29, 31) all point to an important function of the γ subunit.

The sequence similarity between γ and a number of other small membrane peptides is particularly intriguing, as it raises the possibility that γ may belong to a family of ion transport modulators. In this paper, we have determined the topology of the γ subunit and determined its tissue distribution at the protein level. We also present evidence that the peptide may have a direct role in regulating Na,K-ATPase activity. A preliminary report of this work has been published in abstract form (37)

4.4 EXPERIMENTAL PROCEDURES

Cell culture and membrane preparations. Rat α1-transfected HeLa and MDCK cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), LLC-PK cells, in alpha minimal essential medium (α-MEM) containing 10% FBS, normal rat kidney cells (NRK-52E), in DMEM containing 5% calf serum, and cultured glomerular epithelial cells (GEC), as described by Cybulsky *et al.* (12), and generously supplied by Dr. A. V. Cybulsky, McGill University. Membranes from HeLa cells, kidney medulla and axolemma, were prepared and stored as described previously (36), and from other cultured cells by the same procedure used for transfected HeLa cells. Rat glomeruli were isolated according to the procedure described by Kreisberg and Wilson (25), and membranes prepared from them by the same procedure as that used for membranes of kidney medulla. Rat red blood cell membranes were prepared from sodium citrate-treated blood as described by Blostein (6).

Western blots and enzyme-linked immunosorbent assays (ELISA). Unless indicated otherwise, membrane proteins were analyzed by SDS-PAGE and transferred to Polyvynilidine difluoride (PVDF) membranes (BioRad) as described previously (36), except that two-phase polyacrylamide gels were used (8%/15%) to allow good separation of α and γ subunit bands on the same gel. The PVDF membranes were divided in two, with the lower half analyzed with a polyclonal anti-γ antiserum (1:150) obtained by a procedure described previously (30) and the other with antiserum 754 (1:500) which is specific for the N-terminal sequence (amino acids 1-13) of the lamb α1 subunit. The latter antiserum was a generous gift of Dr. W. J. Ball, University of Cincinnati. For ELISA, the plates were first incubated overnight (4°C) with 100 μl microsome suspension comprising 10 μg/ml rat kidney microsomes (permeabilized or not in 0.65 mg/ml SDS as described in ref. 17) in 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3 and 0.03% bovine serum albumin (BSA), pH 9.6, with SDS added in the case of intact microsomes so that the final concentration in all samples was 20 μg/ml, washed 5 times with 250 μl/well buffer A comprising 137 mM NaCl, 1.5 mM KH_2PO_4, 3.2 mM

Na_2HPO_4, 2.7 mM KCl, 0.05% BSA, pH 7.4, blocked for 2 hours at 37°C with 200 µl/well buffer A containing 5% BSA, and then incubated (1 hour, 37°C) first with either anti-γ (1:5,000), anti-α (1:20,000), anti-β (1:500) or an equivalent dilution of non-immune rabbit sera (all diluted in buffer A), then with horseradish peroxidase-linked donkey anti-rabbit antibodies (Bio/Can Scientific) diluted to 1:3,000 in buffer A. Wells were washed extensively 5 times with 250 µl/well buffer A between each step. Peroxidase-linked antibody binding was measured by absorption at 405 nm following 20 min incubation with the ABTS substrate solution (100 µl/well; Boehringer Mannheim).

Antiserum treatment and enzyme assays. Amounts of membranes corresponding to Na,K-ATPase activity of ≈30 nmol P_i/min/ml for rat kidney, axolemma and HeLa membranes or ≈10 nmol P_i/min/ml for red cell membranes were preincubated (10 min at room temperature) in solubilizing buffer (0.3 mg/ml saponin, 1% BSA, 15 mM Tris, pH 7.4). Membranes were diluted 10-fold with 0.3% BSA, 15 mM Tris, 1 mM EDTA, pH 7.4 and incubated (1 hour, 4°C) with anti-γ antiserum (1:100). ATPase and phosphoenzyme assays were then carried out as follows: Na,K-ATPase reactions, as described previously (36) with final ion concentrations of 100 mM KCl + 50 mM choline chloride (baseline activity) or 100 mM NaCl + 10 mM KCl + 40 mM choline chloride (maximal activity). Ouabain (10 µM) was included for axolemma and HeLa cells to inhibit α2/α3 and endogenous α1 activities, respectively. For Na-ATPase reactions, antiserum-treated membranes were further diluted 10-fold with 0.3% BSA + 15 mM Tris, pH 7.4 and assayed as above, except that the final ATP concentration was 1 µM and ion concentrations were 20 mM KCl + 130 mM choline chloride (baseline) or varying amounts of NaCl as indicated, with choline chloride added to maintain a constant (150 mM) chloride concentration. For phosphoenzyme determinations, membranes were further incubated 15 min at 37°C (*c.f.* hydrolysis assay conditions) and assays were carried out in the presence or absence of 80 µg/ml oligomycin as described by Blostein (4), with final concentrations as follows: 4 µg/ml antiserum-treated kidney microsomes, 1 µM ATP, 4 mM $MgSO_4$, 1 mM EGTA, 100 mM NaCl + 50 mM choline chloride. Baseline values were determined in the absence of oligomycin and sodium and in the

presence of 50 mM KCl and 100 mM choline chloride.

Trypsin treatment and N-terminal sequencing. Unless otherwise indicated, intact pig kidney microsomes were purified on a metrizamide gradient, incubated (2mg/ml) overnight at 0°C in the presence or absence of 10 mM RbCl or 2 mM $MgCl_2$ and incubated first with TPCK-trypsin (1:4 w/w) for 2 hours at 37°C, with trypsin inhibitor added (5:1 w/w) to stop the digestion. The microsomes were washed three times by centrifugation at 250,000 x g for 1 hour, and resuspension in 0.25 M sucrose, 0.03 M histidine, 1.0 mM tris-EDTA, pH 7.5, with trypsin inhibitor (10 μg/ml) present in the first two washes. The final pellet was dissolved in 2% SDS and the protein precipitated with methanol (4:1 v/v) overnight at –20°C. Sequencing was carried out as described previously (34)

4.5 RESULTS

Gamma subunit protein distribution in various tissues and cell lines expressing the rat α1 enzyme was assessed using a polyclonal rabbit antiserum raised against SDS-PAGE-purified rat γ subunit. Fig. 4-1A is a representative Western blot of seven tissues expressing the rat α1 subunit. Aliquots comprising similar amounts of Na,K-ATPase activity were applied to each lane. Following transfer to PVDF, the upper half of the blot was probed with an anti-α antiserum, the lower half with the anti-γ antiserum. The intensities of bands reactive with anti-α1 antiserum (upper lanes) indicate also that similar amounts of the catalytic α subunit from each tissue were analyzed. Since the specific activity of the red blood cell enzyme was lower than that of other tissues, and to insure that equivalent activities were loaded on the gel, an excess of red blood cell membranes were analyzed. Accordingly, the anomalous migration of the red blood cell α1 band is likely due to protein overloading. Since γ subunit mRNA is relatively abundant in human pancreas as well as kidney (24), it would have been interesting to analyze pancreas for the presence of γ subunit protein. However, the specific activity of Na,K-ATPase in this tissue is extremely low (26) and in our preparation, less than 5% that of red blood cells, precluding meaningful analysis by Western blotting. Bands reactive with anti-γ antiserum and appearing as doublets as reported previously (27), were detected only in tubules of the kidney; none could be visualized in axolemma, heart, kidney glomerulus, cultured glomerullar cells, red blood cells or rat α1-transfected HeLa cells, even following longer exposure (not shown). Furthermore, as indicated in Fig. 4-1B, although the γ subunit is detected in kidney tubules of rat, pig and dog, none could be detected in cultured epithelial cells of tubular origin from the same species (NRK-52E, MDCK, LLC-PK).

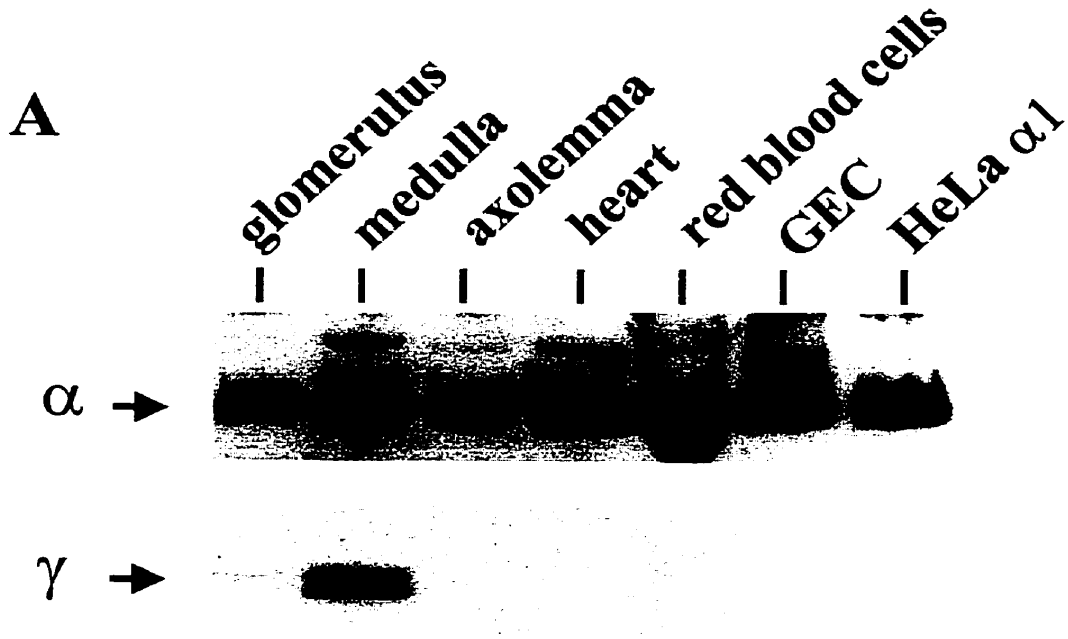
Membrane topology of γ and the side-specificity of its interaction with anti-γ antibodies. The transmembrane orientation of the putative single transmembrane γ subunit chain was determined by treating intact right-side out pig kidney microsomes with trypsin and then analyzing the products following SDS-PAGE and N-terminal

sequencing. As shown in Fig. 4-2, trypsinization led to the appearance of two distinct bands, one of mobility ≈8 kDa and the other, ≈16 kDa (Fig. 4-2A). The N-terminal sequence of the 8 kDa fragment has been previously reported (34) and as shown in Fig. 4-2B is now known to be identical to a sequence in human γ subunit (24) which follows a tryptic cleavage site, showing that the N-terminus is trypsin-sensitive and extracellular. The 16 kDa fragment is derived from the N-terminus of the β subunit, and serves as a control showing that the microsomes are right-side-out and intact; Capasso *et al.* (7) showed that if trypsin had access to the cytoplasmic domain, it would have cleaved the β subunit at ^{4}Lys, and the N-terminal residue would be ^{5}Ala, not ^{1}Ala, as is the case here. The yields of residues obtained in these experiments are indicated in the legend to Fig. 4-2.

As described below, when intact microsomes are digested with trypsin, the higher molecular species of the γ subunit doublet disappears. Either the cleavage results in loss of the entire polypeptide, presumably by a process of internalization and degradation or, more likely, the product is of lower molecular size and becomes indistinguishable from the lower band. The evidence for this is that the epitope(s) recognized by the antiserum requires permeabilization of the microsomes. Thus, the reactivities with anti-γ antiserum of microsomes which were either untreated or permeabilized by SDS in the presence of bovine serum albumin according to the method of Forbush (17) were compared in an enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 4-3, the relative absorbance (OD_{405}) for intact compared to permeabilized microsomes was similar with either anti-γ antiserum or an anti-α antiserum (antiserum 754; see ref. 36) which is known to react with the amino terminus of the α subunit (15.1 ± 1.3% and 11.7 ± 0.4% for anti-γ and anti-α, respectively). In addition, the data are consistent with experiments showing that the unpermeabilized microsomal Na,K-ATPase activity (presumably due to the presence of fragmented membranes) represents 15.4 ± 1.4% of the total activity (not shown). That the increase in antibody binding is secondary to different amounts of protein in wells containing intact versus SDS-treated microsomes is unlikely since: (i) antibody reactivity with intact and SDS-treated microsomes was similar using an

Fig. 4-1 **Tissue distribution of γ subunit protein.**

Immunoblotting was carried out as described in Experimental Procedures. A) Rat tissues (kidney medulla, axolemma, heart, kidney glomerulus, red blood cells), cultured glomerular epithelial cells (GEC) and rat α1-transfected HeLa cells; B) Membranes from rat, pig and dog kidney tubules and from cultured tubule cells of rat (NRK-52E), pig (LLC-PK) and dog (MDCK). Membranes comprising similar amounts of Na,K-ATPase activity (≈3 nmol P_i/min) were added to each well.


A
glomerulus
medulla
axolemma
heart
red blood cells
GEC
HeLa α1
α
γ

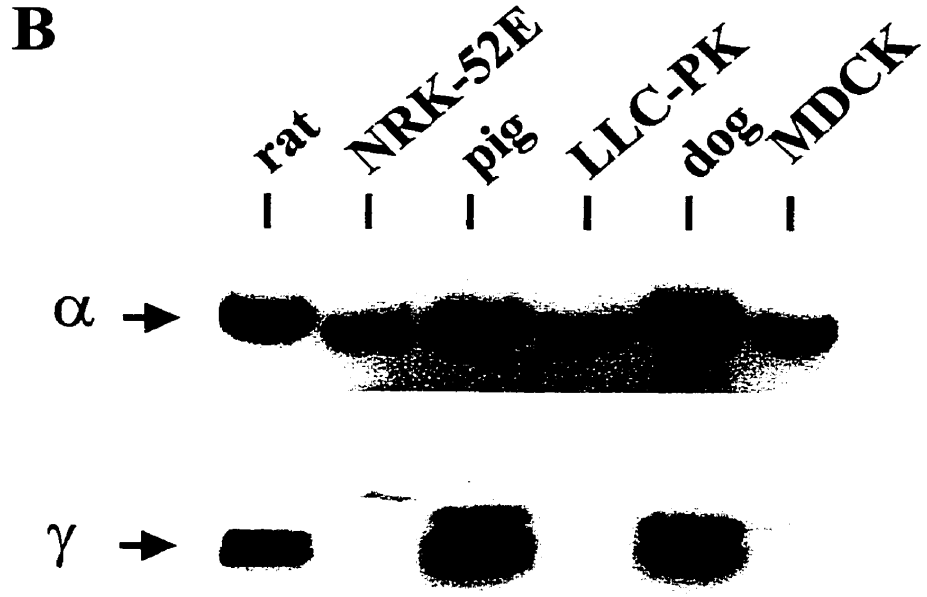
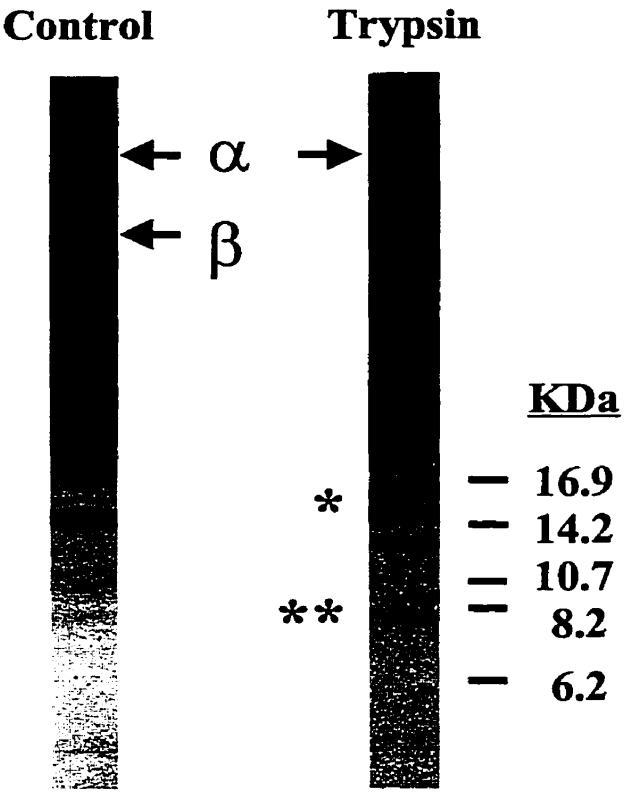

B
rat
NRK-52E
pig
LLC-PK
dog
MDCK
α
γ

Fig. 4-2 **Topology of the γ subunit.**

A) Intact pig kidney microsomes were treated with or without trypsin as described in Experimental Procedures and the resulting peptides analyzed by electrophoresis on a 10% polyacrylamide gel and Coomasie blue staining as described by Capasso et al., 1992 (ref. 24). Lanes are as indicated; B) The N-terminal sequences of the 8 kDa and 16 kDa peptides are compared to those of human γ subunit (from ref. 7) and pig β1 subunit. The yields (pmol per cycle) of residues obtained in the sequences are as follows: for the 16 kDa band: A, 36.6; R, 3; G, 8.2; K, 7.5; A, 10.7; K, 6; E, 3.3; E, 2.5; G, 10.8; and for the 8 kDa band: G, 9; D, 2.5; V, 3.5; D, 1.4; P, 2.8; F, 4.9; Y, 3.1; Y, 3.3; D, 1; Y, 1.7.

A



B

* Sequence found: ARKAKEEG

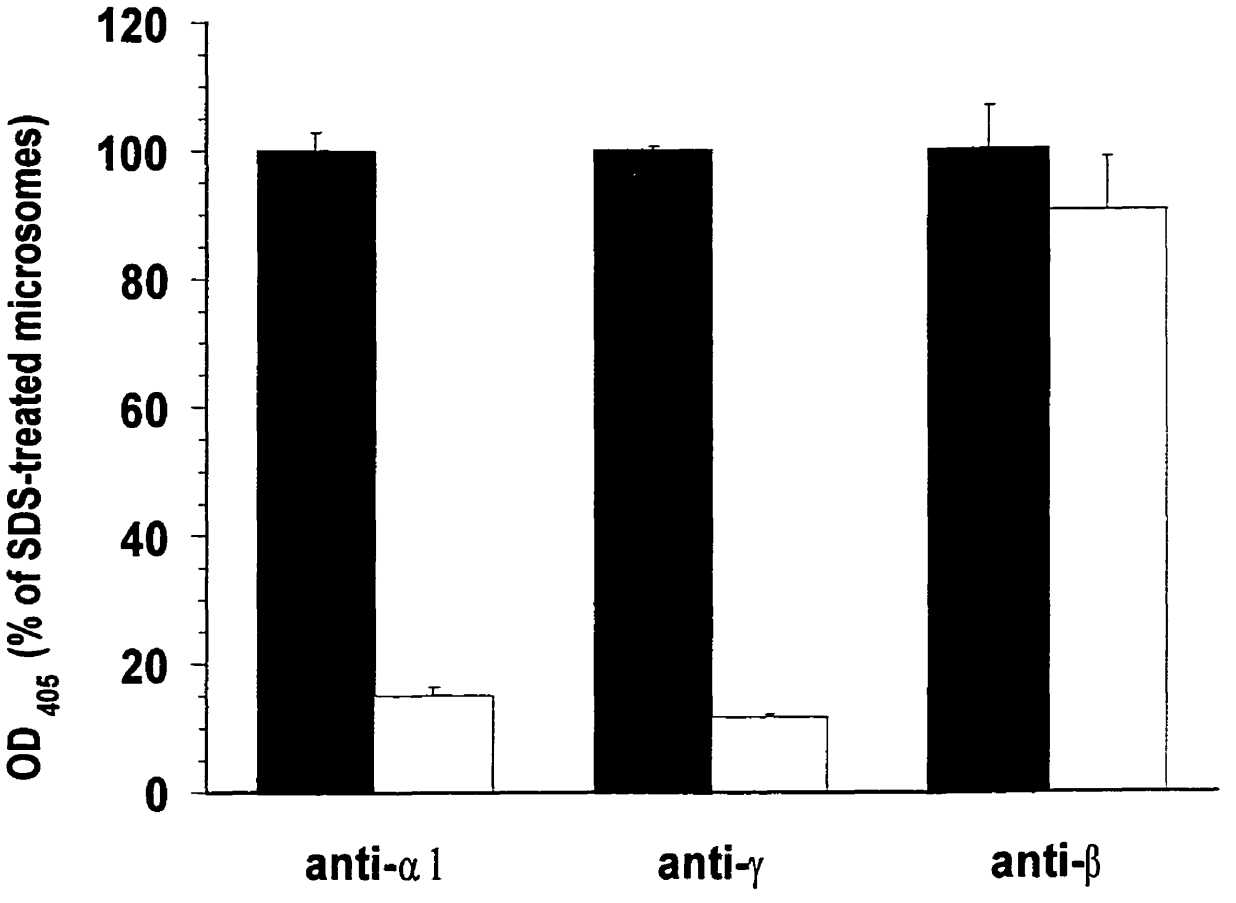
β subunit: ARKAKEEG...

** Sequence found: GDVDPFYYDY

γ subunit: MAAAKGDVDPFYYDY...

Fig. 4-3 **Sidedness of antibody reactivity with γ subunit.**

ELISA plates were coated with SDS-permeabilized (*solid bars*) or intact (*open bars*) rat kidney microsomes and analyzed using either anti-γ subunit antiserum, anti-α1-N-terminus antiserum, or anti-β1-C-terminus antiserum as described in Experimental Procedures. Values shown are expressed as percentages, each representing the difference in OD_{405} obtained with immune and non-immune rabbit serum. With immune serum, OD_{405} values for permeabilized microsomes were 1.39 ± 0.06, 1.13 ± 0.01 and 1.21 ± 0.08, with γ, α and β-specific antisera, respectively. With non-immune serum at dilutions equivalent to those with the respective antisera, values for permeabilized microsomes were 0.19 ± 0.01, 0.17 ± 0.01 and 0.51 ± 0.01, and for intact microsomes, 0.20 ± 0.02, 0.17 ± 0.01 and 0.53 ± 0.01.


120
100
80
60
40
20
0
OD 405 (% of SDS-treated microsomes)
anti-α 1
anti-γ
anti-β

antiserum that recognizes an epitope on the extracellular side, namely amino acids 290 to 302 of the β1 subunit (antiserum 940; see ref. 35), and (ii) OD_{405} values obtained with equivalent dilutions of non-immune serum are not significantly different between permeabilized and intact microsomes (see legend to Fig. 4-3).

Effect of anti-γ antiserum on Na,K-ATPase activity. Based on evidence of a close association of the γ subunit with the Na,K-ATPase α–β dimer (18, 27), experiments were carried out to determine whether anti-γ antibodies can perturb the association in a manner that would have functional ramifications. Accordingly, we tested the effect of anti-γ antiserum on the following: (i) Na,K-ATPase activity under V_{max} conditions as a function of pH; (ii) Na-ATPase activity with sufficient Na^+ to substitute for K^+ at extracellular sites, as well as with Na^+ reduced to bind at only high affinity cytoplasmic sites and (iii) the level of phosphoenzyme measured at 0°C in the absence of K^+, without and with oligomycin present.

As shown in Fig. 4-4A, pre-treatment of permeabilized kidney tubule microsomes with anti-γ antiserum results in partial (31.2% ± 3.2%) inhibition of Na,K-ATPase activity. The antiserum had no effect on non-Na,K-ATPase (baseline) activity, and increasing its concentration (to 1:25 v:v) had no further effect (not shown). In addition, as shown in Fig. 4-4B, the inhibition was not observed in cells which lack γ as shown in experiments with transfected HeLa cells, axolemma and red blood cells. Fig. 4-4A also shows that a significant decrease in the maximal level of phosphoenzyme (EP_{max}) measured in the absence of K^+ with oligomycin present to trap the enzyme in the E_1P conformation could not be detected. In the absence of oligomycin, antibody treatment caused a significant decrease (32.1% ± 7.6%) in phosphoenzyme. The decrease in ratio of V_{max}:EP_{max} indicates that antibody binding decreases the catalytic turnover of the enzyme. The observation (Fig. 4-4A) that oligomycin increases the level of phosphoenzyme to a greater extent in the anti-γ- compared to the control non-immune serum-treated enzyme also suggests that anti-γ alters the steady-state equilibrium between dephospho- and phosphoenzyme in favor of the latter. In support of this conclusion is the

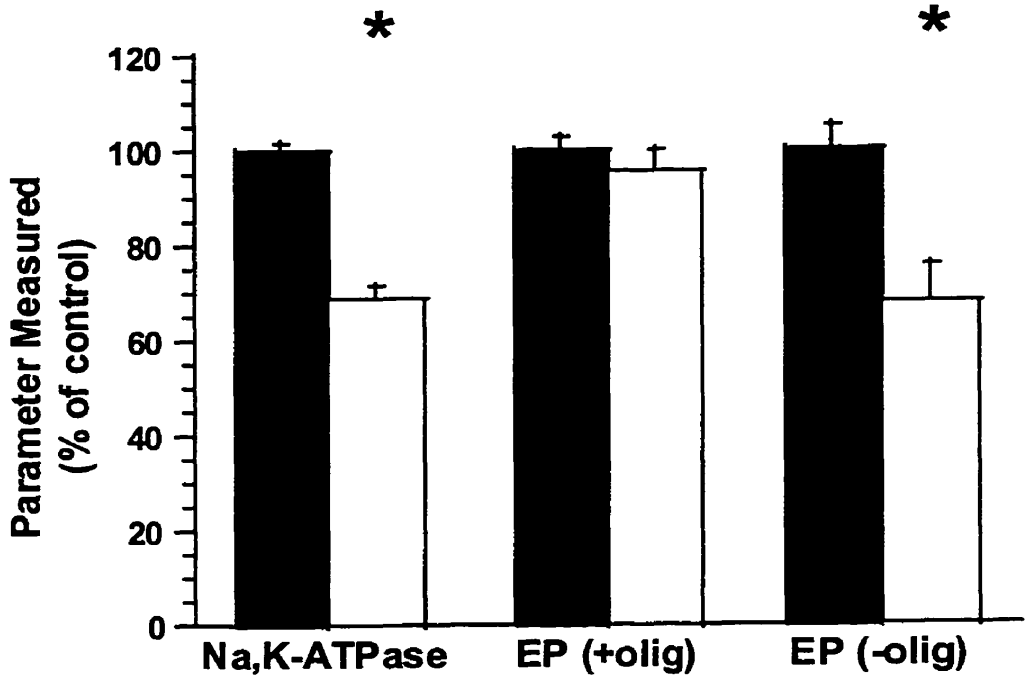
observation (Fig. 4-5) that anti-γ inhibition of Na-ATPase is reduced when the Na^+ concentration is decreased to levels (≈2 mM) that are considered to limit the rate of the $E_1 \rightarrow\rightarrow E_2P$ transition (5). In fact, at the lowest concentrations used (≈0.6 mM), a moderate but significant activation is observed. Comparison of Figs. 4-5 and Fig. 4-4A also shows that at high Na^+ concentration, Na-ATPase is less inhibited than Na,K-ATPase.

To gain further insight into the part of the reaction cycle affected by anti-γ treatment, we tested the effect of antiserum pre-treatment on Na,K-ATPase activity measured at acidic versus alkaline pH. According to Forbush & Klodos (19), the pH-dependence of Na,K-ATPase is limited partly by the rate of K^+ deocclusion at acidic pH, by the rate of the $E_1P \rightarrow E_2P$ transition at neutral pH, and by phosphorylation at pH above pH 8.0. The results shown in Fig. 4-6 indicate that the inhibition observed at pH 7.4 (31.2% ± 3.2%) increases to 40.3% ± 3.0% at pH 6.2 and decreases to 22.2% ± 2.3% at pH 8.9.

Effect of Rb^+ and Mg^{++} on tryptic cleavage of the γ subunit. It was shown previously that the Na,K-ATPase binds and occludes K^+ or its congener, Rb^+, and the conformation effected by $K^+(Rb^+)$ occlusion protects the enzyme from complete digestion by trypsin. Thus, a 19 kDa fragment of the α subunit remains virtually trypsin-resistant (23). Based on the premise that the conformation of an associated subunit may also be protected by K^+ or Rb^+ occlusion, as has been observed for the β subunit (7), we tested the effect of Rb^+ on trypsin digestion of the γ subunit. For these experiments, treated intact right side-out pig kidney microsomes were exposed to trypsin following an overnight pre-incubation (0°C) in the presence or absence of 10 mM RbCl. Fig. 4-7 shows two bands of apparent molecular weight 7.9 and 9.1 kDa on these 10% gels (c.f. 9.5 and 10.9 kDa on 16% gels reported in ref. 30). Only the upper band of the γ subunit is trypsin-sensitive and that digestion is much more extensive in the presence of Mg^{++}. Microsomes that were pre-incubated in the presence of Rb^+ were partially protected against digestion of the upper band.

Fig. 4-4 **Effect of anti-γ antibody binding on Na,K-ATPase and phosphoenzyme.**

Saponin-permeabilized membranes were pre-treated with 0.01 vol either non-immune serum (control; *solid bars*) or anti-γ antiserum (*open bars*), and Na,K-ATPase activity or phospohoenzyme levels were measured as described in Experimental Procedures. Results are averages ± S.D. of at least 3 separate experiments. A) Na,K-ATPase and phosphoenzyme without and with oligomycin as indicated. Fold increases in phosphoenzyme effected by oligomycin were 1.7 in non-immune serum treated samples and 2.4 in anti-γ-treated samples; B) Na,K-ATPase of kidney, axolemma, α1-transfected HeLa and red blood cells. Differences between non-immune- (control) and anti-γ-treated kidney membranes are significant. $p < 0.01$ (*) using the Student's *t* test.


*
*
120
100
80
60
40
20
0
Parameter Measured
(% of control)
Na,K-ATPase
EP (+olig)
EP (-olig)

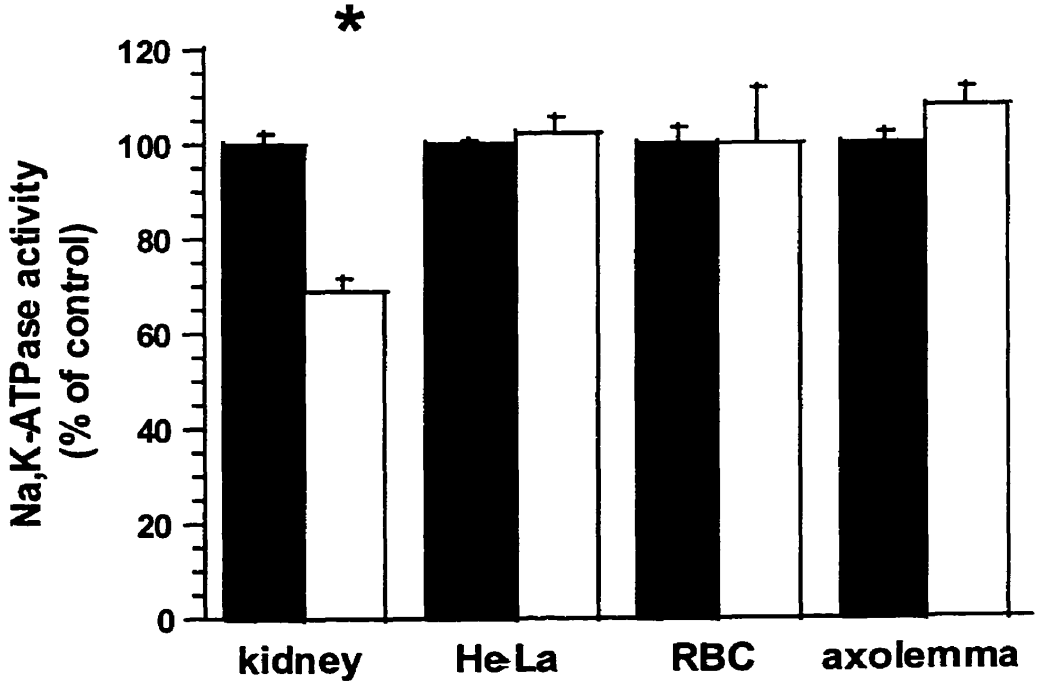

*
120
100
80
60
40
20
0
Na,K-ATPase activity
(% of control)
kidney
HeLa
RBC
axolemma

Fig. 4-5 **Effect of anti-γ antibody binding on Na-ATPase as a function of Na^+ concentration.**

Na-ATPase measurements were carried out at 1 μM ATP and varying Na^+ concentrations on non-immune serum-treated (control) and anti-γ serum-treated membranes as described in Experimental Procedures. Concentrations of Na^+ take into account the amount of Na^+ (150 mM) present in serum. Results are averages ± S.D. of at least 3 separate experiments. Differences between non-immune- (control) and anti-γ-treated membranes are significant. $p < 0.01$ (*) and $p < 0.02$ (**) using the Student's *t* test.

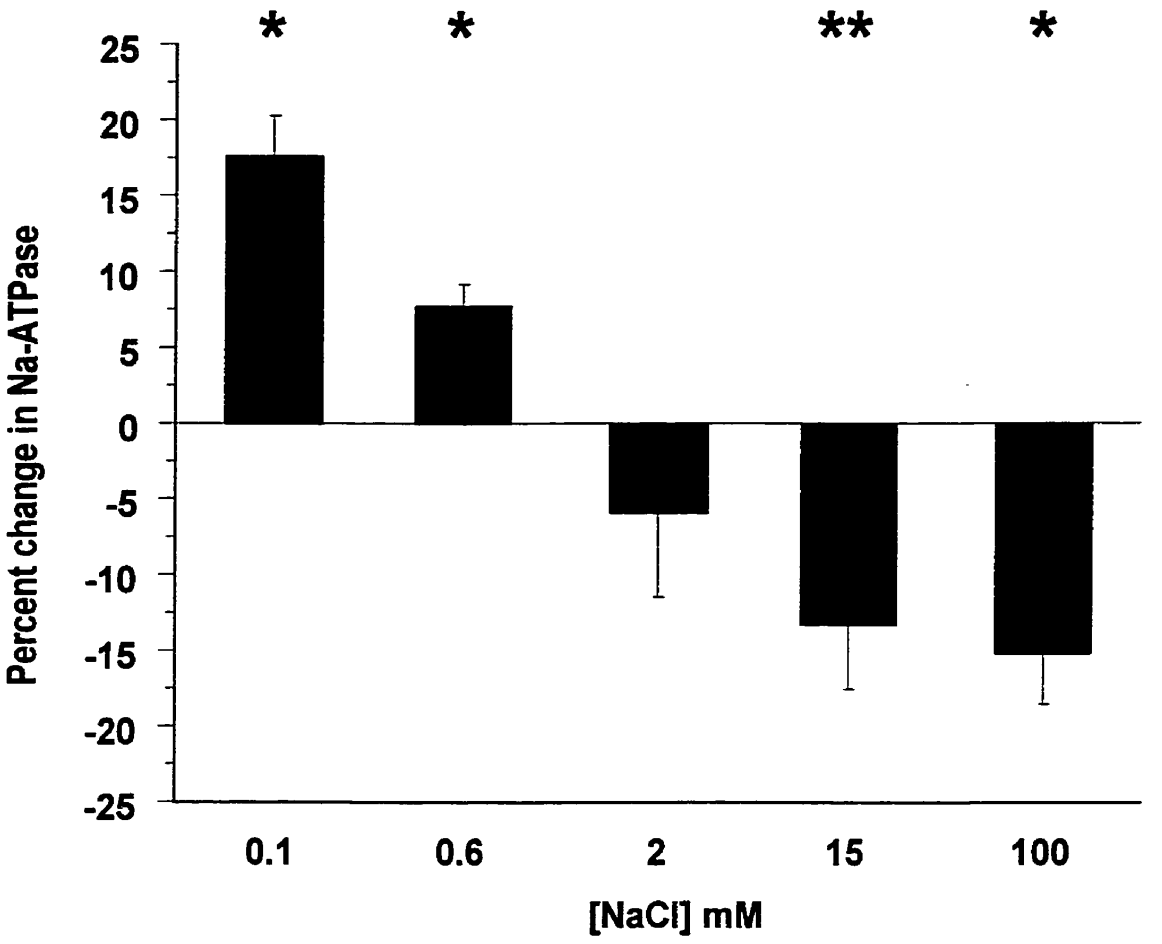

*
*
**
*
25
20
15
10
5
0
-5
-10
-15
-20
-25
Percent change in Na-ATPase
0.1
0.6
2
15
100
[NaCl] mM

Fig. 4-6 **Effect of pH on anti-γ antiserum-mediated inhibition of Na,K-ATPase.**

Rat kidney microsomes were pre-treated at pH 7.4 with either non-immune serum or anti-γ subunit antiserum, and Na,K-ATPase assays were carried out at pH 6.2, 7.4 or 8.9 using 30 mM MES-tris (pH 6.2) or Tris-HCl (pH 7.4 and pH 8.9) as described in Experimental Procedures. Results are expressed as percent inhibition and are averages ± S.D. of at least 3 separate experiments. Differences between pHs are statistically significant. $p < 0.01$ using the student's *t* test.

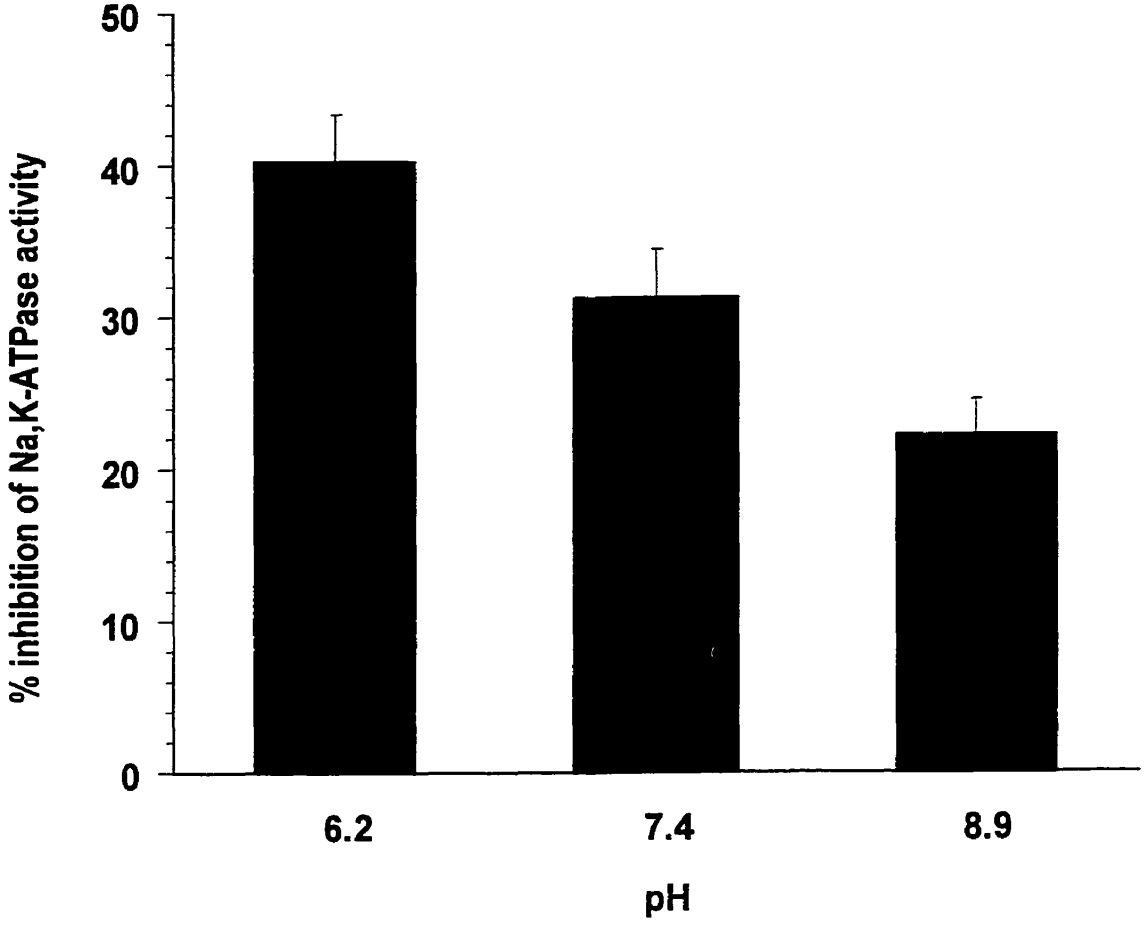
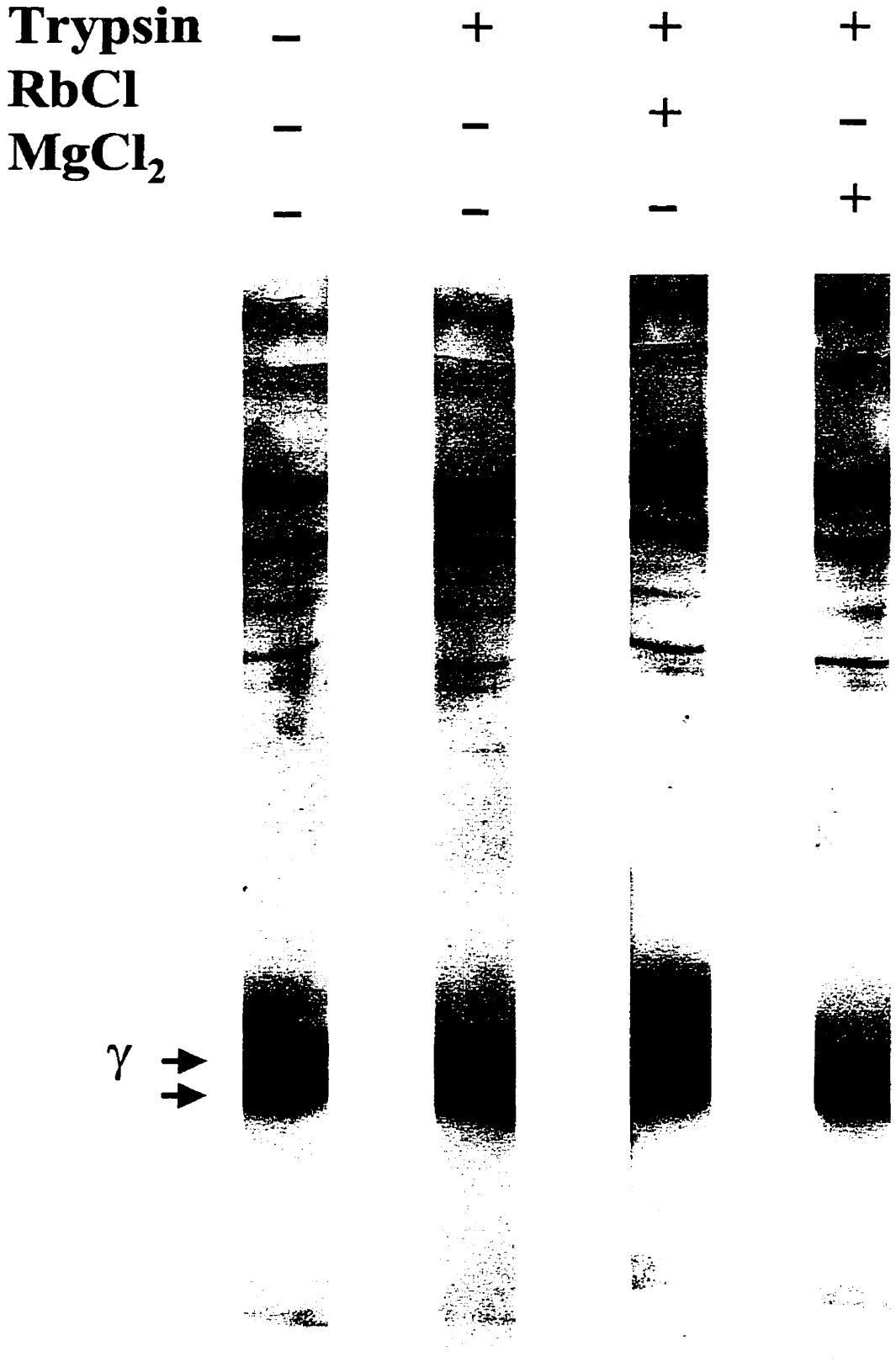

% inhibition of Na,K-ATPase activity
50
40
30
20
10
0
6.2
7.4
8.9
pH

Fig. 4-7 **Effect of trypsin treatment on γ subunit protein.**

Intact pig kidney microsomes were pre-treated with or without 10 mM RbCl or 2 mM $MgCl_2$, and digested with trypsin as described in Experimental Procedures. Electrophoresis (10% polyacrylamide gel) and Western blotting were carried out as described in Capasso et al., 1992 (ref. 24). The two bands of the γ subunit migrate at rates corresponding to molecular weights of 7.9 and 9.1 kDa.


Trypsin – + + +
RbCl – – + –
MgCl2 – – – +
γ

4.6 DISCUSSION

Since its discovery nearly 20 years ago, the tissue distribution and function of the γ subunit of the Na,K-ATPase have remained unknown. That it has an important role in enzyme function is an attractive possibility since its sequence is so highly conserved between species (6). Until recently, there was no clear evidence to support this notion. In this study, we show, for the first time, that the γ chain is not ubiquitous, at least in the rat. Moreover, among the tissues and cell lines examined, it is apparent in only kidney tubules. In this tissue, we have defined unequivocally its topology and obtained evidence of its involvement in Na,K-ATPase activity, consistent with the recent findings of Béguin *et al.* (3).

Initially, the sequence of the γ subunit could not be determined since its amino terminus is apparently blocked, preventing the use of the Edman degradation method of sequencing (7, 10). In 1987, Collins and Leszyk succeeded in sequencing a tryptic fragment of the sheep γ subunit (10), and more recently, Shainskaya and Karlish (34) determined the N-terminal sequence of an 8.1 kDa tryptic fragment which we now know is identical to part of the human γ subunit (24) which follows a trypsin cleavage site (Fig. 4-2; see also Table III, band c of ref. 34). The sequence homology between the tryptic fragment and human γ subunit (Fig. 4-2B) as well as the removal of the blocked NH_2-terminus after trypsin treatment of intact right side-out microsomes show that the N-terminus of the γ subunit is on the extracellular side of the membrane. The same orientation was reported recently (3) and is not unexpected, since it is consistent with studies showing that positive residues of transmembrane proteins are on the cytoplasmic side (39). Phospholemman, another small membrane protein with homology to the γ subunit, has also been shown to have this topology (31).

Although the close association of γ with the α and β subunits of the purified kidney Na,K-ATPase and its presence in a roughly 1:1 stoichiometry (3, 9, 22) with these subunits has led to the general belief that γ is an obligatory component of the Na,K-ATPase, its function appears to be more subtle in nature. The present study not only

confirms the earlier conclusion (3, 33) that the γ subunit is not a necessary subunit, but shows that it is not widely distributed and that its expression is not maintained during culture of renal tubular cells.

It could be argued that the γ subunit is, in fact, present in all tissues, and that Western blotting is too insensitive or that the protein is digested by proteases during membrane preparation. These possibilities are unlikely for the following reasons. Gamma subunit protein remained undetected when (i) gels were overloaded and Western blots, overexposed, and (ii) intact cells (HeLa, MDCK, LLC-PK) were dissolved in sample buffer and applied directly (not shown). In addition, the measured α:γ molar ratio of ≈1 (3, 9, 22) indicates that if γ is present in other tissues, its molar ratio in these tissues would be a small fraction of the ≈1:1 ratio of kidney.

The tissue specificity of γ subunit protein expression is consistent with the recent reports that γ mRNA is expressed only in the kidney and pancreas of human (24) and predominantly in the kidney of *Xenopus* (3). On the other hand, Mercer *et al.* (27) reported the presence in several rat tissues of a 1.5 kb γ-cDNA-reactive mRNA species, with an additional, smaller (0.8 kb) species predominant in the kidney, suggesting either that protein expression is translationally regulated in the rat, or that only the smaller mRNA leads to the mature protein.

Physiologically, this tissue distribution of γ has interesting implications. While in most tissues, the α1 isoform of the Na,K-ATPase has a housekeeping function, its role in the kidney is also specialized. In the nephron, the sodium pump is involved in Na^+ reabsorption, which in turn, regulates reabsorption of solutes such as glucose and amino acids (extensively reviewed in ref. 14). The kidney must therefore have multiple ways of regulating sodium pump activity, which implies kidney-specific modulators of Na,K-ATPase. Although there is extensive literature on the subject, and the effects of some of these modulators is partly understood (reviewed in ref. 15), it is also clear that many pathways of regulation have yet to be discovered and/or defined. The γ subunit may be a candidate for such regulation, as evidenced by its high degree of homology with several

other membrane peptides, namely PLM (Phospholemman), CHIF (CHannel Inducing Factor) and Mat-8 (Mammary tumor, 8 kDa). These proteins have been shown to induce ion currents across oocyte membranes (2, 28, 29), and are believed to be members of a family of ion channels or ion channel regulators. In light of this, it seemed reasonable to expect that the γ subunit might have a role in regulating the Na,K-ATPase in the kidney. Evidence in support of this notion has been obtained in studies of the effects of anti-γ antiserum on kinetic behavior.

There are several points of evidence supporting the conclusion that γ shifts the equilibrium between E_1 and E_2 in favor of the former such that anti-γ counteracts this effect. One is that anti-γ displaces $E_2(K) \leftrightarrow E_1$ in favor of $E_2(K)$ since inhibition of Na,K-ATPase activity by anti-γ is greater at acidic pH under which condition the $E_2(K) \leftrightarrow E_1$ reaction sequence becomes more rate-limiting (19). Second, the oligomycin experiment suggests that the $E_1 \leftrightarrow\leftrightarrow E_2P$ equilibria are poised towards E_2P in the presence of anti-γ. A third point is the reduced inhibition by anti-γ at high pH under which condition $E_1 \rightarrow\rightarrow E_2P$ becomes a major rate-limiting process (19). Fourth, when the reaction is carried out in the absence of K^+ (Na-ATPase activity), inhibition is observed only with sufficient Na^+ ions to act as K^+ congeners at extracellular sites. As the Na^+ concentration is reduced so that mainly cytoplasmic sites are occupied, inhibition is no longer observed; with Na^+ further reduced to levels (<2 mM) which limit the $E_1 \leftrightarrow\leftrightarrow E_2P$ transition (5), anti-γ becomes stimulatory. Relevant to the notion that γ alters the poise in the $E_1 \leftrightarrow E_2P$ equilibria in favor of E_1 is a recent experiment (Table II of ref. 13) showing that, under the same conditions as those used in this study, the oligomycin-stimulated increase in steady-state level of phosphoenzyme in the rat α1 enzyme transfected into HeLa cells (which lack γ) is 2.3-fold, which is similar to that of anti-γ-treated kidney enzyme (see legend to Fig. 4-4).

The aforementioned effect of γ on the $E_1 \rightarrow\rightarrow E_2P$ process may be relevant to the γ-mediated changes in voltage dependence of apparent affinity for extracellular K^+ reported recently in experiments with *Xenopus* (3) since (i) $E_1 \rightarrow\rightarrow E_2P$ is the major

voltage-sensitive phase of the reaction cycle (36), and (ii) an increase in rate of E_2P formation would alter the apparent affinity for K^+-activated dephosphorylation.

It should be noted also that the effects of the anti-γ antiserum are highly specific. First, the inhibitory effect was not observed with membranes isolated from tissues where γ protein is not expressed (Fig. 4-1A). Second, an effect on V_{max} was not observed with anti-α1-N-terminal serum. Third, a dose response curve (not shown) showed that inhibition increases with antiserum concentration and reaches a maximum of about 30% at 1:100, consistent with saturation of the binding sites with antibody rather than a non-specific effect.

The role of the gamma chain may be more complex than evident from studies with anti-γ. In particular, anti-γ inhibition of Na-ATPase at high Na concentration has two possible explanations: (i) anti-γ acts synergistically with cytoplasmic Na^+ to stabilize the $(Na_2)E_2$.ATP form of the enzyme since, as shown by Apell *et al.* (1), Na-ATPase becomes inhibited by Na^+ acting at high concentration on the cytoplasmic side to stabilize this E_2 form; or (ii) anti-γ inhibits the $E_2P \rightarrow E_2$ step which is activated by Na^+ acting at high concentration as a congener of K^+ at the extracellular side. If the latter holds true, an effect of γ, other than stabilization of E_1 state(s), is invoked and may cast doubt on the physiological significance of the kinetic effects. The observation (40) that the phosphoenzyme form of the kidney enzyme is predominantly in the K^+-sensitive (E_2P) conformation compared to the red blood cell enzyme also appears to contradict an E_1-stabilizing role of the γ subunit. However, those studies were carried out with enzyme from two different species (human red blood cells versus guinea pig kidney) and, more importantly, were performed at 0°C. Therefore it remains to be determined whether other, as yet unknown tissue-specific regulatory functions of γ are more important. At the very least, however, our results suggest that the γ subunit is in very close proximity to the α subunit and intimately involved with the catalytic function of the enzyme.

There are precedents for effects of specific antisera on membrane transport systems. Well-documented examples are effects of specific antibodies on cation transport

of genetically low-K^+ (LK) red blood cells of sheep and goats (reviewed in ref. 16). In these cells, one antigen, L_p, renders the Na,K-ATPase susceptible to non-competitive inhibition by intracellular K^+, while another, the L_l antigen, stimulates the K/Cl cotransport of LK red cells. The evidence for the effects of these antigens rests largely on the effects of isoimmune antisera. Thus, antisera to these antigens have been shown to either stimulate Na,K-ATPase activity (anti-L_p) or inhibit K^+/Cl^- cotransport (anti-L_l), presumably through the disruption of L antigen:transporter interactions. Similarly, the effects observed using our anti-γ serum may be the result of disruption of $\alpha\beta$–γ interactions secondary to antibody binding.

One of the more puzzling aspects of the γ subunit is that it migrates as a doublet on SDS-PAGE (27). This doublet is not a consequence of multiple isoforms or differential splicing, since it is formed in rabbit reticulocyte lysate in the presence of a single mRNA species (27). As in the kidney of rat, dog and pig (Fig. 4-1B), two polypeptide species are also observed in amphibian (*Xenopus laevis*) Na,K-ATPase (3). It seems fortuitous, however, that in *Xenopus*, the two species are products of translation initiated at the two initiator methionines identified in the cDNA; in the rat, a single initiator methionine is present (27), leaving open the possibility of posttranslational modification. Interestingly, the upper band of γ is trypsin-sensitive while the lower is not (Fig. 4-7). Complete trypsin digestion of the upper band requires Mg^{++}, but a partial decrease in immunological reactivity is also seen in the absence of the cation. Since both bands are the products of a single mRNA message (27), this differential trypsin effect may reflect a post-translational modification at or near the cleavage site. Since trypsin cleavage occurs at lysine residues which can be hydroxylated, acetylated or ribosylated post-translationally (11), this is an attractive possibility. Supporting this conclusion is the observation that the difference in mobility is retained after the bands are excised and eluted from a polyacrylamide gel and re-electrophoresed (30). Different modifications of the N-terminus, whether through proteolysis or covalent alteration seems unlikely since

the N-termini of both bands are similarly blocked to sequencing[1]. Covalent modification of the γ subunit could also explain the discrepancy between the observed migration of the protein on SDS-PAGE and the predicted molecular weight, and why the bands migrate at different rates depending on the percent acrylamide in the gel. Since the tryptic fragment resulting from cleavage of the γ subunit (Fig. 4-2A) migrates at a molecular weight of 8kDa as discussed above, it is expected to be indistinguishable from the intact lower band, which migrates at 7.9 kDa. Although sometimes difficult to discern and quantify, an increase in antiserum reactivity of the lower band was observed in some experiments (not shown), particularly when rat microsomes were used. Fig. 4-7 also shows that membranes incubated in the presence of 10 mM Rb^+ (a congener of K^+), are protected from the Mg^{++}-independent hydrolysis. This result is reminiscent of the Rb^+-imparted protection from tryptic digestion of both the α (23) and β (7) subunits, and presumably indicates that the γ subunit is protected from tryptic digestion when the pump is in the $E_2(K)$ state, and therefore involved in occlusion of K^+. Relevant to this conclusion is the observation (30) that the γ subunit is an integral part of the complex of peptide fragments found in so-called 19-kDa membranes.

Taken together, these results indicate that γ may have a role in regulating the renal Na,K-ATPase, possibly by stabilizing E_1 conformations. With γ-specific antiserum, it is shown for the first time that the γ subunit protein is not ubiquitous. The absence of the γ subunit in most tissues, and its similarity to putative ion channel modulators, show that its presence is not required for normal function of the Na,K-ATPase, but rather that it may act as a modulator of activity. Furthermore, the protective effect of Rb^+ ions on tryptic digestion of the γ subunit suggests that γ may be involved in K^+ occlusion/deocclusion, which is intimately linked to, and dependent upon the $E_2 \leftrightarrow E_1$ conformational equilibrium.

[1] S.J.D. Karlish, unpublished observation

4.7 ACKNOWLEDGMENTS

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CHAPTER 5

EXPRESSION AND FUNCTIONAL ROLE OF THE γ SUBUNIT OF THE NA,K-ATPASE IN MAMMALIAN CELLS

5.1 PREFACE

This manuscript has been published in the Journal of Biological Chemistry (Therien, A.G., S.J.D. Karlish, and R. Blostein. *J. Biol. Chem.* 274: 12252-12256, 1999). It is an extension of the previous chapter, in that it describes experiments combining biochemical and molecular techniques aimed at further characterization of the functional role of the γ subunit.

Dr. Steven J.D. Karlish provided his unpublished results and insightful suggestions.

5.2 ABSTRACT

The functional role of the γ subunit of the Na,K-ATPase was studied using rat γ cDNA-transfected HEK-293 cells and an antiserum (γC33) specific for γ. Although the sequence for γ was verified and shown to be larger (7237 Da) than first reported, it still comprises a single initiator methionine despite the expression of a γC33-reactive doublet on immunoblots. Kinetic analysis of the enzyme of transfected compared to control cells, and of γC33-treated kidney pumps shows that γ regulates the apparent affinity for ATP. Thus, γ-transfected cells have a decreased K'_{ATP} as shown in measurements of (i) K'_{ATP} of Na,K-ATPase activity and (ii) K^+-inhibition of Na-ATPase at 1 μM ATP. Consistent with the behavior of γ-transfected cells, γC33 pre-treatment increases K'_{ATP} of the kidney enzyme, and K^+-inhibition (1 μM ATP) of both kidney and γ-transfected cells. These results are consistent with previous findings that an antiserum raised against the pig γ subunit stabilizes the $E_2(K)$ form of the enzyme (Therien *et al.*, J. Biol. Chem.(1997) **272**: 32628-32634). Overall, our data demonstrate that γ is a tissue (kidney)-specific regulator of the Na,K-ATPase that can increase the apparent affinity of the enzyme for ATP in a manner which is reversible by anti-γ antiserum.

5.3 INTRODUCTION

The Na,K-ATPase is the sodium pump protein responsible for maintaining the electrochemical gradient present across the membranes of most animal cells (11). It consists of at least two subunits, α and β, each of which exists as one of several isoforms (α1, α2, α3, α4 and β1, β2, β3; for review, see ref. 3). The α subunit, also known as the catalytic subunit, contains the binding sites for the enzyme's nucleotide and cation substrates, as well as the catalytic and regulatory (PKC and PKA) phosphorylation sites. The role of the β subunit is less clear, but it is required for normal processing and expression of the enzyme, and may have a role in regulating the interaction of cations with the α subunit (6). The different isoforms of the pump are expressed in a tissue- and developmental-specific fashion, and are believed to be distinct in both function and modes of regulation (3).

A small single transmembrane protein called the gamma subunit was originally believed to be a third subunit of the pump. It was discovered by Forbush *et al.* in 1978 (10) and later cloned in rat, mouse, cow, sheep (16), human (14), and *Xenopus laevis* (2), and has sequence homology to a family of channel-inducing peptides (1, 19, 20). Although its function has remained elusive, experiments in *Xenopus* oocytes have shown that the γ subunit alters the K^+-affinity of the pump in a voltage- and Na^+-dependent fashion (2) and may induce cation channel activity (17). Our recent western blot analysis using an anti-gamma antiserum indicated that γ protein is present in only the kidney medulla, but not in other tissues tested (red cells, heart, brain, kidney glomerulus) including cultured cell lines derived from cells of the kidney tubule. We showed that the antibodies bound to the cytoplasmic tail of γ and stabilized the E_2 form of the enzyme, presumably by disrupting α–γ interactions (23).

In this report we show that expression of γ in cells devoid of this protein results in a significant increase in apparent affinity for ATP and that the gamma-transfected cells resemble the α1β1γ kidney enzyme in that this effect is abrogated by antiserum raised

against a 10-residue peptide of the carboxy terminus of the gamma subunit.

5.4 EXPERIMENTAL PROCEDURES

Antibodies. γC33 is a rabbit polyclonal antiserum raised against a peptide representing the C-terminal 10 amino acids of the γ subunit. In the experiments reported herein, γC33 was used and a control non-immune serum was obtained from the same rabbit prior to immunization. The peptide, KHRQVNEDEL, was synthesized at the Alberta Peptide Institute, University of Alberta, and used either as the free peptide for competition studies, or linked to KLH and emulsified with Freund's adjuvant before injection into rabbits. Antibody 6H is a mouse monoclonal antibody specific for the α1 isoform of the Na,K-ATPase, and was a generous gift from Dr. Michael Caplan, Yale University. Horseradish peroxidase-labeled secondary antibodies (donkey anti-rabbit) were purchased from Bio/Can Scientific.

5'-RACE and pREP4-γ cDNA Synthesis. 5'-Rapid Amplification of cDNA Ends (5'-RACE) was carried out using Clontech's Marathon-ready cDNA from rat kidney and following manufacturer's instructions. Appropriate primers (see below) were synthesized and the γ subunit gene sequence was amplified by PCR. The 5'-end primer contained a site for Hind III endonuclease (bold-face), a Kozak sequence (underlined; see ref. 15) and the first 24 bases of the γ subunit gene as determined by 5'-RACE (GGGGGGG**AAGCTT**GCCGCCACCATGACAGAGCTGTCAGCTAACCAT). The 3'-end primer contained a Bam HI endonuclease site (bold-face) and bases complimentary to the last 24 bases of the γ subunit gene as determined by Mercer *et al.* (16) (GGGGG**GGATCC**GTCACAGCTCATCTTCATTGACCT). The resulting DNA was then cleaved with these endonucleases and ligated into the corresponding sites of pREP4 vector (Invitrogen) to make pREP4-γ. Sequencing of the recombinant plasmid was carried out using Pharmacia's T4 sequencing kit. pREP4 and pREP4-γ DNA used for transfections was purified using Qiagen's affinity columns according to the manufacturer's instructions.

Transfections, Tissue Culture and Membrane Preparations. HEK-293 cells at 50% confluency in a 14 cm culture plate were transfected with pREP4 or pREP4-γ using Boeringher Mannheim's FuGENE 6 reagent, and following the manufacturer's instructions. Cells were selected for 10 days, divided among 5 x 14 cm plates, and allowed to grow to confluency (about 3 weeks) in D-MEM containing 10% newborn calf serum and 400 μg/ml hygromycin B. Cellular membranes from transfected cells and from rat kidney medulla were prepared by the procedure described elsewhere (23).

Western Blots. Western blot analysis and densitometry measurements were carried out as described previously (24) with the following modifications: 10% polyacrylamide gels were run on a Protean II gel electrophoresis apparatus (BioRad), transferred to PVDF membranes (Millipore) and blotted with 6H antibodies and γC33 antiserum, both at dilutions of 1:10,000.

Enzyme Assays. Na,K-ATPase and Na-ATPase assays were carried out at 37°C in a final volume of 100 μl as described previously (23). For Na,K-ATPase assays, final concentrations of reactants were: 100 mM NaCl, 10 mM KCl, 40 mM choline chloride, 4 mM $MgSO_4$, 1 mM EDTA, 30 mM Tris-HCl (pH 7.4), and varying concentrations of ATP as indicated. Na,K-ATPase activities shown represent the ATPase activities inhibited by 5 mM ouabain, and ranged from 1500 to 4500, 130 to 180 and 110 to 130 nmol P_i/mg/min for kidney, HEK-pREP4 and HEK-pREP4-γ membranes, respectively. For Na-ATPase assays, final concentrations of reactants were: 20 mM NaCl, 20 mM choline chloride, 2 mM $MgSO_4$, 1 mM EDTA, 5 mM EGTA, 20 mM histidine-Tris (pH 7.4), and 1 μM ATP. To determine effects of K^+ on Na-ATPase, choline chloride was replaced by the indicated concentrations of KCl. For assays of effects of anti-γ, membranes were pre-incubated for 1 hour at 4°C in the presence of immune (γC33) or non-immune (pre-immune) sera at a ratio of 1:100. For experiments of K-inhibition of Na-ATPase, the sera were dialyzed for 48 hours at 4°C against three changes of 1000 vol 5 mM Imidazole (pH 7.4). When present, the 10-mer peptide was used in the antiserum preincubation at a concentration of 20 μM. K'_{ATP} values were calculated by analyzing

ATP-activation curves using the Michaelis-Menten formulation. All experiments shown are representative of at least three separate experiments, and each data point shown is the mean ± S.D. of the difference between triplicate determinations carried out in the absence and presence of ouabain.

5.5 RESULTS

We showed previously that the γ subunit protein is expressed in a tissue-specific manner. Of the various rat tissues analyzed by western blotting (kidney medulla, kidney glomerulus, red cells, heart, axolemma), γ was detected in only the kidney medulla (23). In more recent experiments (not shown) this analysis has been extended to additional tissues of the rat, namely lung, small intestine, stomach and spleen. The γ protein could not be detected in these tissues except for a trace amount in spleen (relative to α, amounting to ≈ 2% of that present in the kidney medulla). The kidney-specific presence of γ also holds true with mouse tissues (kidney, axolemma, heart) analyzed similarly[1].

Expression of the γ subunit in mammalian cells. Our earlier evidence for a modulatory role of the γ subunit on the conformational equilibrium of the Na,K-ATPase reaction was inferred from studies of the effects of an anti-γ antiserum on enzymatic activity. To evaluate directly the functional role of γ, it was essential to transfect cDNA encoding γ into mammalian cells devoid of γ. An additional goal of such experiments was to establish the basis for the existence of γ as a doublet in the rat (16) as in the *Xenopus* kidney (2). Accordingly, we first used 5'-RACE to ascertain that the previously reported cDNA of the rat γ subunit comprised the full-length sequence and, if not, whether the doublet in western blots is secondary to the presence of an additional start codon in the mRNA for the γ subunit as is the case of *Xenopus* kidney (2). The resulting sequence shown in Fig. 5-1 confirmed the presence of a single initiator methionine. However, the γ cDNA thus obtained encodes a protein of 66 rather than 58 residues as originally reported (16), and corresponds to that subsequently revised by Minor *et al.* (11). The calculated molecular weight is 7237 Da. The dichotomy may be due to either a cloning artifact or, possibly, an isoform variant[2].

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[1] A. Therien, R. Daneman, and R. Blostein, unpublished observations.

[2] R.W. Mercer, personal communication.

Efforts to express γ in HeLa and HEK cells using a standard stable transfection system resulted in levels of expression which, compared to the kidney, were considered to be too low (γ:α = 0.1) given the relatively modest effects of anti-γ on the kidney enzyme. In an effort to increase the level of expression, we used a system which combines the advantages of both 'classical' (transient and stable) expression systems and in which the plasmid pREP4 is used. In addition to a hygromycin resistance gene, this plasmid contains an origin of replication which allows it to remain expressed episomally for several weeks in the nuclei of primate and canine cells. Thus, hygromycin can be used to select for cells which contain multiple copies of the gene (rather than just one). Accordingly, we subcloned the gene for γ (revised sequence shown in Fig. 5-1) in pREP4 and transfected HEK-293 cells with both recombinant and wild type plasmids. Membranes were made from the transfected HEK-pREP4-γ and control HEK-pREP4 cells and the amount of γ subunit protein relative to α subunit protein was estimated by comparison to kidney membranes using western blot analysis of both the γ and α subunits.

The blots shown in Fig. 5-2 indicate that the γ doublet is present in both the kidney and HEK-pREP4-γ membranes but not in control HEK-pREP4 membranes. The densities of the γ subunit doublet and α subunit band of HEK-pREP4-γ were compared to those of kidney using several dilutions and varying times of exposure to film. We determined that pREP4-γ membranes contain 34 ± 12% (S.E.M.) of the amount of γ present in kidney after normalizing for α1 densities. Assuming that the γ:α ratio of kidney is 1:1 (2, 7, 12), this indicates that the stoichiometry of the γ:α proteins in HEK-pREP4-γ ≈1:3. That this ratio reflects γ associated with α was confirmed in western blots of immunoprecipitates using the antibody 6H (not shown).

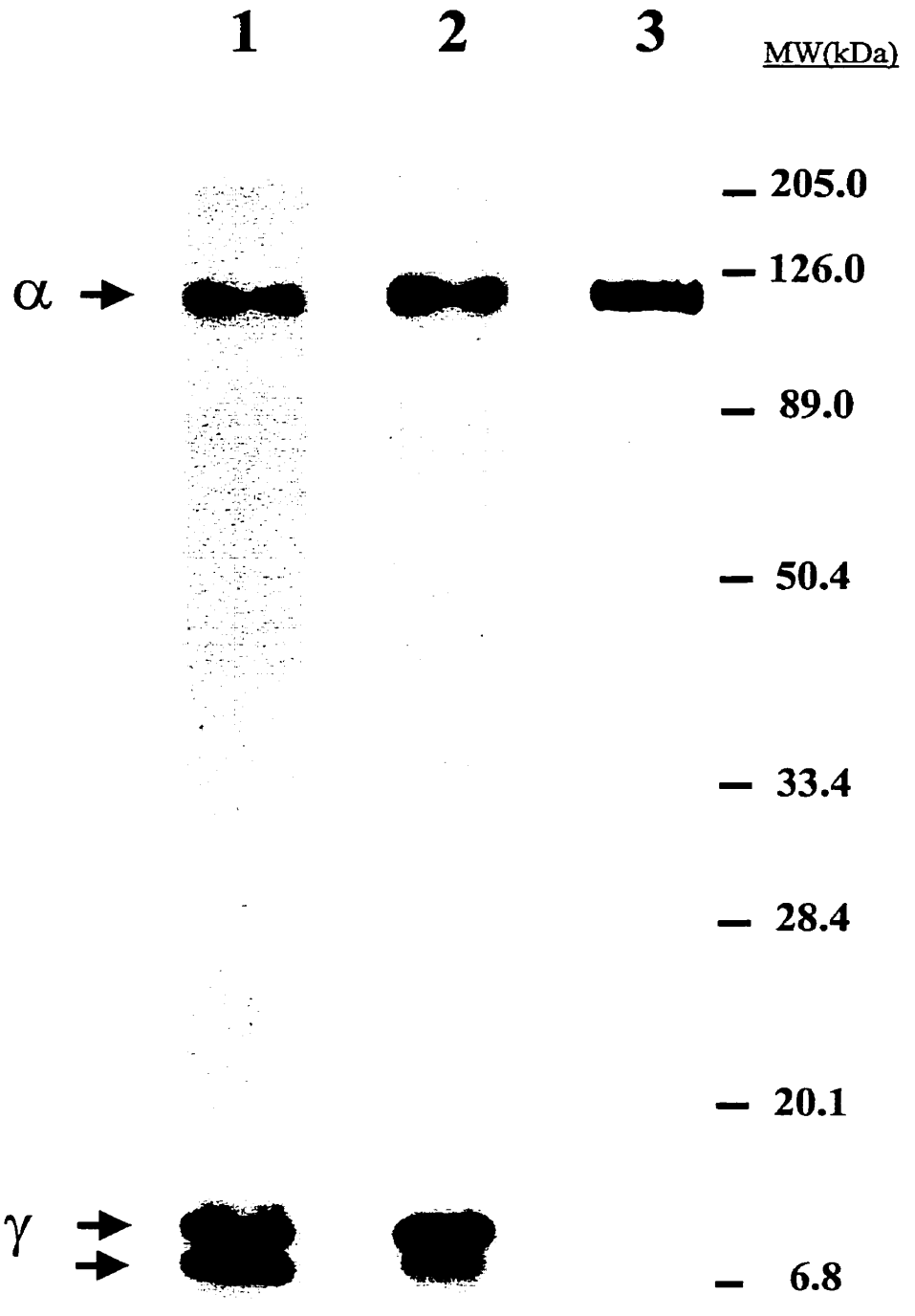
Fig. 5-1 **5'-untranslated and coding regions of rat γ subunit cDNA and deduced amino acid sequence.**

Nucleotide sequence was determined by 5'-RACE analysis as described in "Experimental Procedures".

```
CACAGGAAGATCGTGGAGAAGCCCTGGTGGCTGGGGAAATGACAGAGCTGTCAGCTAACCATGGT

        (5'-untranslated region)          M  T  E  L  S  A  N  H  G      9

GGCAGTGCCAAGGGGACGGAGAATCCCTTCGAGTATGACTATGAAACCGTCCGCAAAGGAGGCCTG

 G  S  A  K  G  T  E  N  P  F  E  Y  D  Y  E  T  V  R  K  G  G  L     31

ATCTTCGCGGGCCTTGCCTTCGTCGTGGGACTCCTCATTCTCCTCAGCAAAAGATTCCGCTGTGGG

 I  F  A  G  L  A  F  V  V  G  L  L  I  L  L  S  K  R  F  R  C  G     53

GGCAGTAAGAAGCATAGGCAGGTCAATGAAGATGAGCTGTGA

 G  S  K  K  H  R  Q  V  N  E  D  E  L stop                            66
```

Fig. 5-2 **Western blot analysis of rat kidney, HEK-pREP4-γ and HEK-pREP4 membranes.**

Immunoblotting was carried out as described in "Experimental Procedures". Lane 1: 2.0 μg rat kidney membranes; lane 2: 30 μg HEK-pREP4-γ membranes; lane 3: 30 μg HEK-pREP4 membranes.


1
2
3
MW(kDa)
205.0
126.0
89.0
50.4
33.4
28.4
20.1
6.8
α
γ

Functional effects of γ. We showed earlier that binding of antibodies raised to the γ polypeptide doublet associated with the pig kidney Na,K-ATPase binds to the cytoplasmic tail of the γ subunit (23). This binding was associated with partial inhibition of the Na,K-ATPase activity. Moreover, inhibition varied as a function of conditions which affect the rate-limiting step(s) during steady-state hydrolysis, for example, varying pH. Thus, the inhibition (≈30%) observed under conditions of optimal concentrations of substrates and at pH 7.4, decreased as pH increased, and increased as pH decreased. We concluded that the antiserum caused a shift in the $E_1 \leftrightarrow E_2(K)$ equilibrium towards $E_2(K)$.

In order to maximize the inhibitory effect of the antiserum, particularly for tests of the effect of γ in the transfected cells in which the γ:α ratio is lower than in the kidney medulla, we tested the prediction that inhibition would be greater at suboptimal ATP concentrations, under which conditions the $E_2(K) \rightarrow E_1$ sequence becomes even more rate-limiting (21). For these experiments, a 10-residue peptide representing the C-terminus of the γ subunit was synthesized and used for the production of γC33 antisera. This allowed confirmation of the specificity of the anti-γ effects and provided free 10-mer peptide for competition studies. Fig. 5-3A shows a representative experiment on the effects of anti-γ (serum γC33) on Na,K-ATPase activity of renal enzyme at near-saturating (1 mM) and subsaturating (10 μM) concentrations of ATP. As predicted, inhibition increases as the ATP concentration is lowered, from 36 ± 4% inhibition at 1 mM ATP to 70 ± 11% at 10 μM ATP (averages of several experiments). In addition, the presence of excess amounts of free peptide corresponding to the C-terminus of γ during the preincubation reversed completely the inhibition observed at both ATP concentrations; no effect on the activity of non-immune serum-treated enzyme was observed. Fig. 5-3B is a Lineweaver-Burke plot of a representative experiment showing the effect of ATP concentration on activity. It shows that pre-treatment of the enzyme with antiserum γC33 caused a 1.8-fold increase in K'_{ATP} (for values, see inset of Fig. 5-4 below). V_{max} for γC33-treated enzyme was 78 ± 7% that for non-immune serum-treated enzyme. The critical implication of this result is that anti-γ reverses an increase in affinity effected by the γ subunit. This was tested in HEK-pREP4-γ cells and HEK-pREP4 cells.

We first compared the effect of γC33 on HEK-pREP4-γ, HEK-pREP4 cells and on kidney enzymes, all assayed at 10 μM ATP. The experiment (not shown) indicated that γC33 caused 33 ± 2% and 82 ± 15% inhibition of HEK-pREP4-γ and kidney enzymes, respectively, and had no effect on the activity of HEK-pREP4 cells. This inhibition is consistent with the aforementioned relative amounts of γ in kidney versus HEK-pREP4-γ cells. Experiments were then carried out to determine whether the γ subunit has any effect on K'_{ATP}. The plots shown in Fig. 5-4 indicate that the HEK-pREP4-γ enzyme has a significantly higher affinity for ATP compared with control HEK-pREP4 enzyme (for K'_{ATP} values, see inset). The γ-mediated 1.3-fold decrease in K'_{ATP} in these cells, while modest, is, in fact, similar to the effect of γ in the kidney membranes taking into account the lower α:γ ratio in the transfected cells (approximately one-third that of kidney membranes). This being the case, we used a more sensitive assay of ATP affinity to magnify the effect of γ and to determine whether anti-γ antiserum can reverse its effects. This assay takes advantage of the fact that K^+ inhibits Na-ATPase activity at very low (1 μM) ATP concentration under which condition the (low affinity) ATP-activated K^+-deocclusion reaction becomes rate-limiting. Accordingly, this inhibition decreases as the affinity for ATP at its low affinity binding site increases (8). As shown in Fig. 5-5A, K^+ is less effective at inhibiting Na-ATPase activity of pumps of γ-transfected membranes compared to control membranes. Experiments were then carried out to test and compare the K^+-inhibition and effect of anti-γ thereupon, of the enzyme of kidney medulla, HEK-pREP4-γ and HEK-pREP4. Fig. 5-5B shows the percentage inhibition at 0.2 mM KCl of these pumps in the presence of non-immune versus immune serum. Whereas pre-incubation of kidney and pREP4-γ pumps with γC33 effects 2.1-fold and 1.5-fold increases in K^+-inhibition, respectively, no γC33-mediated change is detected for HEK-pREP4 pumps.

Fig. 5-3 **Effect of γC33 antiserum and γC33-reactive peptide on ATP affinity of renal pumps.**

A) Rat renal membranes were assayed for Na,K-ATPase activity at 100 μM or 1 mM ATP after preincubation in the presence of γC33 antiserum (IS) or non-immune rabbit serum (NIS) and in the absence or presence of peptide representing the C-terminal 10 amino acids of the γ subunit (used to generate γC33). Differences between non-immune and immune serum-treated enzyme are significant ($p < 0.01$ using Student's t test). B) Rat renal membranes were assayed for Na,K-ATPase activity at different ATP concentrations after pre-incubation in the presence of γC33 (empty circles) or non-immune serum (filled circles). Lineweaver-Burke plots of a representative experiment are shown.

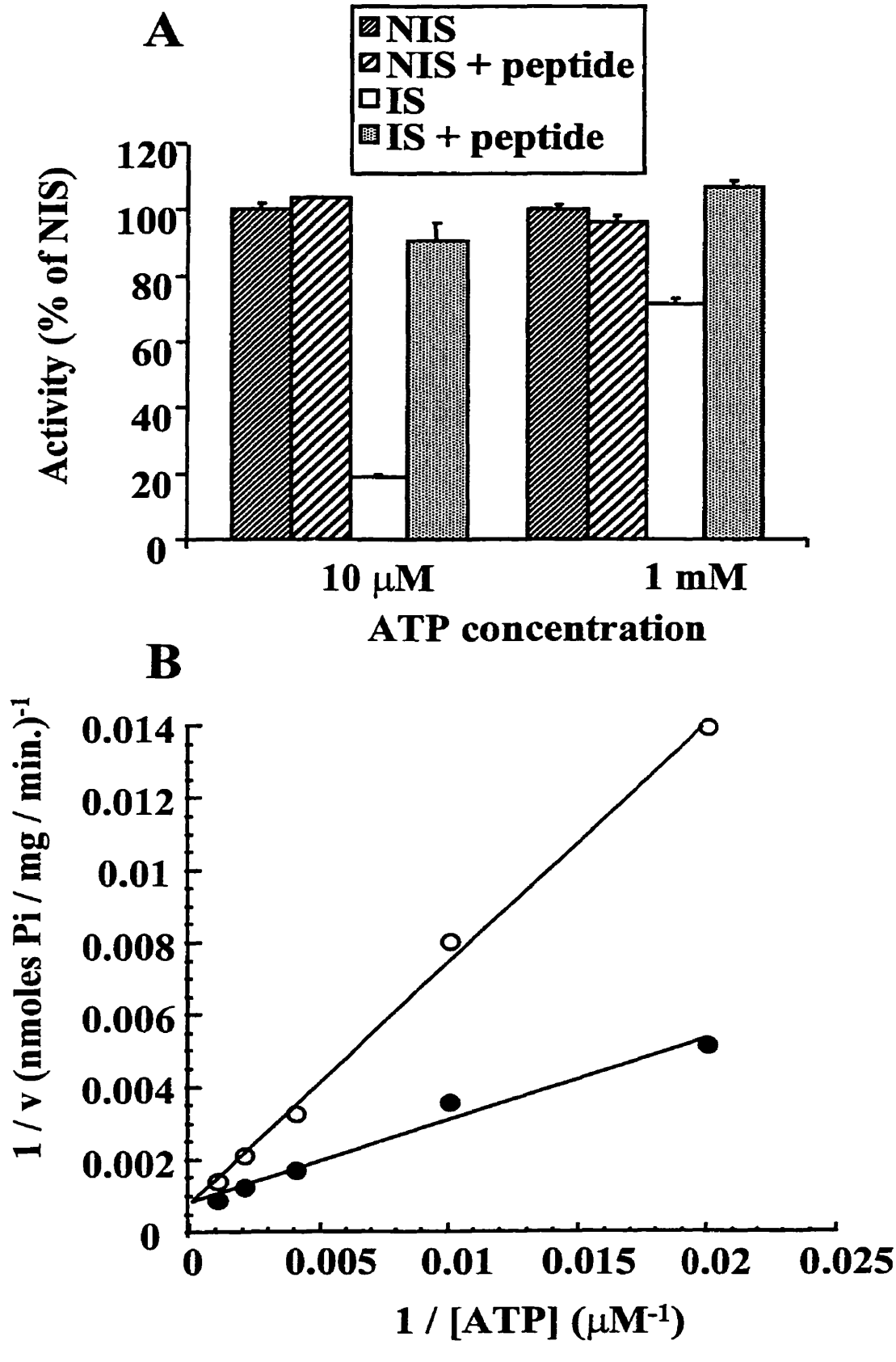

A
NIS
NIS + peptide
IS
IS + peptide
Activity (% of NIS)
120
100
80
60
40
20
0
10 μM
1 mM
ATP concentration
B
1 / v (nmoles Pi / mg / min.)-1
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
0
0.005
0.01
0.015
0.02
0.025
1 / [ATP] (μM-1)

Fig. 5-4 **Effect of γC33 on ATP affinity of HEK-pREP4 and -pREP4-γ pumps.**

Membranes isolated from HEK-pREP4-γ (filled circles) and HEK-pREP4 (empty circles) were assayed for Na,K-ATPase activity at varying ATP concentrations. Lineweaver-Burke plots of a representative experiment are shown, with V_{max}'s of the two membrane preparations normalized to 1.0. *Inset:* Table summarizing K'_{ATP} values for γC33- and non-immune serum-treated kidney enzyme, and HEK-pREP4-γ and HEK-pREP4 enzymes. IS represents γC33 and NIS represents non-immune serum. Differences between non-immune and immune serum-treated renal enzyme ($p < 0.01$), as well as between pREP4- and pREP4-γ-transfected cells ($p < 0.02$) are significant using Student's *t* test.

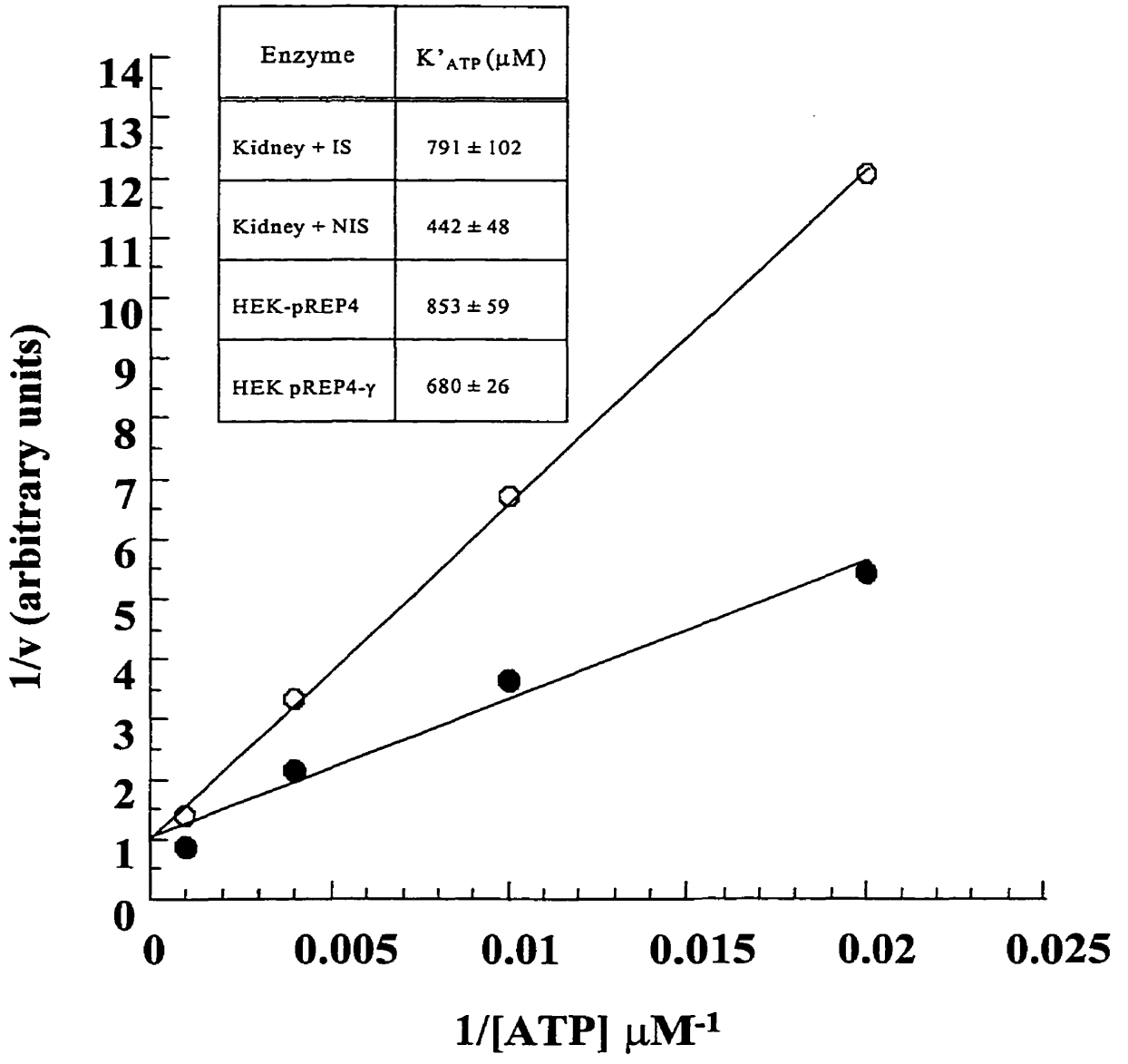
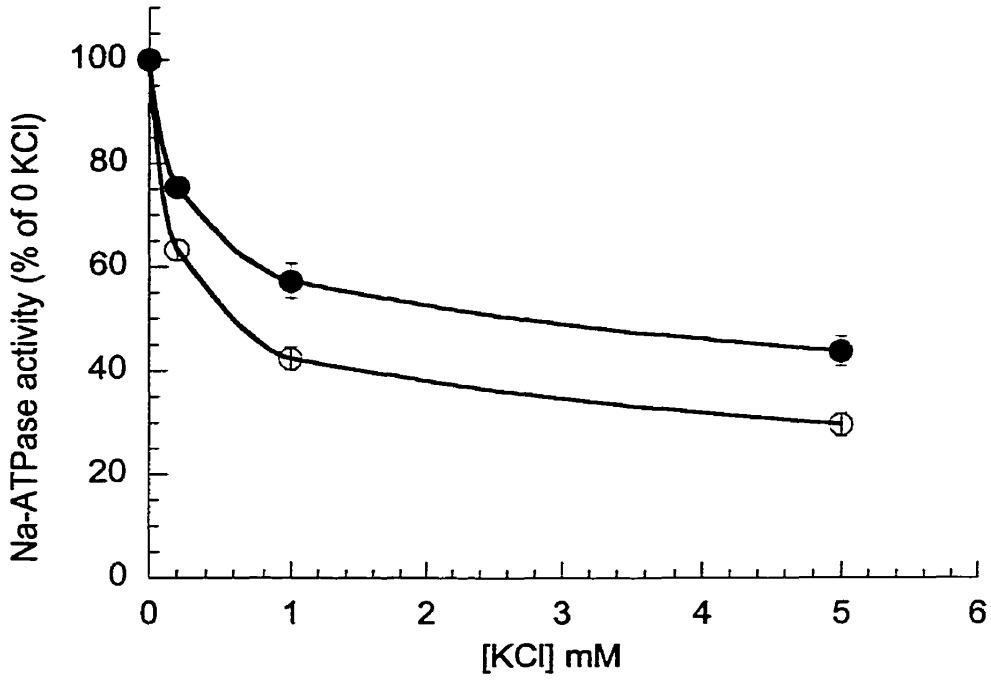

Enzyme
K'ATP (μM)
Kidney + IS
791 ± 102
Kidney + NIS
442 ± 48
HEK-pREP4
853 ± 59
HEK pREP4-γ
680 ± 26
1/v (arbitrary units)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
0
0.005
0.01
0.015
0.02
0.025
1/[ATP] μM-1

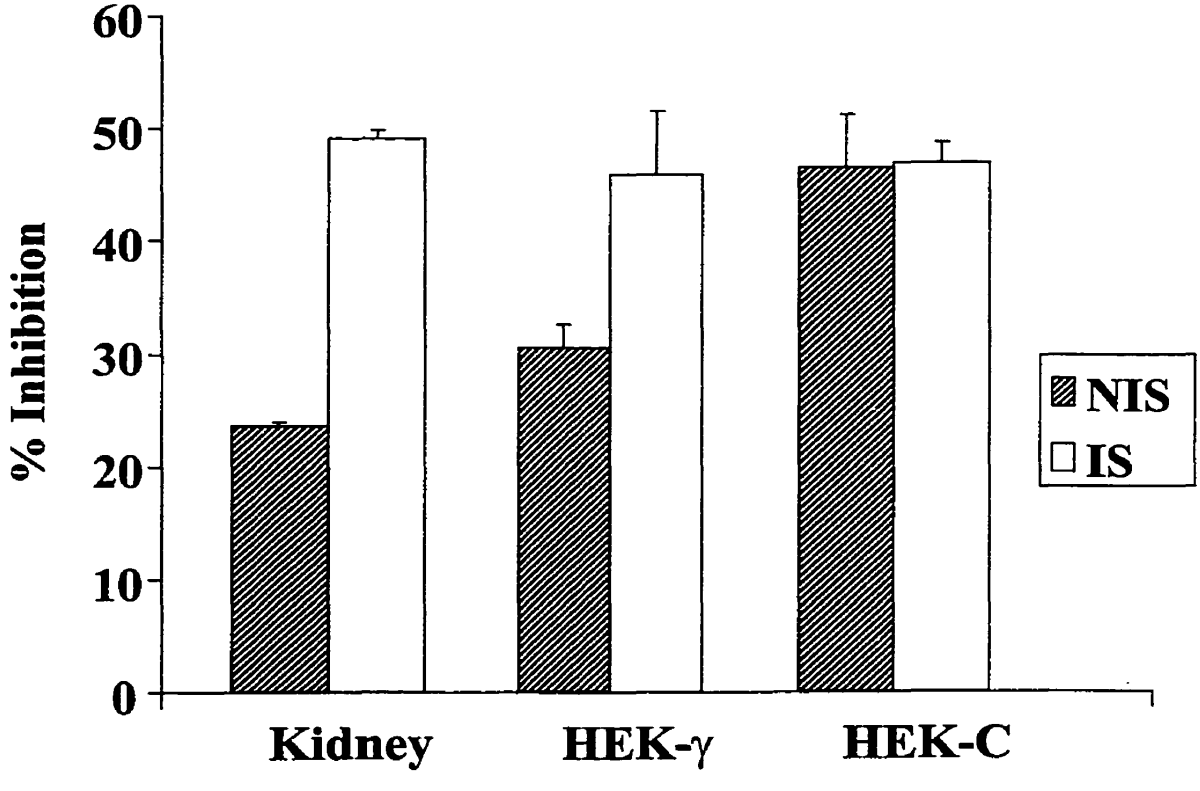
Fig. 5-5 **K^+-inhibition of Na-ATPase activity of rat kidney, HEK-pREP4 and HEK-pREP4-γ pumps and the effect of γC33.**

Membranes were assayed for Na-ATPase activity in varying concentrations of KCl as described in "Experimental Procedures". A) K^+-inhibition profile of pREP4- (empty circles) and pREP4-γ- (filled circles) transfected cells. B) Inhibition of Na-ATPase activity in the presence of 0.2 mM KCl after preincubation with γC33 or non-immune serum. Differences between non-immune and immune serum-treated kidney ($p < 0.01$) and HEK-pREP4-γ enzymes ($p < 0.02$) are significant using Student's *t* test.

A


Na-ATPase activity (% of 0 KCl)
100
80
60
40
20
0
0
1
2
3
4
5
6
[KCl] mM

B


% Inhibition
60
50
40
30
20
10
0
Kidney
HEK-γ
HEK-C
NIS
IS

5.6 DISCUSSION

The successful transfection of the γ subunit into mammalian cells with sodium pumps devoid of this subunit has enabled the direct analysis of the functional role of this Na,K-ATPase-associated protein. Although the γ subunit does not appear to be necessary for normal Na,K-ATPase activity (2, 12, 22), its role as a modulator of function is consistent with its appearance in a tissue (kidney) -specific manner.

Recently, Béguin *et al.* (2) have shown that the rat γ subunit lowers the affinity of the pump for K^+ in cRNA-injected *Xenopus* oocytes, at least in the absence of Na^+. A γ-mediated decrease in K'_{ATP} could explain this increase in $K'_{0.5}$ for K^+ since, as a first approximation, ATP- and K^+- affinities are inversely related (9). However, that result may be confounded by the use of cRNA synthesized using the original sequence for rat γ (16). In a recent report, the human γ subunit was shown to induce cation channel activity in *Xenopus* oocytes (17), consistent with several reports of other channel-inducing membrane peptides (1, 18, 19). These proteins have homology with the γ subunit, but are generally larger and some contain possible PKA and PKC phosphorylation sites at their C-terminal ends that are not present in the γ subunit (1, 16, 19, 20). Although we have no information regarding such a role in our transfected cells, it should be pointed out that the sequence of the putative human γ subunit reported in the aforementioned study contains 30 extra amino acids at its N-terminus (17) which are absent in rat γ (c.f. Fig. 5-1). Whether the rat γ subunit also has channel function and/or this extended N-terminus confers a particular functional role in forming channels in *Xenopus* oocytes remains to be determined.

The N-terminal sequence of the rat γ subunit reported here and by Minor *et al.* (11) is different from the one originally reported (5). That it is the correct sequence is substantiated by the following observations. First, the γ subunit doublet present in membranes of transfected cells corresponds in size to that of kidney membranes (Fig. 5-2). Second, the presence of a lysine residue at position 13 (Fig. 5-1) is in accordance with

the finding that the upper band of the rat γ subunit is cleaved by trypsin (treatment of intact right-side-out microsomes; ref. 23). Third, preliminary results using MALDI-TOF mass spectroscopy indicate that the pig kidney gamma subunit has a length of between 64 and 67 residues[3], consistent with a length of 66 reported here and in ref. 11.

The presence of two distinct bands of gamma has been the subject of some controversy. Whereas Mercer *et al.* first showed that a single RNA species could yield two protein products evidenced on western blots using an artificial translation system (16), Béguin *et al.* showed that in *Xenopus laevis*, the two bands were secondary to the presence of two distinct start codons (2). Our results with 5'-RACE analysis precludes the presence of distinct ATG codons for the rat protein, but indicate instead that post-translational modifications are involved, since transfection of HEK-293 cells with a gene containing single start and stop codons yielded two bands of similar mobilities to those of the kidney γ subunit. In addition, preliminary mass spectroscopy results are consistent with the notion that the difference between the two bands is the result of post-translational modifications[3]. The differences in the ratio of the densities of upper to lower band between gamma subunits of kidney and transfected HEK (see Fig. 5-2) suggest tissue-specific variations in post-translational modifications. Whether each band has some distinct role remains to be determined.

Overall, our results suggest an interaction between the Na,K-ATPase and the C-terminal tail of the γ subunit which regulates ATP affinity, and which is reversible upon binding of antibodies to γ. The finding that γ increases the apparent affinity for ATP in γ-transfected cells is completely concurrent with the effect of anti-γ on the αβγ pump of the kidney tubule. Moreover, under conditions in which K^+-sensitivity of Na-ATPase at low ATP concentration is used as a sensitive marker of differences in ATP affinity, the reversal of the γ effect by anti-γ is similar with the enzyme of γ-transfected cells and the kidney medulla. These similarities underscore our earlier interpretation of the effect of γ from analysis of the effects of the anti-γ antiserum. Whether the increased apparent

[3] A. Shainskaya and S.J.D. Karlish, unpublished observations.

affinity for ATP is, in fact, a true increase in affinity, or a reflection of an alteration in conformational equilibrium towards E_1 form(s) is unclear and requires further analysis, as does the question of whether the difference in ATP affinity can be evidenced in a change in apparent affinity for extracellular K^+. Whatever the case, it is the change in ATP affinity which is likely to be of major physiological relevance.

The increase in apparent affinity for ATP effected by γ is approximately 2-fold as evidenced in either the effect of anti-γ on the kidney enzyme or of γ transfected into HEK cells, extrapolating the ratio of γ:α in HEK-pREP-γ to that of the kidney. Such a change in apparent affinity may be of critical physiological importance. While other physiological functions may be served by the γ subunit (as suggested recently in ref. 13), a near 2-fold shift in ATP-affinity is a potentially important regulatory mechanism. The gamma subunit may serve to preserve the pumping activity in cells or conditions in which the ATP level falls suddenly. Relevant to this notion is the observation that the renal outer medulla is highly prone to becoming anoxic because it works on the brink of anoxia even in normal circumstances (4, 5). That the γ subunit effect is reversible upon addition of anti-γ antibodies further underscores its physiological relevance. It may be hypothesized that some cytosolic factor, like the anti-γ antibodies, bind to the γ subunit and disrupt its interactions with the enzyme. Mutational analysis of the C-terminal 10 amino acids which comprise the epitope reactive with anti-γ may provide information on specific residues involved in α–γ interactions.

5.7 ACKNOWLEDGMENTS

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CHAPTER 6

GENERAL DISCUSSION

Following the discovery of the Na,K-ATPase in 1957, several reports on the kinetics on its interactions with its various ligands have been published. In general, it has been observed that the kinetic behaviour of the sodium pump depends on the nature of the tissue from which it is isolated (for example, see refs. 459, 464, 476, 494). In some instances, the tissue-specific behaviour of the enzyme is related to the presence of different isoforms in various tissues. However, until recently, considerable controversy has remained regarding the role of isoform diversity in determining the affinity of the Na,K-ATPase for its ligands, in particular nucleotide and cation substrates. The available data supports the notion that there exist factors other than the nature of the catalytic isoform per se that determine the kinetic behaviour of the sodium pump. This thesis describes two aspects of tissue-specific modulation of pump behaviour which are particularly relevant to tissues in which the sodium pump has a highly specialized role and must respond to changing cellular and physiological needs.

6.1 TISSUE-SPECIFIC MODULATION OF CATION ACTIVATION KINETICS

Tissue-specific differences in Na^+- and K^+-activation of the Na,K-ATPase have frequently been described. For example, several groups have compared the Na^+-activation kinetics of the kidney and neuronal (brain, axolemma or pineal gland) enzymes. In general, their studies have shown that Na,K-ATPase of kidney has a lower affinity for Na^+ than that of brain, axolemma or pineal gland (459, 464, 477, 494). Since pumps of neuronal tissues are comprised mostly of $\alpha 2$ and $\alpha 3$ isoforms while kidney pumps contain solely the $\alpha 1$ isoform, these results suggested that $\alpha 1$ has a lower affinity for Na^+ than $\alpha 2$ and $\alpha 3$. However, experiments using cells expressing the individual isoforms of the rat enzyme failed to substantiate this interpretation. Thus, with membranes preparations from both HeLa cells (244) and Sf-9 cells (51), the order of apparent affinities for Na^+ is $\alpha 2 \approx \alpha 1 > \alpha 3$. Furthermore, Rb^+ transport studies on transfected HeLa cells carried out by Munzer *et al.* (351) showed even greater isoform-specific differences in affinity for Na^+, albeit in the same direction, than had been obtained with the porous membrane preparations (244). These authors also showed that following

fusion into the same membrane environment (red blood cell), kidney pumps (α1) have a higher affinity for Na^+ than axolemma pumps (α2 and α3). Taken together, the results of Jewell and Lingrel (244), Munzer *et al.* (351) an Blanco *et al.* (51) are at odds with the aforementioned studies (459, 464, 477, 494). In order to resolve these dichotomies, it was desirable to first characterize the cation-activation kinetics of the enzymes of various tissues and cells containing a preponderance of one isoform or the other (Chapters 2 and 3).

The results presented in Chapter 2 and 3 show tissue-specific, isoform-independent differences in the apparent affinities for both Na^+ and K^+. The most intriguing tissue-specific difference in kinetic behaviour is the high K'_{Na} value associated with the enzymes of some tissues (see Figs. 2-1A and 3-1A). In agreement with the model proposed by Garay and Garrahan (180), we found that the apparent sodium affinity constants (K'_{Na}) of α1 and α3 pumps of various tissues vary linearly as a function of K^+ concentration (Figs. 2-4 and Fig. 3-2). Analysis of our data using this model enabled us to derive kinetic constants for the affinities of the enzymes for Na^+ (K_{Na}) and K^+ (K_K) at cytoplasmic cation binding sites for the various tissues (Table 2-2 and Table 3-1). This analysis shows that differences in Na^+-activation are attributable to distinct affinities for both (i) Na^+ as an activator, and (ii) K^+ as a competitive inhibitor. Overall, the susceptibility of the pump to K^+/Na^+ antagonism determines its apparent affinity for Na^+. Furthermore, there exist dramatic tissue-specific differences in the ratio K_{Na}/K_K, the paramter that describes this antagonism. We also showed that distinct susceptibilities to K^+/Na^+ antagonism are due neither to the presence in these tissues of distinct β isoforms (Fig. 2-5 and Sections 2.6 and 3.6), nor to interactions of the enzyme with the γ subunit (Section 3.6). Our results reconcile differences between various reports of isoform-specific kinetics, and show that the nature of either the α or β isoform is not the only determinant of cation-activation kinetics of the enzyme. Furthermore, our work has provided insight into the structural and mechanistic basis for tissue-specific differences in K^+/Na^+ antagonism, as described below.

As mentioned above, a clue as to the structural basis for these tissue-specific

differences in Na^+-activation kinetics had been provided by Munzer *et al.* (351), who showed that the order of Na^+-affinities for the kidney and axolemma enzymes upon fusion into red blood cells was opposite that obtained previously using broken membrane preparations of the two tissues (476). We hypothesized that the basis for this discrepancy is that components of the membrane of kidney cells tend to increase the susceptibility of the sodium pump to K^+/Na^+ antagonism, and that components of the red cell membrane decrease it, such that fusion of kidney pumps into red cells abrogates K^+/Na^+ antagonism. To test this, we compared the Na^+-activation profiles of kidney pumps before and after fusion into erythrocytes, and showed that the nature of the membrane can alter this kinetic parameter, and therefore that the high susceptibility to K^+/Na^+ antagonism characteristic of the kidney enzyme is reversible (Fig. 3-4). Although the structural basis of this modulation remains unclear, this 'reversibility' is consistent with the conclusion that a loosely-bound membrane component, presumably a lipid or protein, is involved.

Results with the fused kidney pumps allow for interpretation of previous results obtained in this laboratory. In those studies, rat kidney pumps were fused into human red cells, and the kinetic profiles of the ouabain-sensitive (human) and relatively ouabain-resistant (rat) components were compared (350). Although the K^+-activation profiles were identical, the affinity of the rat enzyme for Na^+ was consistently lower than that of the human enzyme. Furthermore, there was a direct correlation between values of V_{max} and K'_{Na} of the exogenous enzyme obtained from different fusion experiments. The finding that the membrane environment affects K^+/Na^+ antagonism, and consequently is a determinant of K'_{Na} values, may explain this phenomenon. In some fusion experiments, it may well be that 'over-loading' of the red cell membrane with kidney membrane components impairs dilution of the kidney components by the red cell membrane. Accordingly, if we assume that, compared to that of the red cell, the membrane environment of the kidney promotes high susceptibility to K^+/Na^+ antagonism (Fig. 3-4), fusion experiments resulting in relatively high numbers of exogenous kidney pumps (high V_{max}) would result in higher K'_{Na} values promoted by the kidney membrane. On the other hand, fusions resulting in fewer exogenous pumps (low V_{max}) would result in lower K'_{Na}

values effected by better dilution into the red cell membrane.

Our finding that the nature of the membrane environment is a major determinant of the kinetic behaviour of the sodium pump is especially relevant to tissues in which the Na,K-ATPase has specialized physiological roles in addition to general 'housekeeping' functions. Since kidney and heart pumps appear to be most susceptible to K^+/Na^+ antagonism (Table 3-1), it is likely that this mechanism of pump modulation is important in these tissues. The specialized role of the Na,K-ATPase in kidney tubules, for example, is to regulate Na^+ and K^+ reabsorption and/or secretion, as described in the Section 1.3.1. As such, there are presumably a number of regulatory mechanisms that enable the kidney to adapt to constant changes in dietary sodium and potassium. The data presented in this thesis suggest that one of these regulatory mechanism involves modulations in K^+/Na^+ antagonism at cytoplasmic cation activation site(s). Such a regulatory mechanism is physiologically plausible, since the ratio of K^+ to Na^+ in the cytoplasm of most cells is normally at least 10, such that under normal circumstances, the renal sodium pump is minimally activated. Thus, a mechanism whereby the susceptibility of the enzyme to competitive inhibition by cytoplasmic K^+ is altered would allow great flexibility in regulation of Na,K-ATPase activity (compare the relative percent maximal activities of pumps of various tissues at 10 mM NaCl and 50 mM KCl in Fig. 3-1A). In addition, such a mechanism would allow short-term regulation of pump activity, not requiring *de novo* pump synthesis. It should be pointed out that alterations in susceptibility to K^+/Na^+ antagonism could be evidenced in changes in either K'_{Na} or the apparent maximal activity under physiological Na^+ and K^+ concentrations. Accordingly, modulators whose mechanisms of action have not yet been defined may, in fact, involve alterations in K^+/Na^+ antagonism. For example, the decrease in K'_{Na} of renal pumps effected by insulin (see Section 1.3.7.b and ref. 152) could conceivably be secondary to a decrease in K^+/Na^+ antagonism mediated by the hormone.

In the heart, the sodium pump is an indirect determinant of contractile strength since cytoplasmic Na^+ affects Ca^{++} levels via the plasma membrane Na^+/Ca^{++} exchanger (56; see also section 1.3.1) . While it is known that hearts of mature rats contain only the

α1 and α2 isoforms (see for example ref. 376), the precise role of these two isoforms remains unknown. By decreasing the levels of the α1 or α2 isoform in heterozygous null mutant mice, James *et al.* (240) have recently provided evidence in favor of distinct roles for the α1 and α2 isoforms in the heart. They observed that hearts of heterozygous α2 null mutants are hypercontractile, while those of heterozygous α1 null mutants are hypocontractile. Their data suggest that α2 promotes weak contractions by maintaining low cytoplasmic Na^+, and hence low Ca^{++} levels, while α1 favors stronger contractions by maintaining comparatively high levels of both cations. These results are at least partly compatible with our observation that heart α1 pumps are highly susceptible to K^+/Na^+ antagonism (Fig. 3-1A and Table 3-1), since these pumps would be expected to maintain a higher cytoplasmic Na^+ concentration. Recent structural studies on arterial smooth muscle support the notion that the two isoforms have distinct roles in myocytes. Blaustein and co-workers (57, 58) have shown the existence of distinct functional regions where the plasmalemmal and sarcoplasmic reticular membranes are in close association. Furthermore, these units, referred to as plasmerosomes, are the sole sites of expression of Na,K-ATPase α2 and α3 isoforms as well as Na^+/Ca^{++} exchangers, while sodium pump α1 isoforms are distributed homogeneously throughout the plasma membrane. Such a pattern of sodium pump isoform expression supports a role of the α2 isoform of heart in regulation of contraction, consistent with the recent observation that the α2 isoform is the target of modulation by cardiac glycosides in the heart (240). Based on our results and those discussed above, we propose specific roles for the two isoforms present in heart. Pumps comprising the α1 isoform appear to keep the heart generally hypercontractile, while the those comprising the α2 isoform act to temper the basal level of contractility imposed by α1. As such, regulation of α2 by various mechanisms, including, perhaps, inhibition by endogenous cardiac glycosides, allows for fine-tuning of the heart's contractility. Alternatively, or possibly in addition, mechanisms that modulate the susceptibility of the α1 isoform to K^+/Na^+ antagonism may be important in regulating heart contractility, as suggested by the reversibility of this parameter in the renal enzyme (Fig. 3-4).

To gain insight into the mechanism of K^+/Na^+ antagonism at cytoplasmic cation binding sites we studied the binding and occlusion of K^+ at these sites using an indirect assay for K^+ binding and occlusion. Karlish *et al.* (266) had previously shown that interactions between the cation and the dephosphorylated enzyme occur while the enzyme is in the E_1 form, presumably at cytoplasmic binding sites. Therefore, we predicted a direct a correlation between K^+-occlusion and the ability of K^+ to compete with Na^+, the physiological ligand for this site. Taking into account tissue-specific differences in the rate of K^+-deocclusion, such a correlation was indeed observed (compare column "K_K" of Table 3-1 with column "k_o" of Table 3-2). These results provide evidence that the site(s) to which Na^+ ions bind and from which K^+ ions are released, is (are) one and the same. Interestingly, Sachs had previously shown that cytoplasmic Na^+ and K^+ compete for the same binding site during K^+/K^+ exchange, in a manner similar to that observed for Na,K-ATPase by Garay and Garrahan (426). Taken together with our results, pumps of tissues such as kidney and heart, that are more susceptible to K^+/Na^+ antagonism, should catalyze K^+/K^+ exchange more readily and be less susceptible to competitive inhibition of this non-physiological exchange by Na^+. This prediction has yet to be tested.

In addition to the tissue-specific differences in K^+/Na^+ antagonism discussed above, our analysis of the K^+-activation kinetics of α1 pumps reveals a strikingly lower affinity of α1 pumps of axolemma for this cation compared to other α1 pumps (Fig. 2-1B). This finding is of potential physiological significance since at such a high $K'_{0.5}$ value, extracellular concentrations of K^+ may be subsaturating, particularly in neuronal tissues (481). Our studies have also revealed tissue-specific differences in the kinetic behaviour of α3 pumps. In particular, α3 pumps of HeLa cells have both a higher apparent affinity for K^+ and a lower apparent affinity for Na^+ than the α3 isoforms of either axolemma or pineal gland (Table 2-1 and Fig. 2-2). Furthermore, the low affinity of these pumps for Na^+ is related to their dramatically high susceptibility to K^+/Na^+ antagonism, similar to our findings on heart and kidney pumps. This finding provides an explanation for the aforementioned increase in the differences in Na^+-affinities between the α1 and α3 isoforms in transport measurements on intact cells (351) compared to

permeabilized membranes (244). Thus, the high susceptibility of α3 pumps of HeLa to K^+/Na^+ antagonism combined with the high K^+ concentration in intact cells causes a dramatic decrease in the affinity of these pumps for Na^+ in transport experiments (Fig. 2-6 and Section 2.6). The kinetic distinctiveness of the α3 isoform of HeLa cells is further underscored by recent experiments from our laboratory showing that HeLa α3 pumps have a much lower (2- to 3-fold) catalytic turnover than α3 pumps of pineal gland or axolemma[1]. The basis for the kinetic distinctiveness of α3 pumps of HeLa is unknown, but it should be pointed out that these pumps are mutated at the two residues flanking TM1 and TM2 to render them relatively ouabain resistant (referred to as α3*; see ref. 244 and Section 1.2.4). That these mutations affect the catalytic behaviour of these pumps cannot be ruled out, although similar mutations did not effect these changes in α2 pumps in the same system (244, 351).

6.2 TISSUE-SPECIFIC REGULATION OF THE APPARENT AFFINITY FOR ATP BY THE γ SUBUNIT

In 1993, Mercer and co-workers (334) cloned and sequenced a small polypeptide, called the γ subunit, which had previously been shown to associate with the Na,K-ATPase. Their study showed also that γ subunit mRNA is expressed in a complex tissue-specific manner, although the functional role of the peptide remained unknown. Accordingly, part of our initial effort to gain insight into the molecular basis for tissue-specific differences in K^+/Na^+ antagonism (Chapters 2 and 3) was to test various rat tissues for the presence of the γ subunit. Although a relationship between susceptibility to K^+/Na^+ antagonism and expression of the γ subunit was not found, our experiments aimed at detecting and quantifying the γ subunit of various rat tissues showed that of all tissues tested, expression of γ protein is restricted to kidney tubules (Fig. 4-1). This intriguing finding prompted us to pursue the question of the functional role of the γ subunit in the renal sodium pump.

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[1] S.E. Daly, L. Segal, and R. Blostein, unpublished observations

In a first series of experiments, we analyzed the topology of the γ subunit and found it to be C-terminus-in, N-terminus-out (Fig. 4-2), consistent with a similar topology of other members of the family of proteins to which the γ subunit belongs, and consistent also with von Heijne's 'positive inside' rule (506). We also sought to gain insight into the structural basis for the migration of the γ subunit as a doublet on Western blots. The expression of a doublet in γ subunit-transfected HEK cells (Fig. 5-2) precludes alternate splicing as a possibility. It has recently been reported that the basis for the doublet in *Xenopus laevis* is the presence of a second initiator methionine (31). To determine whether this observation extends to other species, we sequenced part of the rat γ subunit cDNA using 5'-RACE (Fig. 5-1). Although the resulting sequence was longer and different from the one originally published, it was consistent with experiments (448; see also Fig. 4-2) showing that the N-terminus of the γ subunit is trypsin sensitive (the old sequence was reported to contain a glutamate rather than a lysine at the trypsin-sensitive site; compare sequence from Fig 5-1 with that of ref. 334) and corresponded more closely to observed migration rates in polyacrylamide gels (see Chapter 4). The sequence confirms the presence of a single ATG codon but also reveals an in-frame CTG located four codons downstream of the initiator ATG (Fig. 5-1) which could represent an alternative site for translational initiation. Thus, CTG has been shown to be capable of initiating translation (386), and the presence of a purine at position –3 relative to CTG fulfills the minimal requirements for translational initiation (284). Another possible explanation for the presence of a doublet on SDS-PAGE involves differential post-translational modification, whether chemical modification of residues or cleavage of the N-terminus. Supporting this possibility is the observation that only the upper band of γ is trypsin–sensitive (Fig. 4-7). Interestingly, the migration pattern of the mouse γ subunit doublet is different from that of the rat, even though these two proteins differ only at two positions (Leu^{45} and Ser^{55} of the rat are Ile and Gly, respectively, in the mouse; see ref. 334)

In order to gain insight into the functional role of the γ subunit, we examined the effects of our anti-γ antiserum on the function of the sodium pump of rat kidney. Our

initial finding that anti-γ inhibits Na,K-ATPase catalytic turnover in kidney, but not in tissues that do not express γ (Fig. 4-4), provided good evidence that the effects were specific. The experiments showing that a peptide corresponding to the epitope of the antiserum can abrogate the effect (Fig. 5-3A) confirmed the specificity of the effect. Further analysis of the functional effects of anti-γ suggested that it stabilizes the E_2 form(s) of the enzyme. The pH-dependence of the anti-γ-mediated inhibition of Na,K-ATPase activity, combined with the observation that Rb^+ protects against tryptic digestion of the γ peptide (Figs. 4-6 and 4-7) was consistent with a role of anti-γ in shifting the equilibrium of the K^+-deocclusion reaction ($E_2(K) \rightarrow E_1$) towards $E_2(K)$. Based on the well-documented effects of anti-L_p antigen on the kinetics of the low-K^+ sheep red blood cell Na,K-ATPase, we hypothesized that anti-γ mediates its effects by disrupting interactions between the Na,K-ATPase complex and the γ subunit, such that the role of the γ subunit is to shift the aforementioned equilibrium towards E_1. By transfecting the γ subunit into HEK cells, we were able to show that this was indeed the case.

The experiments on transfected cells showed that the γ subunit stabilizes the E_1 conformation of the Na,K-ATPase by increasing the affinity of the enzyme for ATP at its low affinity site (Fig 5-3, 5-4 and 5-5). I was able to show that anti-γ reverses this increase in affinity in transfected cells, and that the previously observed modest inhibition of Na,K-ATPase activity in the renal enzyme was increased at subsaturating concentrations of ATP, consistent with the hypothesis that anti-γ reverses γ-mediated effects. Although these data are consistent with a role of the γ subunit in increasing the affinity of the sodium pump for ATP, it is plausible that such an increase is secondary to a decrease in the affinity of the enzyme for K^+, since the apparent affinities for these two substrates are inversely related (144). In addition, the report by Béguin *et al.* (31) that the rat γ subunit can alter the enzyme's affinity for K^+ in a voltage- and Na^+-dependent manner would seem to support a role of γ in modulating K^+-affinity. However, recent

experiments in our laboratory argue against this conclusion[2]. While anti-γ antibodies alter the enzyme's affinity for K^+ at subsaturating ATP concentrations, this effect diminishes as the ATP concentration increases; at 1 mM ATP, no anti-γ-dependent effects on K^+-affinity are observed. These results are consistent with a direct effect of γ on ATP affinity, and not on K^+-binding. For similar reasons, it is unlikely that the shift in ATP affinity is secondary to direct effects on the conformational equilibria of the enzyme.

What is the physiological relevance of a regulator of the affinity of the sodium pump for ATP? In most cells, ATP levels are sufficient to saturate the Na,K-ATPase. Therefore, a modest shift in ATP affinity should not have dramatic effects. However, there are cases where ATP levels in intact cells are dramatically lowered (30% to 90%), such as during anoxic shock. The relationship between anoxia, or hypoxia, and cellular ATP concentration has been studied in many tissues (20, 241, 282, 306, 337, 377, 469). Of particular interest is the recent study by Milusheva *et al.* (337) who reported that in rat striatal muscle slices, incubation under hypoxic conditions for a relatively short period of time (30 minutes) can decrease cytoplasmic ATP levels to 1.11 nmol/mg protein. Soltoff and Mandel's approximation of 2.4 μl intracellular volume/mg protein (467), albeit for kidney cells, can be used to estimate a value of less than 500 μM ATP under these conditions, a value well below saturation (for example, see ref. 469). In addition, it has been observed that ATP levels may not be homogeneous in the cytoplasm. Thus, Aw *et al.* (20) observed a near total inhibition of sodium pump-mediated Rb^+ uptake in hepatocytes under conditions where ATP levels dropped a mere 40%, whereas the activity of a cytosolic ATPase (ATP-sulfurylase) decreased proportionately to ATP concentration. Based on these results, these authors suggested that the 'effective' ATP concentration near the plasma membrane may be lower than that nearer the mitochondria, even in the absence of membrane compartmentalization. Combined, these two observations show that anoxia can lead to decreases in ATP available to the sodium pump.

[2] H. Pu, A.G. Therien, and R. Blostein, unpublished observations

It might be argued that in the aforementioned studies, anoxia was induced artificially, and that such conditions may not be relevant to situations *in vivo*. However, recent studies have shown that even in normal, disease-free organisms, at least one tissue, the kidney medulla, must function under near anoxic conditions (reviewed in refs. 80, 105). The role of the kidney medulla in reabsorption of water precludes the presence in this tissue of extensive vascularization and blood supply, since reabsorption occurs by maintaining the extra-nephronic environment at severely hyperosmotic levels. As such, blood supply must be balanced in the medulla to keep the cells in this tissue oxygenated, while maintaining osmotic pressure at required levels. In fact, the oxygen pressure in cells of the medulla has been observed to be between 2.5- and 5-fold lower than in cells of the kidney cortex (79, 80). Accordingly, the kidney medulla represents one case where even under normal physiological conditions, oxygen, and by extension ATP, is at a premium.

As is the case in most segments of the nephron, water and solute reabsorption and secretion in the medulla is under the control of the sodium pump. As such, continued pumping is crucial for proper kidney function. However, Soltoff and Mandel (469) have reported that a reduction in oxygen pressure, a common occurrence in this tissue as described above, leads to drops in sodium pump activity secondary to decreases in ATP concentration. Therefore, the existence of a reversible regulator of Na,K-ATPase ATP affinity would allow for fine tuning of sodium pump activity under ATP-depleted conditions. This regulator should alter the pump's affinity for the nucleotide only moderately, for an excessive increase would effect even greater decreases in ATP concentration (469), leading to compromised cell viability. The data presented in this thesis are consistent with the kidney-specific γ subunit being such a regulator.

In addition to providing information about the functional role of the γ subunit in a mammalian system, our work has provided evidence for specific interactions between γ and the sodium pump complex. Although the precise domain(s) responsible for the aforementioned effects of γ remain(s) unknown, it is interesting that an antiserum directed against the C-terminal 10 amino acids of the polypeptide (Chapter 5) abrogates its

functional effects. Indeed, analysis of the sequence of the antiserum's epitope reveals the presence, in the last four amino acids, of two (in the proteins of *Xenopus*, sheep and cow) or three (in rat, mouse and human) negatively charged residues. What role this negative charge may have is unknown, but one could speculate that it may be involved in electrostatic interactions with the Na,K-ATPase that are reversed upon binding of an antibody molecule. At the very least, our results are consistent with an important role of the cytoplasmic C-terminal tail of the γ subunit in regulating sodium pump activity.

6.3 CONCLUSIONS

The work presented in this thesis has defined and characterized two tissue-specific modes of Na,K-ATPase regulation. It supports the conclusion that components of the membrane play a major role in regulation of the sodium pump, independant of the primary structure of the enzyme itself. Moreover, it provides valuable information on some of the mechanisms involved in modulation of the enzyme in tissues where the role of the pump is highly specialized. Since this study presents novel mechanisms of sodium pump modulation, much work now remains to further our understanding of their physiological relevance and molecular nature.

The data from Chapters 3 and 4 have shown that the membrane environment of the pump is an important determinant of the way cations interact on the cytoplasmic side with the Na,K-ATPase. However, the nature of the precise membrane component(s) involved is yet to be elucidated. Possible approaches for solving this problem include the use of cross-linking reagents, immunoprecipitation, the yeast two-hybrid system, or the recently described split-ubiquitin system (470) to detect novel pump-protein interactions. Alternatively, the role of specific lipids could be investigated through solubilization and reconstitution of the renal enzyme. It is noteworthy, however, that little evidence exists supporting an effect of lipids on kinetic behaviour, even though such effects have been extensively studied (see Section 1.3.3.a).

Of even greater interest are the possibilities offered by the recent discovery of a

functional role of the γ subunit (Chapters 4 and 5). The power of molecular biological techniques, particularly site-directed mutagenesis, should allow for a thorough characterization of the precise role of each domain of the protein. For example, this approach could be used to gain insight into the structural basis for the migration of the γ subunit as a doublet on Western blots. Since only the α1 isoform of the catalytic subunit is present in the kidney, an additional question presents itself: can the γ subunit interact with and modulate the activity of pumps comprising the α2 and α3 isoforms? Expression of γ in HeLa cells expressing these isoforms should answer this interesting question.

In addition to questions stemming from this work, many remain regarding sodium pump regulation that are beyond the scope of this study. For example, despite the presence in the literature of countless reports on the subject, the precise roles and mechanisms of regulation by phosphorylation are still mostly unknown. The involvement of phosphorylation in hormonal regulation should also be the target of further investigation. The role of distinct α and β isoforms with regards to pump regulation and tissue-specific pump kinetics is another question that need to be addressed. It is hoped that the findings presented in this study will further our understanding of sodium pump regulation as a mechanism for adaptation to changing cellular needs.

CHAPTER 7

APPENDICES

7.1 APPENDIX 1

Initial attempts at reversing K^+/Na^+ antagonism of kidney pumps.

In order to determine whether the high K^+/Na^+ antagonism characteristic of kidney pumps could be reversed, the effects of treatments with various agents and in different conditions on the Na^+-affinity of the Na,K-ATPase in the presence of high (100 mM) K^+ were assessed. All assays were carried out, and K'_{Na} values determined, as described in Chapter 2. The results were as follows:

(i) High ionic strength: the K'_{Na} values of pumps of rat α1-transfected HeLa and rat kidney decreased to the same extent when assays were carried out under high ionic strength (1100 mM total, made up with choline chloride);

(ii) Low pH: K'_{Na} values increased to the same extent for pumps of either HeLa cells or kidney when assays were carried out at pH 6.2 compared to pH 7.4;

(iii) Detergent: K'_{Na} values of kidney pumps were unaltered after pre-treatment in the absence of detergent, in the presence of 0.65, 1.0 or 1.65 mg/ml of the ionic detergent sodium dodecyl sulfate (SDS), or in the presence of the non-ionic detergent saponin (0.3 mg/ml);

(iv) Calcineurin: treatment of kidney membranes with 2 units of calcineurin (phosphatase 2B) in the presence of 4 units of calmodulin and 0.1 mM $CaCl_2$ had no effect on K'_{Na};

(v) Anti-γ antiserum: Pre-treatment of kidney membranes with anti-γ antiserum, as described in Chapter 4, had no effect on K'_{Na} values.

Taken together with the observation that K^+/Na^+ antagonism of kidney pumps is reversed upon fusion into red blood cells (Fig. 3-4), these results indicate that modulation of susceptibility to K^+/Na^+ antagonism is the result of relatively tight interactions between the pump and some membrane component which is not the γ subunit.

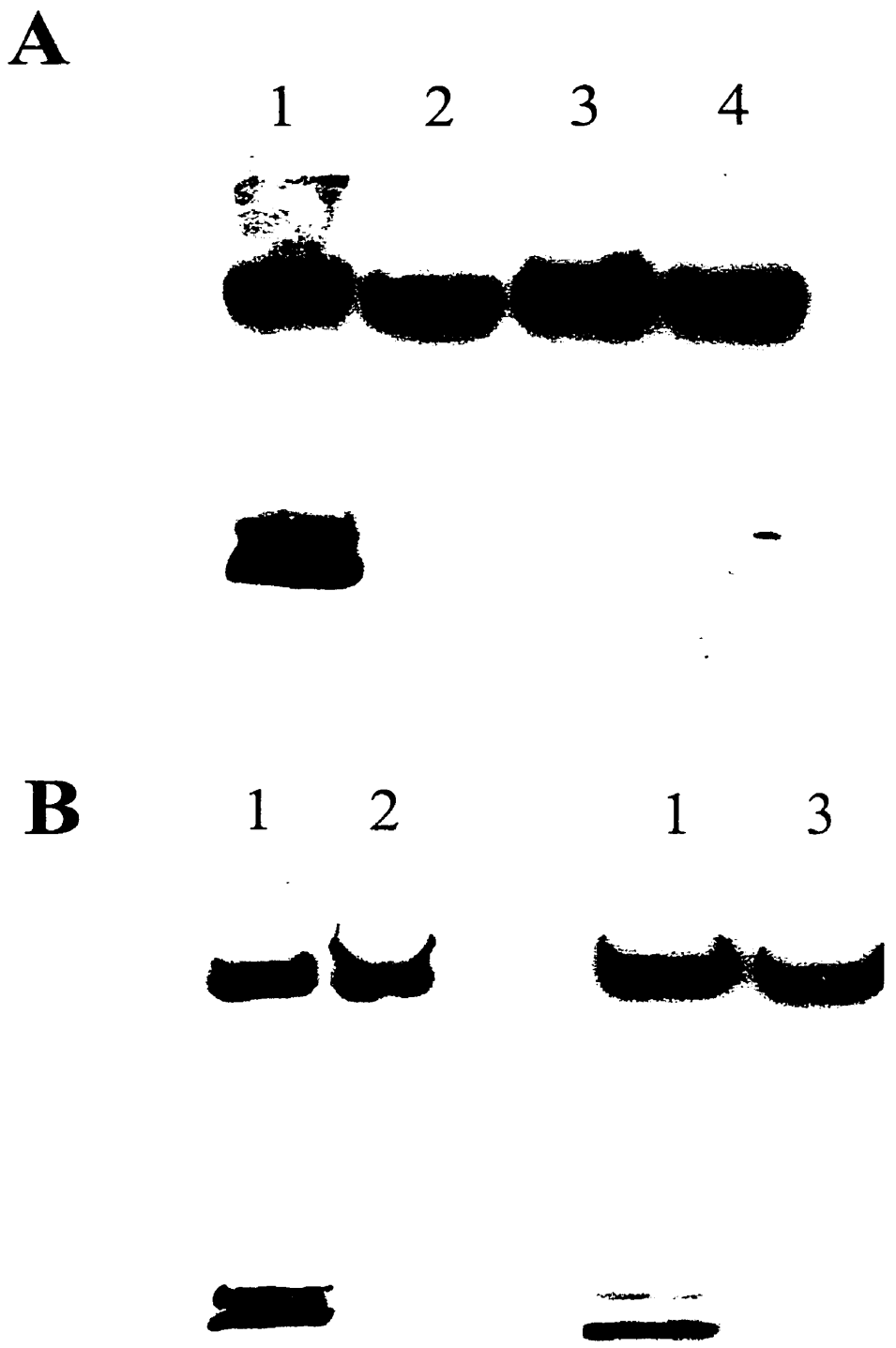
7.2 APPENDIX 2

Tissue-specific expression of the γ subunit in rat and mouse tissues

The Western Blot analysis presented in Chapter 4 is extended to six additional tissues, three from rat, and three from mouse. As shown in Fig. 7-1, the γ subunit is expressed solely in the rat and mouse kidney, and not in rat lung and stomach, or mouse axolemma and heart. Trace amounts of γ were detected in rat spleen, calculated by densitometry analysis to be less than 2% of the amount found in kidney relative to α. The absence of γ subunit in these tissues and others (see Chapter 4), and more importantly, in non-renal tissues of another species, namely the mouse, further underscores its importance as a kidney-specific modulator of the Na,K-ATPase.

Fig. 7-1 **Appendix 2.**

Western blots of membranes of rat and mouse tissues were carried out as described in Chapter 2, using α1-specific antibody 6H (donated by Michael Caplan, Yale University) and γ-specific antisera γC33 (see Chapter 5), both at 1:10,000 dilutions. Amounts of membranes loaded contain equivalent Na,K-ATPase activities ascribed to α1 (resistant to 5 μM, but sensitive to 10 mM ouabain) . A) Rat tissues. Lane 1, kidney; lane 2, lung; lane 3, stomach; lane 4, spleen. B) Mouse tissues. Lanes 1, kidney; lane 2, axolemma; lane 3, heart.


A
1 2 3 4
B
1 2
1 3

7.3 APPENDIX 3

Protocols for membrane preparations

Most experimental methods used in this thesis are described in Sections 2.4, 3.4, 4.4 and 5.4. This appendix includes details on the procedures for the isolation of cellular membranes from tissues and cells used in Chapters 2 to 5.

Kidney medulla, kidney glomeruli, small intestine epithelial cells and heart (see ref. 254). Tissues were first homogenized with 10 strokes of a motor-driven (1000 rpm) teflon-glass homogenizer in SHE buffer (0.25 M sucrose, 0.03 M histidine, 1.0 mM tris-EDTA, pH 7.5) and homogenates were centrifuged for 15 minutes at 6,000 x g. Pellets were discarded and supernatants were centrifuged for 1 hour at 40,000 x g. Pellets were washed once by homogenization in SHE buffer and centrifugation, resuspended in SHE buffer in a final volume of 1.5 ml/mg original tissue and stored at –70°C.

Red blood cells (see ref. 61). Fresh blood collected from rats in 1/10 vol 100 mM EDTA was centrifuged for 5 min at 500 x g. Pelleted red blood cells were washed three times by suspension in 10 vol of 150 mM NaCl followed by centrifugation, and lysed in 10 vol H_2O. Unlysed cells and cellular debris were removed by centrifugation (500 x g) and supernatant containing membranes was centrifuged for 15 min at 40,000 x g. The pellet was washed by resuspension followed by centrifugation (15 min each at 40,000 x g) twice with 1 mM Tris-HCl containing 1 mM EDTA-Tris (pH 7.4), once with 10 mM EDTA-Tris (pH 7.4) and three times with 2 mM Tris-HCl (pH 7.4). The final pellet was resuspended and stored (-70°C) in 1 mM EDTA-Tris (pH 7.4).

Axolemma (see ref. 477). Axolemma tissue was minced using a scalpel blade, suspended in Solution 1 (1 M sucrose, 150 mM NaCl and 10 mM Tris-Cl (pH 7.4)), homogenized with 15 strokes of a Dounce homogenizer (pestle B) and centrifuged in a Beckman SW-28 rotor (15 min at 80,000 x g). The floating "wafer" thus obtained was washed once with Solution 1 and once with Solution 2 (Solution 1 containing 0.85 M instead of 1 M sucrose) by homogenization (10 strokes) followed by centrifugation. The final "wafer" was rehomogenized (10 strokes with pestle A) in 10 mM Tris-Cl

containing 0.5 mM EDTA-Tris (pH 7.4) and left overnight at 4°C with stirring to remove myelin. Myelin-free material was pelleted by centrifugation (30 min at 65,000 x g), resuspended in Gradient Solution (1 M sucrose, 10 mM Tris-Cl, 1 mM EDTA-Tris (pH 7.4)) and centrifuged on a sucrose gradient (0.3 to 1.2 M sucrose; 1 hour at 90,000 x g, no brake). The two bands thus obtained were pooled and centrifuged (1 hour at 65,000 x g) and the resulting pellets washed once in Gradient Solution containing 0.32 M sucrose, and resuspended and stored (-70°C) in the same.

Cultured cells (HeLa, GEC, HEK, LLC-PK, MDCK and NRK-52E). Culture medium was removed from confluent plates and cells were washed twice with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4). Cells were lifted using a cell scraper, washed three times with ice-cold PBS by suspension followed by centrifugation (2 min at 500 x g), suspended in lysis buffer (10 mM Tris-Cl (pH 7.4), 1 mM $MgCl_2$) and allowed to incubate on ice for 20 min. Cells were then homogenized using 50 stokes of a motor-driven (1000 rpm) teflon-glass homogenizer and unbroken cells and cellular debris were removed by centrifugation (2 min at 500 x g). Supernatant was centrifuged (1 hour, 40,000 x g) and the pellet thus obtained washed twice with 1 mM EDTA-Tris (pH 7.4) and suspended and stored (-70°C) in the same.

CHAPTER 8

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