REGULATION OF THE SODIUM POTASSIUM ADENOSINE TRIPHOSPHATASE (Na,K-ATPase) BY FXYD 2

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Submitted June 2006

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy.

Your file Votre référence
ISBN: 978-0-494-27861-1
Our file Notre référence
ISBN: 978-0-494-27861-1

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ABSTRACT

The Na,K-ATPase, or Na^+ pump is an integral membrane protein found in the cells of virtually all higher eukaryotes and is one of the most important systems in cellular energy transduction. Na,K-ATPase catalyzes the electrogenic exchange of three intracellular Na^+ for two extracellular K^+ ions coupled to the hydrolysis of one molecule of ATP. The research described in this thesis concerns the regulation of the Na,K-ATPase by FXYD 2, a member of the FXYD family of small single transmembrane proteins. FXYD 2, commonly known as the γ modulator, is located primarily in the kidney and has a role in modulating the enzyme's apparent affinities for ligands. This study has addressed several aspects of γ structure and function, namely its function in intact cells, the function of the γ transmembrane domain, and delineation of regions of the enzyme's catalytic α subunit with which γ interacts. Transport assays using intact transfected HeLa cells showed that the two γ variants, γa and γb, cause (i) an increase in K^+/Na^+ antagonism, seen as an increase in K'_{Na} at high K^+ concentration, and (ii) an increase in apparent ATP affinity seen as an increase in ouabain-sensitive K^+ influx as a function of ATP concentration. These results are consistent with those obtained earlier with unsided membrane preparations. The present study also showed a γ-mediated increase in steady-state intracellular Na^+ concentration and, in contrast to assays using permeabilized membranes, a γ-mediated increase in apparent affinity for extracellular K^+. Experiments with synthetic γ transmembrane (γ-TM) peptides provided insight into the role of the TM region

such that incubation of these peptides with membranes containing αβ pumps modulated K'_{Na} similarly to transfected full-length γ, indicating that the TM domain alone can cause an increase in K'_{Na} at high K^+ concentration. Results with γ-TM bearing the Gly^{41}→Arg missense mutation associated with familial renal hypomagnesemia provided direct evidence that this mutation prevents γ association with αβ pumps. In a study aimed to identify regions of α critical for the functional effects of γ, interactions of α1/α2 (and the reverse α2/α1) chimeras with γ showed the importance of the carboxy terminus, particularly TM 9. The chimera data also indicate that interactions of transmembrane regions of the catalytic α subunit with FXYD proteins are not necessarily the sole determinants of the kinetic effects of γ on Na^+ affinity since the extramembranous L7/8 loop of α appears to modulate intramembranous α-γ interactions that mediated the increase in K^+/Na^+ antagonism.

RÉSUMÉ

La Na,K-ATPase, ou pompe à sodium, est une protéine membranaire intégrale exprimée par les cellules de presque tous les eucaryotes supérieurs, et est un des systèmes les plus importants impliqués dans la transduction d'énergie cellulaire. La Na,K-ATPase catalyse l'échange électrogénique de trois Na^+ intracellulaires pour deux K^+ extracellulaires grâce à l'énergie fournie par l'hydrolyse d'une molécule d'ATP. Les recherches décrites dans cette thèse concernent la régulation de la Na,K-ATPase par FXYD 2, un membre de la famille des petites protéines membranaires FXYD. FXYD 2, communément admise comme le modulateur γ, est située principalement dans le rein; elle est connue pour moduler les affinités apparentes de l'enzyme pour ses ligands. Cette étude vise plusieurs aspects de la structure et de la fonction de la sous-unité γ, en particulier sa fonction dans les cellules intactes, le rôle du domaine transmembranaire de γ, et les régions de la sous-unité catalytique α de la Na,K-ATPase avec laquelle γ interagit. Les études de transport utilisant des cellules HeLa intactes démontrent que les deux variants, γa et γb, causent (i) une augmentation de l'antagonisme K^+/Na^+, vue comme une augmentation de K'_{Na} à haute concentration de K^+, et (ii) une augmentation de l'affinité apparente pour l'ATP, vue comme une augmentation de l'afflux sensible à la ouabaïne de K^+ en fonction de la concentration d'ATP. Ces deux observations sont compatibles avec les résultats obtenus à partir de préparations de membranes non polarisées. Les résultats ont également montré une augmentation de la concentration cytoplasmique en Na^+

grâce à γ, et contrairement aux résultats obtenus avec des préparations de membranes non polarisées, indiquent une diminution de $K'_{K(ext)}$ provoquée par γ. Les études utilisant des peptides transmembranaires synthétiques de γ (γ-TM) ont fourni de l'information quant au rôle de sa région transmembranaire (TM). L'incubation de ces peptides avec des membranes contenant des pompes αβ induit la modulation de K'_{Na} de la même façon que dans les cellules transfectées avec γ intègre, ce qui semble indiquer que seul le domaine transmembranaire peut causer une augmentation de K'_{Na} à une haute concentration de K^+. Les résultats obtenus avec le γ-TM portant la mutation de $Gly^{41}\rightarrow Arg$ associé à une forme d'hypomagnésémie rénale familiale, indique que cette mutation empêche l'association de γ avec αβ. Une étude visant à identifier les régions critiques de la sous-unité α interagissant avec γ a montré l'importance de l'extrémité C-terminale du peptide, en particulier TM 9; les expériences ont été menées en utilisant des chimères α1/α2 ainsi que les chimères inverses α2/α1. Les résultats indiquent également que les interactions entre les régions transmembranaires de la sous-unité catalytique avec des protéines de FXYD ne sont pas nécessairement les déterminants uniques des effets cinétiques de γ sur l'affinité de Na^+ pour la Na,K-ATPase. Il semble en effet que la boucle extramembranaire L7/8 module aussi les interactions α-γ intramembranaires pour induire une augmentation de l'antagonisme K^+/Na^+.

ACKNOWLEDGMENTS

Throughout this academic experience, there are several people who deserve credit and appreciation for their role in making all this possible. First and foremost, my supervisor, Dr. Rhoda Blostein for guiding, teaching, and inspiring the research in this thesis. I wish to thank her for her insightful discussions, patience and encouragement over the last five years. To all the postdoctoral fellows and technicians in the laboratory who provided me with invaluable technical advice and friendship throughout my experiences in the lab, particularly Dr. Laura Segall, Dr. Delia Susan-Resiga and Rosemarie Scanzano. I also wish to thank my Research Advisory Committee members Drs. John Silvius and John Orlowski for valuable input over the years.

I would also like to thank Drs. Alex Therien and Charles Deber for collaborations and peptide synthesis as well as Dr. Philip Dunham who collaborated on experiments and for useful discussions and suggestions. I thank Drs. Kathleen Sweadner and Jerry Lingrel who have also contributed to this work through gifts of antibodies and cells.

This thesis would not be possible without the support and assistance of my friends and family. To my parents, John and Maria Zouzoulas, who raised an independent, driven child, and never doubted my abilities. To my sister, Demetra Zouzoulas, who always pushed me to excel and believed in me. Their love and support provided me the strength and perseverance I needed to achieve my goals. To my dearest friends (the 11^{th} floor lunch crew and the MBGs) who listened and advised me throughout this thesis, I thank you for keeping me sane!! Each and

everyone listed above and others who have not been named directly deserve my thanks for supporting me through this portion of my life and career.

ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

Chapter 2: The modulatory effects of the two variants of γ, seen previously with unsided membrane preparations (i.e. an increase in K'_{Na} at high $[K^+]$ and K'_{ATP}), are relevant to Na,K-ATPase behaviour under the more physiological conditions that prevail in intact mammalian cells. This study also showed an increase in steady state $[Na^+]_{in}$ and unlike assays using permeabilized membranes, experiments with sided preparations (intact cells) revealed a third effect of γ, a modest decrease in $K'_{K(ext)}$.

Chapter 3: Results showed that solely the transmembrane (TM) domain of γ effects the shift in apparent Na^+ affinity since incubation of synthetic TM peptides with membranes containing αβ pumps modulates K'_{Na} in a manner similar to transfected full-length γ subunit. Using both mutated γ peptides and full-length γ cDNA-transfected HeLa cells, I showed that a specific glycine residue, Gly41, which is associated with a form of familial renal hypomagnesemia when mutated to Arg, abrogates α-γ association.

Chapter 4: An α1/α2 chimera approach to identify regions of interaction of α critical for the functional effects of γ confirmed the functional importance of the carboxy terminal region, particularly TM 9. A significant new finding is that interactions of TM regions of the catalytic α subunit with FXYD proteins are not necessarily the sole determinants of the kinetic effects of γ on apparent affinity for Na^+ since the extramembranous L7/8 loop of α appears to modulate intramembranous α-γ interactions to effect the γ-mediated increase in K^+/Na^+ antagonism.

CONTRIBUTION OF AUTHORS

CHAPTER 2: The effect of the gamma modulator on Na/K pump activity of intact HeLa cells

Zouzoulas, A., Dunham, P.B., and Blostein, R. 2005. *The effect of the γ modulator on Na/K pump activity of intact HeLa cells.* J. Membr. Biol. 204: 49-56.

Except for sodium determinations carried out on extracted samples that were sent for analysis in the laboratory of co-author Dr. P.B. Dunham, I carried out all the experiments and data analysis described in this paper.

Chapter 3: Structure/function analysis of the transmembrane segment of γ

Zouzoulas, A., Therien, A.G., Scanzano, R., Deber, C.M., Blostein, R. 2003. *Modulation of the Na,K-ATPase by the γ subunit: Studies with transfected cells and transmembrane mimetic peptides.* J. Biol. Chem. 278: 40437-40441.

Co-first author Alex Therien, a postdoctoral fellow in the laboratory of Dr. Charles Deber, University of Toronto, synthesized the mimetic peptides and Rosemarie Scanzano performed the kinetic experimens described in figures 3 and 4. I performed the experiments with the transfected cells, namely those shown in Figures 1, 2, 4 and Table 1.

Chapter 4: A structure/function analysis of α chain interaction with the γ subunit

Zouzoulas, A. and Blostein, R. 2006. *Regions of the catalytic α subunit of Na,K-ATPase important for functional interactions with FXYD 2.* 2006. J. Biol. Chem. 281: 8539-8544.

I designed all chimeras and carried out all the experiments described in this paper.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Ca-ATPAse	Calcium-activated adenosine triphosphatase
cAMP	Cyclic adenosine 3', 5'-monophosphate
CCD	Cortical collecting duct
cDNA	Complementary DNA
CFP	Cyan-coloured fluorescent protein
CHIF	Channel inducing factor
cRNA	Coding RNA
C-terminus/terminal	Carboxyl-terminus/terminal
DCT	Distal convoluted tubule
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
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
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N'-tetraacetic acid
EP	Phosphorylated Na,K-ATPase
ER	Endoplasmic reticulum
H,K-ATPase	Proton and potassium-activated adenosine triphosphatase
IC_{50}	Concentration of inhibitor causing 50% inhibition
IMCD	intermedullary collecting duct
$K_{0.5}$	Concentration of substrate causing half-maximal activation
kb	One thousand base pairs
kDa	kiloDaltons
L2/3	intracellular loop between transmembrane 2-3
L4/5	intracellular loop between transmembrane 4-5
L7/8	extracellular loop between transmembrane 7-8
MONaka	modulator of Na, K-ATPase
Mat-8	Mammary tumor-associated 8 kDa protein
mRNA	Messenger RNA
mTAL	Medullary thick ascending loop of Henle
Na,K-ATPase	Sodium and potassium-activated adenosine triphosphatase
N-terminus/terminal	Amino-terminus/terminal
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCT	Proximal convoluted tubules
PFO-PAGE	Perfluorooctanoate- polyacrylamide gel electrophoresis

Pi	Inorganic orthophosphate (PO_4)
Php	Phosphohippolin
PKA	cAMP-activated protein kinase
PKC	Calcium-activated protein kinase
PLM	Phospholemman
PLMS	Phospholemman-like protein in shark
PLN	Phospholamban
PVDF	Polyvinylidene difluoride
RIC	Regulated ion channel homolog
S.D.	Standard deviation
SEM	Standard error of the mean
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SERCA	Sarcoplasmic/endoplasmic reticulum Ca-ATPase
TM	Transmembrane domain
WT	Wild type
YFP	Yellow fluorescent protein

CHAPTER 1

INTRODUCTION

1.1 PREFACE

In 1997, Skou was awarded the Nobel Prize for his discovery of the Na,K-ATPase. The publication of thousands of reports each year relevant to various aspects of Na,K-ATPase structure and function underscores the impact of this discovery. Maintenance of ionic homeostasis, namely a high intracellular K^+ concentration (≈150mM) and low intracellular Na^+ (≈10-20mM), is largely accomplished by the Na,K-ATPase which is the main consumer of ATP in mammalian cells (64). As a result, this transport creates the electrochemical gradient across the cell membrane that is essential for various cellular functions such as fluid reabsorption, electrolyte homeostasis in the kidney and maintenance of the resting potential in excitable cells. The Na^+ gradient created by the Na,K-ATPase is also the driving force for secondary active transport processes necessary for the reabsorption of many solutes.

The focus of this thesis is on regulation of the Na,K-ATPase by FXYD 2, commonly known as the γ modulator. The Introduction provides a brief overview of the structure and mechanism of the Na,K-ATPase and pump regulation with emphasis on modulation of the Na,K-ATPase by γ, a member of the FXYD family of small single transmembrane proteins. This thesis is manuscript-based, in accordance with Section C of the Faculty of Graduate Studies and Research Guidelines for Thesis Preparation. Chapters 2, 3 and 4 are published manuscripts. As such, Chapters 2 to 4 contain their own reference lists, and Chapter 7 contains references for the Introduction and General Discussion. In addition, in compliance with section C, paragraph 2, Chapters 2 to 4 contain a preface which

includes "connecting texts that provide logical bridges between the different papers".

1.2 The Na,K-ATPase

1.2.1. ATP-driven ion pumps

The Na,K-ATPase, also known as the Na^+ pump, is a member of the P_2 subfamily of a larger P-type ATPase family. P-type ATPases differ from the vacuolar or V-type ATPases and from the multimeric, ATP synthesizing, F-type pumps in that they form a phosphorylated intermediate in the course of their reaction cycle. During the reaction cycle, the enzyme undergoes conformational changes of both phosphoenzyme and dephosphoenzyme, i.e. $E_1P \rightarrow E_2P$ and $E_1 \rightarrow E_2$ respectively.

The P-type ATPase family comprises more than 300 members. In recent years many different classification schemes have been proposed that are based on the type of ion transported and similarity in structure. In the particular classification discussed here, P-type ATPases are divided into three groups. Members of the P_1 subfamily transport transition metals or metalloid compounds (Cd, Cu, Ag), while those of the P_2 subfamily transport alkali metal cations such as Na^+, K^+, and other cations, namely Ca^{2+} and H^+. Examples of members of the latter subfamily of ion pumps that transport biologically significant cations are the gastric mucosa H,K-ATPase, a close relative of the Na,K-ATPase, and the Ca-ATPases of plasma membrane and sarco(endo)plasmic reticulum (SERCA). It is interesting to note that the Na,K-ATPase and the gastric H,K-ATPase are not only the closest relatives in terms of amino acid sequence identity, but also are P-type ATPases composed of two essential subunits (23, 47). The P_3 subtype is composed of a sole member, KdpB from *E. coli*, which provides K^+-

accumulation. Classification into these subfamilies is based on the location of key sequences in and around the ATP binding domain.

1.2.2. Structure of Na,K-ATPase

1.2.2.a. The α subunit

The larger of the two subunits is the catalytic α subunit which is composed of approximately 1000 amino acids and has a molecular mass of about 110 kDa. As the catalytic subunit, it contains the binding sites for cations, ATP and ouabain. The α subunit comprises 10 transmembrane domains with both cytoplasmic N-and C- termini (56). Many studies have provided insights into the transmembrane topology of the α subunit (3, 18, 83, 85, 104, 112, 131, 142, 148). They predict an N-terminal segment with four transmembrane domains, a large cytoplasmic domain which comprises approximately 30% of the protein and a C-terminal domain consisting of 6 transmembrane domains. This membrane topology is similar to that predicted for other P_2-type ATPases such as the Ca- and H,K-ATPases (18, 22).

The alignment of the Na,K-ATPase to the high resolution crystral structure of SERCA demonstrates notable structural similarities. A recent review discusses these similarities as well as some of the differences (132). In terms of the high resolution structure of SERCA published by Toyoshima and coworkers (139) there are three cytoplasmic domains of the catalytic subunit that have notable function. These regions correspond to the N or nucleotide-binding domain, the P or phosphorylation domain and the A or actuator domain. The N and P domains

are located in the TM 4-TM 5 loop (L4/5), while the A domain is formed by the N-terminus and the L2/3 loop. The extracellular loops are short with the exception of the L7/8 loop. This loop in the Na,K-ATPase has been shown to provide important contacts for association of α with the β subunit (29, 77, 110, 144).

The catalytic α subunits of P_2-type ATPases also share several other structural similarities. These conserved domains, believed to be involved in communication between ATP hydrolysis and conformational change (the energy transduction domain), ion binding and ATP binding (105) include: (i) the large cytoplasmic loop between transmembrane helices 4 and 5 (L4/5) containing the ATP binding and phosphorylation sites, (ii) a highly conserved hinge region at the junction of the L4/5 loop and TM 5, and (iii) a β-strand region in the minor loop between TM 2 and TM 3 (L2/3). Within these domains exist several highly conserved amino acid sequences, namely (i) the DKTGT(L/I/V/M)(T/I/S) motif in the catalytic loop, which contains the aspartate (D) which defines membership in P-type ATPase family, (ii) a TGE(S/A) sequence in the L2/3 loop prior to the phosphorylation site (89, 91), (iii) a GDGXNDXP motif immediately after the ATP binding domain, and (iv) P(E/V)GL in TM 4 which is essential to energy transduction (24). The TGE(S/A)- and DKTG-containing regions are within the A and P domains described by the SERCA crystal structure (139).

1.2.2.b. The β subunit

The β subunit is a single transmembrane protein with its short N-terminus facing the intracellular space, a single transmembrane domain and a long extracellular C-terminal domain (i.e. type II membrane protein). It is fully glycosylated on all three potential N-glycosylation sites and in addition there are 3 disulfide bridges in the extracellular domain. It is composed of approximately 370 amino acids and has a mass of about 55 kDa.

The function of the β subunit is not entirely clear. It has been shown that β acts as a chaperone to ensure the proper folding, insertion and maturation of the α subunit in the plasma membrane (1, 23, 49, 107) and it appears that both α and β subunits are essential for ouabain-sensitive Na,K-ATPase activity (33). There is evidence that deglycosylation causes no loss in activity (10) and has no effect on β protein levels at the membrane's surface (149), which is in contrast to the β subunit of the gastric H,K-ATPase where removal of glycosylation sites results in the loss of enzymatic activity (72). However, reduction of disulfide bridges by β-mercaptoethanol or DTT results in a loss of enzymatic activity (69, 71, 88). The β subunit may also play a role in stabilizing the K^+-occluded state of the enzyme (88). While the presence of the β subunit may play some role in determining the kinetic characteristics of mature pumps, major kinetic differences are most likely the result of tissue-specific differences in α isoform composition (see 1.2.2.d. below).

1.2.2.c. Tertiary and Quartenary Structure of the Na,K-ATPase

The minimal structure required for Na,K-ATPase activity comprises a membrane-associated complex of the two subunits, α and β, which are present at a 1:1 ratio (64). There is debate regarding the oligomeric structure of the Na,K-ATPase (117). It has been demonstrated that the αβ dimer is capable of catalyzing the complete reaction cycle (143), and that this is the naturally occuring form, at least in the red blood cell (98, 119). However, other evidence supports the idea that in other systems, the enzyme functions as a $(\alpha\beta)_2$ heterodimer or possibly as a $(\alpha\beta)_4$, tetramer (15, 65, 86, 128, 140).

The α and β subunits are synthesized independently and assembled in the endoplasmic reticulum (ER) (1). This involves the interaction of several domains of each subunit. The essential interactions occur between the ectodomain of β and the highly conserved SYGQ motif in the extracellular L7/8 loop of α, (9, 28, 77), and between the transmembrane domain of β and TM 8 of the α subunit (109). In addition, Geering et al. (50) showed that part of the N-terminal tail of β may also be involved in interactions with α, although it is not likely to be essential to proper pump folding. For a detailed review of the role of the β subunit in P-type ATPases, see ref. 47.

1.2.2.d. Isoforms of the Na,K-ATPase subunits

First cloned in sheep (126), the amino acid sequence of α has also been deduced for rat, chicken and human (36, 84, 121, 125, 129). Three major isoforms have been identified, namely α1, α2 and α3. Across species there is an

overall high level of amino acid sequence identity for all isoforms (~ 90%). There is also a high degree of identity among the α isoforms (~80%). Most divergent regions include the N-terminus as well as the large cytoplasmic L4/5 loop. Shamraj and Lingrel (123) identified a fourth α isoform in rat testis, α4, and it is the most divergent, sharing only 78% identity with α1.

The α isoforms are expressed in a tissue- as well as development- specific manner; for review see ref. 16. In terms of expressed protein, in rat, α1 is ubiquitously expressed, α2 is found in adipocytes (57, 75, 90), brain (99), glial cells, choroid plexus (151) and adult heart (90), while α3 is predominantly expressed in nervous tissue and in fetal heart (55). Expression of α4 is restricted to the testis. The tissue expression pattern of the Na,K-ATPase α4 isoform is one of the most restricted, having been identified only in mouse, rat, and human testes and at lower levels in mouse epididymis (123, 141).

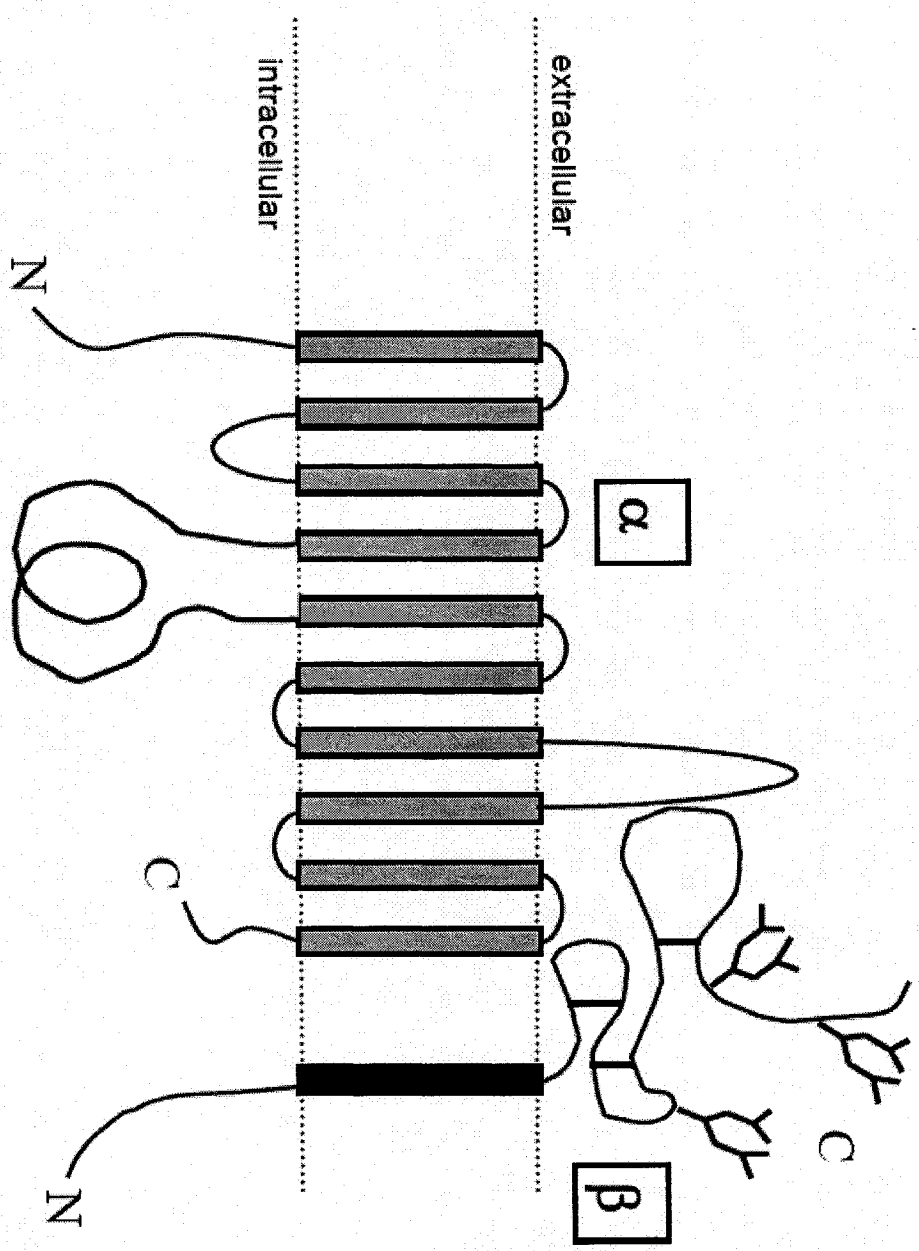
Isoform diversity also exists for the β subunit. The β subunit is also expressed in a tissue- and developmental-specific manner; for review see ref. 16. To-date, three β isoforms have been identified in mammals, namely β1, β2 and β3. These forms of β have been found in many vertebrates such as such as rats, mice, birds and human (14, 51, 76, 97). The β1 isoform is ubiquitous, β2 is found in skeletal muscle, pineal gland and nervous tissue and β3 is found in testis, retina, liver and ling. Unlike the α subunit, amino acid sequence homology is low for the β isoforms. There is only a 34% identity between β1 and β2, whereas sequence identity is 39% for β3 and β1. Highest sequence identity (49 %) is seen

between β2 and β3. The homology of β1 and β2 isoforms is 95% across mammalian species and drops to 60% when nonmammalian species are included in the comparison (16). It is interesting to note that the protein sequence similarity between β2 and the β-isoform of the H,K-ATPase is greater than the similarity between the β2 and β1 isoform of the Na,K-ATPase. The transmembrane domain of the β subunit is the most conserved region among isoforms and species.

Information regarding expression, function and regulation of the Na,K-ATPase isoforms has grown extensively over the years. For more extensive reviews of the Na,K-ATPase structure and mechanism, see refs. 66 and 68. For a review focusing on the functional properties of multiple isoforms and their possible physiological relevance see ref. 16.

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

Scheme of the membrane topology of the α and β subunits of the Na,K-ATPase. The α subunit consists of 10 TM domains (grey rectangles) while the β subunit is a single TM protein (represented by the black rectangle). The sites of glycosylation (♈) and the disulfide bridges (—) of the β subunit are shown.


extracellular
intracellular
N
α
C
C
β
N

1.2.3. Function of the Na,K- ATPase

Enzyme cycle

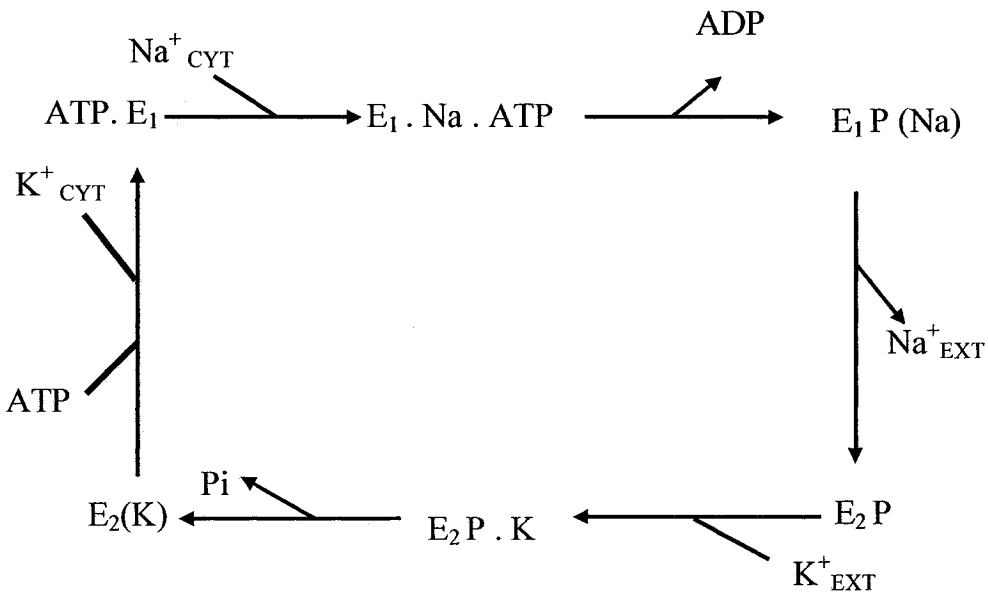
Many studies have been conducted to determine the various transport reactions of the pump, the properties of the reaction intermediates and to identify the amino acid residues and domains that are important for ligand binding and conformational transitions. The pump can be made to function in a variety of non-physiological modes by manipulating experimental conditions. In its normal mode of operation, the Na,K-ATPase exchanges three Na^+ ions from the cytoplasm for two K^+ ions from the extracellular space. Under physiological conditions, the forward reaction operates against the electrochemical gradient of both the transported ions. During its reaction cycle, the Na,K-ATPase proceeds through several different conformations as it interacts with its ligands. The Albert-Post model describing the basic reaction cycle is shown in Fig. 1-2.

Briefly, in the E_1 conformation, the enzyme binds Na^+ and ATP (with high affinity). Hydrolysis of ATP leads to direct phosphorylation of the enzyme and the release of ADP. The three Na^+ ions become occluded within the enzyme which renders them inaccessible from either side of the membrane. The enzyme then undergoes a spontaneous conformational change [$E_1P(3Na) \rightarrow E_2P + 3Na$] as it releases the Na^+ ions into the extracellular milieu. In the E_2P form, the enzyme has a higher affinity for K^+ and a low affinity for ATP. Successive binding of two K^+ ions to E_2P accelerates dephosphorylation and the two K^+ ions become occluded [$E_2P + 2K^+ \rightarrow E_2P(2K) \rightarrow E_2(2K)$]. Deocclusion of two K^+ ions occurs slowly in a spontaneous reaction which is greatly accelerated by low affinity ATP

binding to $E_2(2K)$. Deocclusion is followed by the release of K^+ into the cytoplasm. The enzyme is now poised in E_1 again and ready for another cycle. All steps are reversible. Under normal circumstances, the turnover rate of the enzyme is about 10,000 min^{-1} (see, for example, ref. 64).

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

Occluded cations are represented by brackets. This cycle represents the normal, physiological cycle. Stoichiometry of ion transport not shown; three intracellular Na^+ ions are released to the extracellular space while two K^+ ions are transported to the cytoplasmic side.



1.3 REGULATION OF THE Na,K-ATPase

1.3.1. Overview of Na,K-ATPase regulation

The Na,K-ATPase is a major regulator of intracellular sodium concentration ($[Na^+]_{in}$). Consequently, the sodium gradient created by the Na,K-ATPase also plays an important role in secondary active transport systems such as the Na^+/Ca^{2+}- and Na^+/H^+- exchanger, thus regulating cell volume, cytoplasmic pH and calcium levels. Hence the Na^+ pump is likely to be a major target of regulation. Over the last few decades, most efforts were directed to the study of the structure and function of the Na,K-ATPase enzyme. In more recent years considerable attention has been focused on its regulation, particularly in relation to tissue-specific requirements. To-date known regulators include hormones, cytoskeletal components and other associated membrane components. Whereas long-term changes in pump activity may be the result of altered rates of translation, transcription or RNA stability, short-term changes may reflect modulations in the kinetic properties/substrate affinities, cell surface expression and stability.

Regulators such as hormones can exert their modulatory effects on the Na,K-ATPase through signalling mechanisms. Activation of key signalling molecules like PKA and PKC by hormones initiates signal cascades whose downstream effect is to modulate Na^+ pump activity. The mechanisms by which these hormones and kinases affect Na,K-ATPase activity have been described in detail in Therien and Blostein (134) and a detailed review specific to hormonal regulation and activation of signalling mechanisms in the kidney was written by Feraille and Doucet (38).

The focus of this thesis is the regulation of the Na,K-ATPase by members of the FXYD family of small single TM proteins. Accordingly, the following discussion is focused on the FXYD family of proteins, with special emphasis on the γ modulator which was the first FXYD protein reported to associate with and regulate the Na,K-ATPase, and it is one of the best characterized members to-date.

1.3.2. FXYD family of proteins

1.3.2.a. Primary structure and sequence homology

FXYD proteins belong to a gene family of small, single transmembrane proteins that appear to modulate the Na,K-ATPase and possibly other transporters. Analysis of more than 1000 related ESTs (expressed sequence tag) defined at least seven known members, namely FXYD's 1-7, each possessing a 35 amino acid signature sequence that includes 6 invariant amino acids before, in and after the transmembrane domain in all family members and species. These include the N-terminal FXYD (Phe-X-Tyr-Asp) motif, along with two glycines in the TM domain and a serine in the C-terminal domain. FXYD proteins are not an integral part of the Na,K-ATPase but to-date several have been shown to modulate the catalytic properties of the pump through association in a tissue-specific manner. A detailed analysis of the gene transcripts in the EST database of the members of this family is discussed in length (130). Figure 1-3 shows the rat protein sequences of FXYD family members.

The γ modulator of the Na,K-ATPase is, to-date, the best characterized FXYD member. Other identified members include: Mat-8 (mammary tumor marker) (106), CHIF (corticosteroid hormone induced factor) (6), RIC (42) and PLM (phospholemman) (111). PLMS has been identified in shark rectal glands (94).

Analysis of membrane topology of FXYD members showed that they are type I membrane proteins such that the N-terminus is extracellular and the C-terminus is cytoplasmic. It should be noted that Mat-8 (FXYD 3) can contain two transmembrane domains presumably due to the lack of cleavage of a signal peptide and RIC (FXYD 5) has an unusually long N-terminus.

Fig. 1-3: Rat sequence alignment of FXYD proteins.

Conserved amino acids are underlined

*RIC without N-terminal extension

Table 1-1: Major Tissues Expressing FXYD proteins

FXYD protein	Tissue Distribution	References
FXYD 1 / Phospholemman (PLM)	brain, heart, kidney	(40, 147, 150)
FXYD 2 / γ	kidney	(113, 114, 146)
FXYD 3 / Mammary tumor (Mat-8)	stomach	(32)
FXYD 4 / Corticosteroid Hormone Inducing Factor (CHIF)	kidney, colon	(46, 113, 124)
FXYD 5 / Related to Ion Channel (RIC), Dysadherin	kidney, intestine, lung, heart, spleen	(87)
FXYD 6 / Phosphohippolin (Php)	CNS	(67)
FXYD 7	brain	(12)
PLMS	shark rectal gland	(93)

1.3.2.b. Tissue specific distribution

Initial EST databank analysis also revealed tissue-specific distribution of FXYD family members. A detailed summary of the tissue distribution of FXYD proteins at the mRNA and protein level can be found in a more recent review (45). As seen in Table 1-1, FXYD proteins are abundant in tissues involved in active ion transport such as brain, kidney and heart. While some FXYD members are expressed in the same organ, they do not always show colocalization in the same region of that particular organ. For instance, γ and CHIF are predominantly expressed in the kidney. Immunohistochemistry of rat kidney showed that γ is expressed at highest levels in the thick ascending loop of Henle (TAL) while CHIF is highly expressed along the cortical collecting duct (CCD) principal cells as well as the outer and inner medullary collecting ducts (37).

1.3.2.c. Interactions with the Na,K-ATPase and other ion transport proteins

For many years, the renal Na,K-ATPase was known to contain a proteolipid, later referred to as the γ subunit (27, 116, 118). First considered a contamination of the membrane purification procedure, actual association to the Na^+ pump was convincingly shown by covalent labeling with photoreactive derivatives of ouabain (41). Mercer and co-workers isolated and characterized the cDNAs of γ from several species and in addition, they showed coimmunoprecipitation of α, β and γ demonstrating specific association (102). However, the functional and physiological significance remained unknown for many years. Thus, until the late 1990's FXYD proteins were not identified as a

group of ion transport regulators and their functional effects had not been elucidated.

In the last decade, it has been shown that FXYD's 1-4, 7 and PLMS associate with the Na,K-ATPase. Conflicting data exist in terms of the number of FXYD proteins that may interact with each αβ complex. Coimmunoprecipitation experiments of γ- and/or CHIF-transfected HeLa cells using detergent conditions shown to optimize efficiency ($C_{12}E_{10}$ in the presence of Rb^+/ouabain) indicate that the α:β:FXYD ratio is close to 1:1:1 and that heterodimeric complexes do not exist in these detergent conditions (46, 80). Others have reported the presence of heterodimeric complexes (5) when solubilized in $C_{12}E_8$. It is not known how the protein complex exists in the native membrane.

There is an increasing body of evidence indicating multiple sites of interaction between FXYD proteins and the Na,K-ATPase. Mutagenesis experiments in the Xenopus oocyte system (11, 13) showed that the extracellular FXYD motif is necessary for the efficient association of γ and CHIF with the pump. These authors also showed that the positively charged residues in the cytoplasmic tail are necessary for γ's efficient association and for CHIF's functional effects.

Other experiments using a series of γ-CHIF chimeras (80) coexpressed with the rat α1β1 complex indicated that the stability of α-FXYD associations was dependent on the origin of the FXYD TM segment. Thus, chimeras containing only the TM domain of CHIF are less stable (like CHIF itself) compared to chimeras comprising only γ-TM. It was concluded that residues

A34, I44 and L45 in the TM region of γ are important for the increased efficiency/stability of its association with the Na^+ pump. A helical wheel projection of the aforementioned residues (135) indicates that A34 and L45 face the same direction, while I45 is oriented at an angle of approximately 100°, pointing to multiple sites of interaction with either α and/or β.

The question of where in the αβ complex FXYD proteins interact has been a major issue of investigation. A thermal denaturation experiment suggested that γ may interact with a region that includes TM's 8-10 (34). Later, cryoelectron microscopy of the renal Na,K-ATPase proposed that γ is in a groove surrounded by TM 2, TM 6 and TM 9 of the α subunit (54). Using mutants of α1, Li et al. (78) assessed association of γ, CHIF and FXYD 7 with the Na,K-ATPase and showed that L964 and F967 in TM 9 of the α subunit are important for stable association with FXYD proteins (78). The mutational analysis was also in agreement with a docking model of the Na,K-ATPase/FXYD 7 complex, which predicted the importance of L964 and P967 in subunit interaction (78). Cross-linking experiments also indicate the importance of residues in TM 9 of the α subunit for association with FXYD's (44). Based on their cross-linking data Fuzesi et al. (44) constructed a model showing the TM domain of γ fitting into a groove between TM 2, TM 6 and TM 9 consistent with the above cryoelectron microscopy study, while the cytoplasmic tail of γ interacts with loops L6/7 and L8/9 and stalk S5. Furthermore, they showed an interaction between β and γ. More recent cross-linking experiments (81, 82) show the interaction of C138 in TM 2 of α1 with FXYD (C49 of CHIF or F38C mutant of γ) which fits well with

all predicted models to-date. Modeling of the inhibitory interaction of phospholamban (PLN) with the Ca-ATPase by Toyoshima et al. (138) indicate that PLN fits into a similar groove made of TM 2, TM 6 and TM 9.

Interestingly, recent work on shark rectal gland PLMS showed a cross-link between the C-terminal C74 of PLMS and C254 in the A domain of the α subunit (95). Considering the high degree of homology of the TM region of FXYD proteins, a model was proposed in which PLMS also resides in a pocket made up of TM 2, TM 6 and TM 9 as predicted for γ, CHIF and FXYD 7 (95). However this model places C254 at the opposite face of the protein relative to PLMS. Accordingly, the authors had previously suggested an alternate model in which PLMS is located on the opposite side of α in a cleft comprising TM 1- TM 4. Presumably leucine-isoleucine zipper motifs in M1/M2 of Na, K-ATPase could stabilize interactions as previously suggested by Cornelius and Mahmmoud (30).

The aforementioned studies are indicative of the complexity of the FXYD: Na,K-ATPase interaction. It may turn out that the interactions of FXYD proteins with the Na,K-ATPase do not all occur in the same clefts/grooves of the α subunit.

Phosphorylation of FXYD members may be another means of regulating their association with and modulation of the Na^+ pump. Recently the PLM/Na,K-ATPase interaction was studied using FRET (fluorescence energy transfer) between CFP-Na,K-ATPase and YFP_c-PLMP fusion proteins (17). These studies showed a reduction in FRET following phosphorylation of PLM suggesting that phsophorylation alters PLM's interaction with the pump to relieve inhibition.

PLM and its homologue PLMS are members of the FXYD family known to be phosphorylated by protein kinases. The C-terminus of PLMS is heavily phosphorylated by PKC *in vitro* (94), as is the case for PLM (111).

FXYD 3 or Mat-8 can associate with the Na,K-ATPase as well as with both gastric and colonic H,K-ATPases when expressed in Xenopus oocytes (32). Mat-8 was shown to modulate the glycosylation of β of either the Na,K- or H,K-ATPase and that this modulation was dependent on the presence of Mat-8's signal peptide. Since the expression of Mat-8 is restricted to mucous cells, *in vivo* association occurs only with Na,K-ATPase since H,K-ATPase is absent. PLM is another member that has been shown to coimmunoprecipitate with the Na^+/Ca^{2+} exchanger (NCX) in adult rat myocytes and cause inhibition of NCX1-mediated currents (150). To-date all other FXYD members appear to associate solely with the Na,K-ATPase.

1.3.2.d. Functional interactions of FXYD's with Na,K-ATPase

Table 1-2 summarizes the functional effects of FXYD proteins on Na,K-ATPase activity. It can be seen that the FXYD members modulate the kinetic activity of the enzyme by altering the apparent affinity for its substrates (i.e. Na^+, K^+ and ATP) and suggests a unique and novel mode of regulation of the Na,K-ATPase that involves the tissue-specific expression of an auxiliary subunit. Thus, to-date it is assumed that FXYD proteins are tissue-specific regulatory subunits, which adjust the kinetic properties of the pump to the specific needs of the relevant tissue or cell type.

Next to the γ modulator, CHIF is the best characterized member to-date. The functional effects have been well documented in both Xenopus oocytes (11) and mammalian cells (46). As can be seen from Table 1-2, the effects of CHIF on Na,K-ATPase are opposite to those of γ. Studies in both experimental systems mentioned above have indicated that CHIF increases Na^+ affinity by about 2-fold. In the presence of external Na^+ (90 mM), an increase in K'_K at hyperpolarized membrane potentials was observed such that this decrease in the apparent K^+ affinity is due to increased Na^+ competition at external binding sites. This effect of CHIF on K'_K was not detected in transfected HeLa cells (46). It is possible that the lack of observed effect of CHIF on K'_K in HeLa cells was due to a decreased amount of external Na^+ (30 mM) and a less polarized membrane.

Studies on PLM have also provided some contradictory results that point to the complexity of the functional interactions that FXYD proteins have with the Na,K-ATPase. For instance in Xenopus, expression of PLM results in a decrease in the apparent affinity for Na^+ and K^+ with no effect on the maximal rate (31). However, recent studies using sarcolemma membranes obtained from hearts of PLM KO mice showed a lower maximal rate with no change in apparent affinity for Na^+ (59). Furthermore, other recent work with purified complexes of α-β-PLM from Pichia pastoris (79) showed an increase in affinity for Na^+, opposite to the results obtained in Xenopus oocytes. Whether or not phosphorylation states of PLM can account for these differences is unclear.

Co-expressing FXYD 5 with the α1 and β1 subunits of the Na,K-ATPase in Xenopus oocytes resulted in a 2-fold increase in pump activity, measured either as maximum outward current or ouabain-sensitive $^{86}Rb^{+}$ uptake (87).

Detailed reviews of the structure/function relationship between Na,K-ATPase and FXYD proteins have recently been published (45, 48).

Table 1-2: Effects of FXYD proteins on Na,K-ATPase activity (modified from ref. 45)

FXYD protein	Experimental System	Kinetic Parameter	Fold effect (FXYD/control)	References
FXYD 1 / Phospholemman (PLM)	Xenopus oocytes[a]	$K_{0.5(Na)}$ $K_{0.5(K)}$ V_{max}	1.8 (α1); 1.51 (α2) 1.4 (α1); 1.42 (α2) No change	(31)
	Choroid plexus, neutralizing antibody	$K_{0.5(Na)}$ V_{max}	No change 1.4	(40)
	KO mouse-cardiac SLM	$K_{0.5(Na)}$ V_{max}	No change 2.1	(59)
	Cardiac SLM	V_{max}	2.8 (phosphorylated)	(43)
	Cardiac myocytes	V_{max}	1.36 (α1) (phosphorylated)	(127)
FXYD 2 / γ	Xenopus oocytes[b]	$K_{0.5(K)}$ (at – 150 mV) $K_{0.5(K)}$ (at – 50 mV) $K_{0.5(Na)}$	0.8 (γa or γb) No change 1.3 (γa); 1.2 (γb)	(11)
	Renal microsomes ± C-terminal γ-antibody	K_{ATP}	0.5	(136)
	Mammalian cell membranes	K_{ATP} $K_{0.5(Na)}$ $K_{0.5(K)}$	0.5 (γa or γb) No change 1.5 (γa)[c] 1.5 (γa) 1.5 (γb) 1.6(γa)[c] No change (γa or γb)	(114, 137) (2, 4) (4) (114) (4) (114)
	Mammalian cells	K_{ATP} $K_{0.5(Na)}$ $K_{0.5(K)}$	0.8 (γb) 1.7–1.9 (γa or γb) 0.64	Ch.2
	Reconstitution with γ-TM peptide	$K_{0.5(Na)}$	2–3	Ch.3
	KO mouse Kidney membranes	$K_{0.5(Na)}$ $K_{0.5(K)}$ K_{ATP}	1.3 No change 1.3	(62)

FXYD protein	Experimental System	Kinetic Parameter	Fold effect (FXYD/control)	References
FXYD 3 / Mammary tumor (Mat-8)	Xenopus oocytes	$K_{0.5(Na)}$ $K_{0.5(K)}$	1.2 1.1–1.4	(32)
FXYD 4 / Corticosteroid Hormone Inducing Factor (CHIF)	Xenopus oocytes	$K_{0.5(Na)}$ $K_{0.5(K)}$ (at –50 mV)	0.6 1.5	(11)
	Mammalian cells	$K_{0.5(Na)}$ $K_{0.5(K)}$	0.3 No change	(46)
FXYD 5 / Related to Ion Channel (RIC), Dysadherin	Xenopus oocytes	V_{max}	2	(87)
FXYD 6 / Phosphohippolin (Php)		n.d.	n.d.	
FXYD 7	Xenopus oocytes	$K_{0.5(Na)}$ $K_{0.5(K)}$	No change 1.3–1.9 (α1 and α2)	(12)
PLMS	Proteolytic digestion of C-terminus of PLMS	V_{max}	1.4 (α3)	(93)

[a] $K_{0.5(K)}$ measured in the presence of extracellular Na^+

[b] Effects on $K_{0.5(K)}$ seen in both the presence and absence of extracellular Na^+

[c] In this study the γ subunit was expressed as a doublet which showed effects on both $K_{0.5(Na)}$ and $K_{0.5(K)}$, or as a single band which showed only the $K_{0.5(K)}$ effect.

1.3.3. The γ modulator

1.3.3.a. Discovery of the γ modulator

First noticed by Rivas et al. (118), the γ modulator was only considered to be a part of the Na,K-ATPase complex following photolabelling experiments with 2-nitro-5-azidobenzoyl (NAB) derivatives of ouabain carried out with purified pig kidney membranes (41), also suggesting that γ may be part of the ouabain-binding site. It was later demonstrated that γ associated in equimolar amounts with both α and β subunits (26, 53, 116). Molecular cloning and sequencing of γ have been reported for rat, mouse, sheep and cow (102), Xenopus (13) and human (70). Sequence homology is high overall (~75%) and increases when mammalian sequences are compared (93%).

The γ modulator contains all the conserved motifs typical of the FXYD family of proteins, namely $F^{18}XYD$ in the extracellular domain, $G^{30}G^{41}$ in the transmembrane region and $S^{47}X(R/K)F(R/K)C$ following the transmembrane domain. The presence of positively charged amino acids near S^{47} make it a likely target for phosphorylation by PKC. Interestingly, it has been reported that γ could be phosphorylated by PKC in the presence of detergent (92).

1.3.3.b. Membrane Topology and Distribution

Early studies using N-terminal or C-terminal anti-Flag-labelled γ binding to oocytes were able to detect only the N-terminus of epitope tagged γ. This provided evidence that γ is a type I membrane protein with a single transmembrane domain and its N-terminus facing the extracellular space (13).

This was further supported by Therien et al. (136) who showed trypsin sensitivity of the N-terminus of γ in intact right-side out pig kidney microsomes.

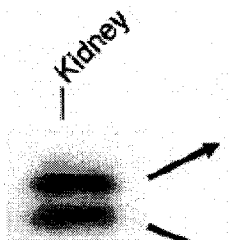
In 1993, Mercer et al. (102) using an immunoprecipitation approach demonstrated that γ was a component of the Na,K-ATPase and that its distribution pattern parallels that of α1 in the nephron. Later, expression of γ protein was assessed in a variety of tissues, including rat glomerular cells, medulla, axolemma, heart, red blood cells, lung, stomach, spleen and several cell lines derived from kidney. Using anti-γ raised to the C-terminus, Therien et al. (136) showed that γ was detected primarily in the kidney and was absent from cultured cells derived from renal tissue.

1.3.3.c. γ variants

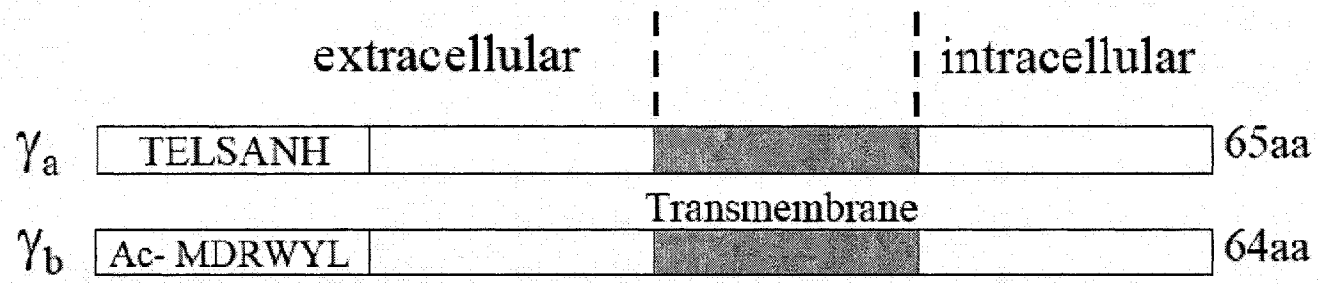
The γ modulator appears as a doublet on SDS-PAGE. Doublets of γ have been observed in kidney membranes derived from rat (4, 73), dog (102) and pig (108). Human γ expressed in Sf-9 cells also migrated as a doublet (103). The presence of the doublet led to the suggestion that γ is post-translationally modified (4) and in an earlier study (102), doublets were also observed in *in vitro* expression in the presence but not absence of pancreatic microsomes supporting this notion. However, further studies using mass spectrometry indicated that the two bands seen on SDS-PAGE of rat kidney are splice variants which differ in their primary sequence (73). The upper band, γa (7184 kDa), corresponded closely to the published sequence (137) and the lower band was referred to as γb (7337 kDa). Tryptic peptide mapping and sequencing by mass spectrometry

revealed that the chains are identical except for their seven N-terminal residues. In γa, the N-terminal residues are TELSANH whereas in γb they are replaced by Ac-MDRWYL. Additional bands observed on Western blots following transfection are probably the result of cell-specific post-translational modifications (73). A third variant had been reported by Jones et al. (61) based on their cloning and sequencing of the mouse γ gene. Analysis of the structure of the mouse gene showed that it encodes three tissue-specific mRNAs that have distinct extracellular N-termini but common C-terminal and transmembrane regions.

Fig. 1-4: rat γ variants



	M.W. (Da)	Transmembrane organization	Posttranslational Modifications in kidney
γa	7184.0	N-terminus out C-terminus in	None
γb	7337.9	Same	N-terminal acetylation



1.3.3.d. Expression and cell surface delivery of γ in various cell systems

Yeast

In earlier studies with pump devoid yeast cells, expression of α and β subunits with and without γ failed to show any γ-dependent effects on overall Na,K-ATPase activity. This suggested that the α and β subunits are the minimal structural unit required for cation transport and that γ is not an obligatory "subunit" (120).

Amphibian oocyte

Beguin et al. (13) using cRNA-injected Xenopus oocytes showed that γ associates with the Na,K-ATPase α and β subunits in or close to the ER and is transported to the membrane within 48 hours. These authors showed that association of γ with the Na,K-ATPase is critical for γ stability and delivery to the membrane. The expression of γ did not affect cell surface expression or maximum transport activity of the Na^+ pump, as seen by the induction of similar pump currents when expressing either αβ or αβγ. However, modulation of kinetic properties by γ was observed as described in section 1.3.3.f. below.

Insect cells

In Sf-9 cells expressing the human γ subunit, single and double immunofluorescence staining demonstrated that γ could be delivered to the plasma membrane independently of and with the other pump subunits and that this increased Na^+ and K^+ uptake (103). Furthermore, coimmunoprecipitation of Sf-9 cells coexpressing γ and α subunits provided evidence for a direct interaction

between the two subunits. Similar results were obtained when α, β and γ were co-expressed.

Expression during embryonic development

Expression of γ was investigated in preimplantation mouse development, where the Na,K-ATPase plays an important role in cavitation (60). Gamma mRNA accumulated continuously throughout all stages of cell development. The γ protein localized at the blastomere surface up to the blastocyst stage, and unlike α and β subunits which accumulate at the basolateral surface, γ was concentrated primarily at the apical surface of the polarized trophectoderm layer. When treated with antisense RNA complementary to γ, cavitation was delayed and there was a reduction in ouabain-sensitive $^{86}Rb(K^+)$ uptake. However Na,K-ATPase activity was unaffected as demonstrated by direct phosphorylation assays on membrane preparations.

In a study of the expression pattern of the α, β and γ subunits during *in vitro* induction of pluripotent murine embryonic stem cells into neuronal cells, no mRNA for γ was detectable at any stage (52). This further supports the tissue-specific distribution and expression of γ.

In a more recent study, the expression of α, β and γ was characterized in the developing Xenopus embryo (35). Each Na,K-ATPase subunit displayed a distinct tissue-specific and developmentally regulated expression pattern. During pronephric kidney development, all three proteins were uniformly expressed along the entire nephron.

Regulation of γ expression and cell growth

Expression of the γ modulator has been shown to be regulated by osmolarity. A study by Capasso and Rivard (19) using cultured inner medullary collecting duct (IMCD) cells showed that γ was absent in cells in isotonic medium, but displayed a time- and osmolarity-dependent appearance in cells adapted to survive in hypertonic conditions (i.e. > 24 hours with NaCl concentrations ≥ 500 mOsm). The regulation is transcriptionally regulated and involves signalling through the PI3K and JNK2 kinase pathways since kinase inhibitors abolished γ expression in hypertonic cells. In a later study these authors showed that the upregulation of the γ modulator was Cl^- dependent (21). Synthesis of the γ modulator is also regulated at the translational level in IMCD cells (20).

A role of γ in cell growth is suggested by the induction of γa in cultured rat kidney epithelial-derived NRK-52E cells in response to hypertonicity which was accompanied by a decrease in cell growth (145). Expression of γa was greater in cells treated with sucrose than NaCl (500 mOsm). Other stress inducers such as heat shock (43 °C for 1 hour), heavy metal (30 μM $HgCl_2$ for 24 hours) and oxidative stress (1 mM hydrogen peroxide for 24 hours) also resulted in upregulation of γa. A significant reduction in Na,K-ATPase activity was noted for cell adapted to hyperosmotic conditions (αβγ) compared to control (αβ). Effects on cell growth and maximal activity were abrogated when expression of γ was knocked down using small interfering RNA duplexes (145). Interestingly, γ expression due to hypertonicity was also induced in cell lines from other tissues

such as C6 (glioma), PC12 (pheochromocytoma), and L6 (myoblasts). Taken together, their data suggest that expression of γ may be affected *in vivo* by the ionic conditions of the kidney and may have adaptive value (145).

1.3.3.e. Specificity of γ interaction with the Na,K-ATPase: γ-α association

In studies of coexpression of γ with α and β using the Xenopus oocyte, γ required association with the Na,K-ATPase for stable expression at the cell surface (13). Furthermore, γ does not interact alone with either α or β subunits but rather requires the assembly of functional pumps (αβ). Coexpression studies revealed that γ could interact with all α isoforms but not with other transporters like the glucose transporter (GLUT2), the rat epithelial Na channel (rENaC) and the gastric H,K-ATPase. Similar results were obtained for both human and rat γ (13). No association of γ with the colonic H,K-ATPase could be detected (25).

1.3.3.f. Functional effects of the γ modulator

Evidence that γ plays a direct role in modulating Na,K-ATPase activity was first obtained in studies with Xenopus oocytes and mammalian cells.

Studies using membrane fragments isolated from γ-transfected cells as well as rat kidney enzyme microsomes have established that γ serves at least three distinct functions in regulating the pump. Specifically, the distinct effects of γ on the Na,K-ATPase are (i) an increase in the apparent affinity for ATP (114, 115, 137) (ii) an increase in the affinity for external K^+ (see Chapter 2 and ref. 11) and (iii) an increase in K^+/Na^+ antagonism, seen as an increase in K'_{Na} at high K^+

concentrations (114, 115). This last effect on Na^+ affinity is underscored in recent studies showing that renal Na,K-ATPase from γ knock-out mice displayed higher affinity for Na^+ than that from wild type animals (62).

Therien et al. showed that antiserum raised against the C-terminal sequence of γ (anti-γ) partially inhibited Na,K-ATPase activity while no effect was detected with membranes from tissue or cultured cells which lacked endogenous γ (136). They showed that anti-γ increasingly inhibited Na,K-ATPase as a function of pH (inhibition increases at acidic pH under which condition the $E_2(K) \rightarrow E_1$ step becomes more rate-limiting), and that the oligomycin-stimulated increase in phosphoenzyme due to trapping of the enzyme in the E_1 state(s) was greater in the presence of anti-γ. Taken together, their findings suggest that γ shifts the steady-state equilibrium towards E_1 state(s) and that binding of the antibody abrogates these effects. These functional effects were also characterized using mock- and γ-transfected HEK cells to measure (i) K'_{ATP} of Na,K-ATPase activity and (ii) K^+ inhibition of Na-ATPase at 1 μM ATP which indicated that γ increased the apparent affinity for ATP (137); an effect again counteracted by anti-γ.

Pu et al. also looked at the behaviour of both splice variants in an attempt to detect functional differences, if any, between them (114). These studies showed that there was no detectable differences between γa or γb with respect to its effect on K'_{ATP}. These authors also showed that γ decreases sensitivity to vanadate compared to mock-transfected α1-HeLa cells (I_{50} for vanadate was

increased ~2-fold). These results are consistent with the aforementioned conclusion (136, 137) that γ shifts the E_1 to E_2 equilibrium in favor of E_1 form(s).

Using electrophysiological approaches to measure Na^+ current in oocytes Beguin et al. (11) showed that in the absence of Na^+, γa and γb both produced an ~2-fold increase in affinity for external K^+ in the high negative membrane potential range (≤ -100 mV). In the presence of extracellular Na^+, γa and γb caused an increased affinity of the Na,K-ATPase for K^+ when the membrane was hyperpolarized (≤ -100 mV) and decreased K^+ affinity at lower membrane potentials (≥ - 50 mV). Using α1 mutants Li et al. showed that F956 and E960 were essential for the transmission of the functional effect of γ on the apparent K^+ affinity of Na,K-ATPase and that the relative contribution of these residues to the K^+ effect differs for different FXYD proteins, probably reflecting the intrinsic differences of FXYD proteins on the apparent K^+ affinity of Na,K-ATPase. In contrast, F956 and E960 were not involved in the effect of γ or CHIF on the apparent Na^+ affinity (78).

Another affect of γ on Na,K-ATPase activity is observed as a decrease in the apparent affinity for Na^+ at cytoplasmic activation sites. Indirect evidence for this effect was seen by Therien et al. (133) in their studies directed towards determining tissue- versus isoform-specific differences in cation activation. Those experiments showed that the rat kidney enzyme exhibits high K^+/Na^+ antagonism when compared to other tissues. Later a detailed analysis of K'_{Na} as a function of K^+ concentration provided strong evidence in support of the conclusion that the increase in K'_{Na} reflects K^+/Na^+ antagonism since this effect is

only seen at high K^+ concentrations and is the result of an increase in affinity for K^+ at cytoplasmic activation sites rather than a direct effect on K_{Na}. Pu et al. (114) showed that this holds true for both variants.

Structure/function studies provide evidence for multiple sites of γ-αβ interaction involving both TM segments and the extramembranous domains of γ. Pu et al. (115) showed the C-terminus is essential for the modulation of K'_{ATP} and that there is long range modulation by the N-terminus since N-terminal deletion abrogated the K'_{ATP} effect of γ. These authors also showed that the TM region is important for K^+/Na^+ antagonism. Using a series of γ-CHIF chimeras in which extracellular, TM and cytoplasmic domains were interchanged, it was shown that the effects of the FXYD-αβ complex on the apparent Na^+ affinity are determined by the origin of the TM domain and that the effect on K'_{Na} is mediated by residues other than those that determine the stability of the complex (these residues were described in section 1.3.2.b.) (80). In summary, to-date it appears that the TM segment mediates the effects of γ and CHIF on Na^+ affinity, while the extramembranous segments mediate the effect of γ on apparent ATP affinity.

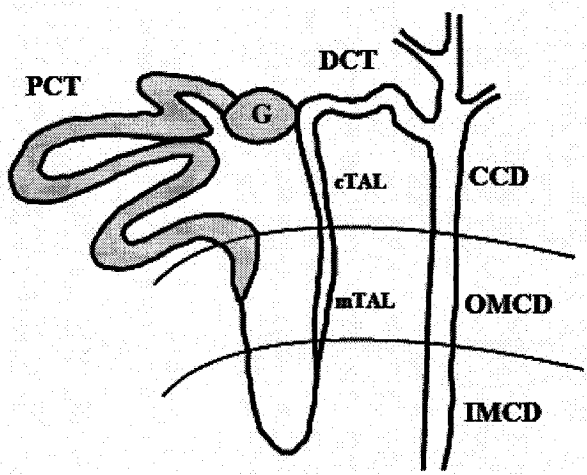
1.3.3.g. Localization of the Na,K-ATPase α subunit and γ in the nephron

Immunocytochemical approaches consisting of double label immunofluorescence studies (114) using an antibody directed towards the C-terminus of γ that is able to recognize both variants, and antibodies raised against the α subunit showed basolateral colocalization, with considerable overlap in the inner and outer medulla. Most notable staining for γ was in the latter. Expression

of α without γ can be seen in some tubule segments of the inner medulla. Taking advantage of the fact that the γ variants differ only in their extracellular N-terminus, antibodies raised against the two distinct N-termini were used to distinguish between the splice variants. Using these antibodies Pu et al. (114) demonstrated a partially overlapping but distinct expression pattern for γa and γb. Both are in the medullary thick ascending limb (mTAL) and in the proximal tubules. The γa variant is specific to the CCD and to cells close to the macula densa (MD), while γb expression is specific to the cTAL. Strong staining for both α and γ subunits was also seen in the distal convoluted tubule (DCT) (4, 146). Similar findings are reported elsewhere (5), are reviewed in refs. 37 and 113 and summarized in Fig 1-5.

Fig. 1-5: γ variant distribution along the nephron

Figure adapted from Farman et al. (37)



	PCT	mTAL	cTAL	MD	DCT	CNT/CCD	OMCD	IMCD
γa	+	+++	-	++	+	+	-	-
γb	+	+++	++	-	+	-	-	-

1.4 Goal of this study

The mechanisms of Na,K-ATPase regulation are diverse and include the modulation by its association with other intrinsic membrane proteins. Amongst such regulators are FXYD proteins that comprise a gene family of small, single transmembrane proteins that modulate the kinetic behaviour of the Na,K-ATPase in a tissue-specific manner. This dissertation concerns the regulation of the Na^+ pump by the γ modulator that exists as two main variants, γa and γb, with distinct as well as overlapping expression along the nephron. Specifically, my research addresses several aspects of γ structure and function, namely its function in intact cells, the role of the γ transmembrane domain, and the regions of the sodium pump's catalytic subunit with which γ interacts. Specific goals are outlined below.

The first goal of my research was to determine whether the modulatory effects of the two variants of the γ modulator of the Na,K-ATPase, γa and γb, seen previously with unsided membrane preparations, are relevant to pump behaviour under more physiological conditions that prevail in intact mammalian cells. The approach was to use intact γ-transfected cells, and to measure γ effects on steady-state $[Na]_{in}$ and pump-mediated $^{86}Rb(K)$ influx as a function of varying intracellular ATP and Na^+ as well as extracellular K^+ (see Chapter 2).

Another objective of my work was to gain insight into regions of the transmembrane (TM) domain of γ that are important for (i) trafficking/association with αβ pumps, (ii) the structural basis for the γ-mediated increase in intracellular K^+/Na^+ antagonism seen as an increase in K'_{Na} at high K^+ concentration and (iii) the basis for the abrogation of γ modulation by the γG41R mutation associated

with renal magnesium wasting. For this purpose, synthetic TM peptides of γ_{wt} and γ_{mutant} were tested directly in functional assays. Accordingly, effects on K'_{Na} at high K^+ concentration as well as association of γ_{wt}- and γ_{mutant} with $\alpha\beta$ pumps were determined using γ_{wt}- and γ_{mutant}-transfected HeLa cells (see Chapter 3).

Another aim of my research was to determine the region(s) of α that not only interact(s) with the γ subunit, but are also important for the modulatory effects of γ. Since initial findings showed that γ associated with all three major isoforms of α (α1, α2 and α3) but increased the K'_{Na} of α1 and α3 and not α2, it was hypothesized that regions important for function are not necessarily those which are important for association of γ with $\alpha\beta$ pumps. Accordingly, I used an α1/α2 chimera approach to identify regions of α critical for the functional effects of γ, in particular K^+/Na^+ antagonism apparent as an increase in K'_{Na} at high K^+ concentration (see Chapter 4).

CHAPTER 2

The Effect of the γ Modulator on Na,K-ATPase Activity of Intact Mammalian Cells

2.1 PREFACE

This manuscript has been published in the Journal of Membrane Biology and is titled "The Effect of the Gamma Modulator on Na/K Pump Activity of Intact Mammalian Cells" (Zouzoulas, A., Therien, Dunham, P.B., and Blostein, R. *J. Membr. Biol.* 204, 49-56, 2005). The goal was to determine whether the modulatory effects of the two variants of γ, seen previously with unsided membrane preparations, are relevant to Na,K-ATPase behaviour under the more physiological conditions that prevail in intact mammalian cells.

2.2 ABSTRACT

This study concerns the modulatory effects of the gamma modulator of the Na/K pump, in particular whether the effects seen in previous experiments with isolated membranes are relevant to Na/K pump behavior in intact mammalian cells. For this purpose, HeLa cells previously transfected with the rat Na/K catalytic subunit were used. The results show that both variants of the regulator, γa and γb, decrease the apparent affinity of the pump for Na^+ and cause a modest increase in apparent ATP affinity as seen in measurements of ouabain-sensitive $^{86}Rb(K^+)$ influx into cells in which ATP was varied using antimycin A and glucose. Equivalent results had been obtained previously in our analyses of Na,K-ATPase activity of membrane fragments, i.e., an increase in $K_{0.5(Na)}$ at high K^+ concentration and a decrease in K'_{ATP}. Comparison of clones of γ-transfected and mock-transfected cells (with similar V_{max} values) indicated that γ causes a modest ≅30% increase in the steady-state concentration of intracellular Na^+. Furthermore, for both γa and γb, values of intracellular Na^+ were similar to those predicted from the kinetic constants, $K_{0.5(Na)}$ and V_{max}. Finally, there was a γ-mediated increase in apparent affinity for extracellular K^+, which had not been detected in assays of permeabilized membranes.

Key words: Na/K pump — γ Modulator — Transport— Na,K-ATPase

2.3 INTRODUCTION

The Na/K pump, or Na,K-ATPase, is an integral membrane protein found in virtually all cells of higher eukaryotes. It comprises two subunits, a large catalytic α subunit and a smaller highly glycosylated β subunit necessary for the proper folding, insertion and maturation of α in the plasma membrane. The αβ dimer is the minimal structural unit required for Na/ K pump activity.

The Na,K-ATPase catalyzes the ATP-driven electrogenic exchange of three intracellular Na^+ ions for two extracellular K^+ ions coupled to the hydrolysis of one molecule of ATP. It plays a key role in chemiosmotic balance and in maintenance of the resting membrane potential. Furthermore, the Na^+ gradient generated by this pump has a critical role in regulating intracellular pH and Ca^{++} via Na^+/H^+ and Na^+/Ca^{++} exchange systems, and in the uptake of many solutes via Na^+-coupled co-transport processes.

Some of the main factors contributing to the modulation of the Na/K pump are the enzyme's substrates (Na^+, K^+ and ATP), cytoskeletal components, endogenous digitalis-like inhibitors and hormones and the signalling cascades that hormones activate (24). Regulation may also occur through the association of the Na/K pump with other intrinsic membrane proteins such as members of the FXYD family. This family comprises small Type I membrane proteins comprising a single transmembrane domain in which a PFXYD motif in the extramembranous N-terminus is invariant in cells of all mammals (22). To date, seven FXYD proteins have been identified. Several have been shown to modulate the kinetic behaviour of the pump in a tissue-specific manner (see refs. 5, 6, 10).

The γ modulator (FXYD2) of the renal Na,KATPase was discovered over 25 years ago (8), and the cDNA cloned later by Mercer et al. (17). It is the best characterized member of the FXYD family. It exists as two main variants, γa and γb. Mass spectrometry indicated that γa and γb differ only in the N-terminus (14). In rat kidney, γa has TELSANH and γb has Ac-MDRWYL. Some regions of the renal tubule have one or the other variant and other regions have both (2, 19, 29). Both variants are particularly abundant in the medullary thick ascending limb.

Previous studies from our laboratory as well as others, using membrane fragments isolated from γ-transfected rat α1-HeLa or rat NRK cells, have shown that γ serves at least two distinct functions in regulating Na,K-ATPase activity. These are (i) an increase in the apparent affinity for ATP (25, 26) and (ii) a decrease in apparent affinity for Na^+ (1, 19). The latter was shown to be due primarily to an increase in apparent K^+ affinity at cytoplasmic Na^+ activation sites (19).

A point mutation, G41→R, in the transmembrane region of γ has been associated with autosomal dominant renal magnesium wasting (16). Studies have shown that this mutation alters γ association with the Na/K pump through abnormal trafficking of γ (16). Trafficking of αβ pumps to the cell surface is not altered (20).

The present study addresses the question of how the effects of γa and γb inferred from the experiments on unsided membrane preparations are manifested in the intact cell.

2.4 EXPERIMENTAL PROCEDURES

Mutagenesis and Transfection- Rat γa and γb cDNAs were subcloned into the pIRES expression vector and transfected into HeLa cells stably expressing the rat α1 subunit of the Na,K-ATPase kindly provided by Dr. J.B Lingrel as previously described (19). The point mutation G41R was introduced into γb-pIRES using the QuikChange Site Directed Mutagenesis Kit (Stratagene). Control rat α1-HeLa cells were also transfected with the pIRES empty vector.

Transport Assays- Na/K pump activity was measured by determining unidirectional $^{86}Rb(K^+)$ influxes. Assays were carried out in the presence of a low (10 μM) ouabain concentration to inhibit endogenous human pump activity and ± a high (5 mM) ouabain concentration to inhibit activity of pumps containing the rat α1 subunit and determine baseline activity (rat Na/K pumps have a very low sensitivity to ouabain). To determine the apparent affinity for intracellular Na^+, $^{86}Rb(K^+)$ influxes (nmoles/mg/min) were determined in cells grown to 70–80% confluence on 24-well plates and assayed with various Na^+ concentrations and constant (4 mM) K^+ concentration in media containing monensin (to raise Na^+ permeability) as described previously (18) with minor modifications (9). Briefly, to vary intracellular Na^+, cells were preincubated for 15 minutes at 37°C with 12 μM monensin and 10 μM bumetanide added to solutions with various Na^+ concentrations. For the determination of the kinetic constants for Na^+ activation, values of $^{86}Rb(K^+)$ influxes expressed as functions of added extracellular Na^+ concentration were fitted to a simple one site Michaelis-Menten model (9) since it

was not technically feasible to determine intracellular Na^+ at each concentration of medium Na^+. The apparent affinity constant thus obtained is only a measure of the apparent affinity for Na^+ and is therefore represented as $K_{0.5(Na)app}$ (see Table 2-1), whereas the apparent affinity for Na^+ calculated from previous experiments with membrane fragments and precisely known Na^+ concentrations (Na,K-ATPase assays) is expressed as $K_{0.5(Na)}$ (see Table 2-2). For the determination of $K_{0.5(K)}$ and V_{max} for K^+ activation, extracellular K^+ activation of $^{86}Rb(K^+)$ influxes was analyzed with cells equilibrated with monensin and the indicated Na^+ concentration and varying K^+ concentrations. The data were fitted to a cooperative model (c.f. ref. 13):

$$V = V_{max} [cat]^n / (K_{0.5(cat)} + [cat]^n) \qquad (1)$$

where "cat" is either Na^+ or K^+. An average value for the Hill coefficient (n = 1.3) was used for all experiments with varying extracellular K^+ since no significant difference between n values of control and γ-transfected cells was seen ($1>p>0.1$). Assays were carried out with cells transfected with γ (γa and/or γb or γbG41R) and control mock-transfected cells. All assays were carried out on two clones of each type of transfected cell line. Data for the Na^+ and K^+ activation profiles were expressed as percentages of V_{max}.

Determination of Intracellular Cation Concentrations- Cells were grown in triplicate to 70–80% confluence on 100 mm tissue culture dishes and washed as described previously (18). Following treatment with 3 mL 0.05% Triton X-100, the extract was analyzed for Na^+ and K^+ content by atomic absorption and for

protein by the Lowry method. Determinations were carried out on two clones of each type of transfected cell line.

Procedures for varying intracellular ATP and measuring $^{86}Rb(K^+)$ influx concurrently- To reduce intracellular ATP concentration in a graded and reproducible manner, cells cultured in 24-well plates were incubated for 2 h at 37°C with gentle agitation in medium containing (in mM) 40 NaCl, 2 KCl, 108 choline chloride, 5 HEPES-Tris, 1.5 PO_4-Tris, 0.5 $MgCl_2$, 1 $CaCl_2$, pH 7.4, 1 μM ouabain, 0.1 μM antimycin A and D-glucose ranging from 0.05–1 mg/mL. The medium was aspirated and replaced by the same medium containing 12 μM monensin, 10 μM bumetanide (to inhibit NaKCl2 cotransport) and 10 μM ouabain (as in flux assays). Following a preincubation of 15 min, one set of triplicate wells was taken for the determination of ATP and protein and a second set was taken for the determination of transport activity as described above. For each experiment, assays were carried out in triplicate, with either γa-, γb -, or γbG41R-transfected rat α1-HeLa together with control mock transfected cells assayed concurrently.

Measurement of ATP concentration- Following preincubation as described above, the medium from each well was aspirated and the cells were immediately chilled on ice and lysed with 0.5 mL 5% TCA. Following gentle agitation for 10 min on ice, the 24-well plate was centrifuged and the supernatant adjusted to pH 7.6 with 2 M Tris. To each assay vial were added 2.5 mL diluent (in mM: 50 glycine, 1

Tris, 5 $MgSO_4$, 0.5 EDTA, 0.1% BSA, 0.1% NaN_3), 200 μL of sample supernatant or standards (solutions containing 0.001 nmol–1.2 nmol ATP in 5% TCA neutralized to pH 7.6), 50 μL luciferin (357 μM) and 10 μL luciferase (1.5–3 $x10^6$ units/mL). The light emissions were measured by liquid scintillation spectrometry using a wide channel of the single-photon monitor of the Beckman Coulter LS 6500 Multi-Purpose liquid scintillation counter. Protein remaining in the wells was solubilized with 0.5 mL 0.5 M NaOH and measured by the Lowry method. The concentration (mM) of intracellular ATP was estimated by assuming 8 μL water/mg protein (18).

Estimation of intracellular Na^+ concentration- To estimate intracellular Na^+ concentration in γ- and γG41R-cells, we used the following relationship (based on ref. 11):

$$L \cdot ([Na]_{out} - [Na]_{in}) = V_{max} \cdot [Na]_{in}^3 / (K_{0.5(Na)} + [Na]_{in})^3 \qquad (2)$$

where L is the rate constant of the Na^+ leak in s^{-1}, $[Na]_{out}$ and $[Na]_{in}$ represent extracellular and intracellular Na^+ concentrations, respectively, and the other parameters have their usual meaning. L was first calculated using the kinetic parameters of V_{max} and $K_{0.5(Na)}$ of the Na/K pump and the measured $[Na]_{in}$ of the control (α1-C) mock-transfected cells. This value of L thus calculated was then used to estimate $[Na]_{in}$ in γ- and γG41R-transfected cells using values of V_{max} of the same cells. Since the Na^+ concentrations are precisely known in experiments carried out with membrane fragments, $K_{0.5(Na)}$ values used were calculated from earlier Na^+-activation profiles of membrane Na,K-ATPase carried out at high (100

mM) K^+ concentration (19) using Eq. 1. with n = 3. Iterative techniques using Excel software were used to solve the above non-linear Eq. 2.

Data Analysis- Curve fitting was carried out using the KaleidaGraph computer program (Synergy software). Data are presented as mean ± SEM of the number of experiments shown in parenthesis. One-way ANOVA, or, where required, a nonparametric ANOVA, followed by appropriate pair-wise comparisons were carried out with GraphPad Instat software.

2.5 RESULTS

The effect of the two γ variants on Na/K pump behaviour of intact cells was assessed by comparing the kinetics of Na^+ and K^+ activation of ouabain-sensitive $^{86}Rb(K^+)$ influx into γa- and γb-transfected α1-HeLa cells assayed concurrently with control mock-transfected HeLa cells. γbG41R-transfected α1-HeLa cells were similarly assayed. As described in our earlier studies (18), intracellular Na^+ was fixed at a range of concentrations by preincubation with monensin.

Effect of varying Na^+

In the representative experiment depicted in Fig. 2-1, cells were equilibrated with varying concentrations of Na^+ and extracellular K^+ concentration was kept constant at 4 mM. Figure 2-1 as well as the results of replicate experiments summarized in Table 2-1 show that both γ variants decrease the apparent affinity for Na^+, whereas a difference between γbG41R and control could not be detected. This is consistent with the results of earlier biochemical assays of Na,K-ATPase activity using permeabilized membranes. In those experiments (19), the precise Na^+ concentrations were known and the data were thus fitted to a 3-site cooperative model (Eq. 1) (see Table 2-2). The differences in kinetic constants seen with intact cells ($K_{0.5(Na)app}$) compared to membranes ($K_{0.5(Na)}$) are due presumably to deviations of intracellular Na^+ from equilibrium values, particularly at low concentrations of added Na^+ (18). V_{max} values of the cells used for the determination of $[Na]_{in}$ are shown in Table 2-2. However, the

variations between them are not considered meaningful since they probably reflect differences in transfection efficiency of the different cDNAs. A key question is whether the decrease in apparent affinity for Na^+ seen with both γ variants in intact cells results in an increase in the steady-state intracellular Na^+ concentration ($[Na]_{in}$) of the cultured HeLa cells. To address this issue, we measured $[Na]_{in}$ in γa- and γb- as well as γbG41R-and mock-transfected rat α1-HeLa cells. The results shown in Table 2-2 indicate that, for all γ's (γa, γb and γbG41R), the measured $[Na]_{in}$ values are similar to those calculated from V_{max} of the same cells and $K_{0.5(Na)}$ obtained from our earlier Na,K-ATPase assays of permeable membranes (19) for which Na^+ concentrations are known precisely.[1] As evident in equation 2, V_{max} and $K_{0.5}$ have opposite effects on $[Na]_{in}$. Therefore, in γb cells which had a V_{max} similar to the control, $[Na]_{in}$ is ≅ 30% higher than the mock control. In contrast, in γa, the higher V_{max} compared to control offsets a significant effect of $K_{0.5(Na)}$ on $[Na]_{in}$.

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[1] Appropriateness of using $K_{0.5(Na)}$ from ATPase assays carried out at 100 mM K^+ is indicated by data showing that $K_{0.5(Na)}$ values for at least the two isoforms, α1 and α2, are 16.5 ± 0.1 (Table 2-2) and 21.9 ± 2.25 (A. Zouzoulas and R. Blostein, unpublished data), respectively, which are notably similar to $K_{0.5(Na)}$ determined in flux experiments, i.e. 17.2 ± 1.12 and 19.68 ± 0.96, respectively (values from ref. 18).

Fig. 2-1: Activation of ouabain-sensitive $^{86}Rb(K^+)$ influx by intracellular Na^+. Ouabain-sensitive $^{86}Rb(K^+)$ influxes were measured in cells equilibrated with the indicated concentrations of Na^+ as described in Experimental Procedures and with 4 mM extracellular K^+. Data are expressed as percentages of V_{max} calculated as described in Experimental Procedures. In the representative experiment shown, $K_{0.5(Na)app}$ (mM) was determined as described in Experimental Procedures. Values for control- (○), γa- (□) and γb- (◇) transfected cells were 8.9, 18.9 and 18.7, respectively.

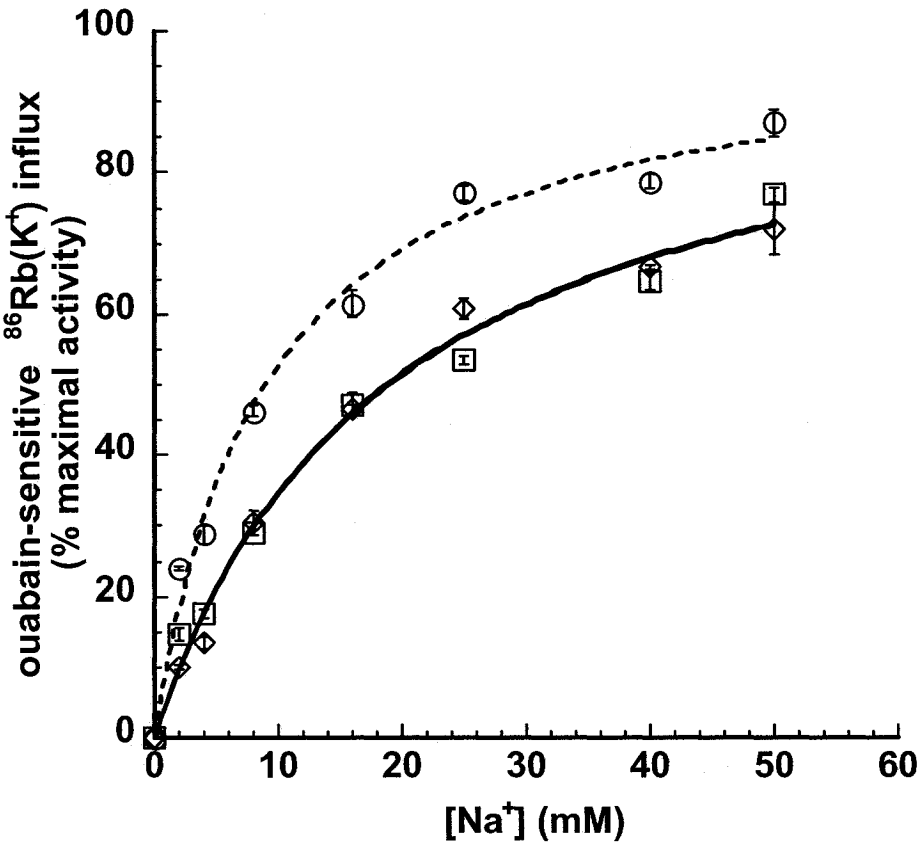

100
80
60
40
20
0
ouabain-sensitive $^{86}Rb(K^+)$ influx
(% maximal activity)
0
10
20
30
40
50
60
$[Na^+]$ (mM)

Table 2-1: Effects of γ on apparent cation affinities

	α1-C	α1-γa	α1-γb	α1-γbG41R
$K_{0.5(Na)app}$ (mM)	8.7 ± 0.4 (24)	14.9 ± 1.2*** (12)	17.2 ± 0.8***† (14)	8.0 ± 0.5* (6)
$^{a}K_{0.5(K)}$ (mM)	0.62 ± 0.02 (14)	0.40 ± 0.02*** (7)	0.40 ± 0.02*** (7)	0.58 ± 0.01* (3)

Numbers in brackets indicate the number of separate experiments. V_{max} values are shown in Table 2-2.

$^{a}K_{0.5(K)}$ was determined with cells equilibrated with 20 mM Na^{+} as described in Experimental Procedures.

$^{***}p < 0.001$ compared to control, $^{*}p > 0.05$ compared to control, $^{\dagger}p > 0.05$ compared to γa

Table 2-2: Effect of γ on steady-state parameters of Na^+ transport

Transfected α1-HeLa cells	[a]$K_{0.5(Na)}$ (mM)	V_{max} (nmoles/mg/min)	$[Na]_{in}$ (measured) (mM)	$[Na]_{in}$ (calculated) (mM)
mock	16.5 ± 0.1	37.8 ± 0.9 (24)	10.4 ± 0.6 (11)	- -
γa	20.7 ± 0.3***	42.2 ± 1.9* (12)	11.7 ± 0.6* (9)	12.2
γb	21.9 ± 0.5***[§]	36.5 ± 1.7* (14)	14.5 ± 0.5***[†] (7)	13.9
γb-G41R	16.2 ± 0.3*	30.7 ± 0.8** (6)	10.7 ± 0.6* (7)	11.4

Numbers in parentheses refer to the number of separate $[Na]_{in}$ (each in triplicate) and V_{max} determinations. The measured value of $[Na]_{in}$ for mock cells was 10.4 mM and this value was used to calculate the leak constant needed for estimation of intracellular Na^+ concentration (see Experimental Procedures and equation 2).

[a]calculated from data obtained by Pu et al. (19) using equation 1 and n = 3, and are based on assays carried out at 100 mM K^+.

***p< 0.001 compared to control, ** p< 0.01 compared to control, *p > 0.05 compared to control, [§]p > 0.05 compared to γa, [†]p < 0.05 compared to γa

Effect of varying extracellular K^+ concentration

In previous studies using unsided membrane fragments, we failed to detect a significant effect of γ on K^+-activation kinetics of Na,K-ATPase of rat α1-transfected HeLa cells (ref. 19 and experiments not shown). In contrast, Arystarkhova et al. (1) reported a γ-mediated decrease in K^+ affinity in their experiments with γ-transfected NRK52E cells. Similarly, treatment of rat renal membranes (αβγ pumps) with anti-γ (C-terminal) antibodies increased K'_K (25). In the latter study, the increase in K'_K diminished with increase in ATP concentration, consistent with the consecutive model of ATP-accelerated K^+ deocclusion following K^+-activated dephosphorylation of E_2P (c.f. ref. 7) whereby an increase in ATP affinity should decrease apparent K^+ affinity and vice versa. Although a statistically significant effect of γ on $K_{0.5(K)}$ was not detected in our initial flux studies with intact cells (19), this issue was addressed further by performing a large number of experiments with cells equilibrated with Na^+ at various concentrations. The results of these flux studies comprising control versus γa and γb assayed concurrently (summarized in Table 2-1; typical experiment shown in Fig. 2-2) indicate that both variants cause a modest but significant increase in apparent affinity for K^+. Similar percentage decreases (≅ 30%) in $K_{0.5(K)}$ were observed over a range of Na^+ concentrations tested as shown in Table 2-3. As in the experiments carried out at 20 mM Na^+ and shown in Table 2-1, a difference between the effects of γa and γb was not detected.

Fig. 2-2: Activation of ouabain-sensitive $^{86}Rb(K^+)$ influx by extracellular K^+. Ouabain-sensitive $^{86}Rb(K^+)$ influx was carried out as described in Experimental Procedures. Data are expressed as percentages of V_{max}. In the representative experiment shown, the cells were equilibrated with 20 mM Na^+. $K_{0.5(K)}$ (mM) for control- (○), γa- (□) and γb- (◇) transfected cells were 0.51, 0.35 and 0.38, respectively.

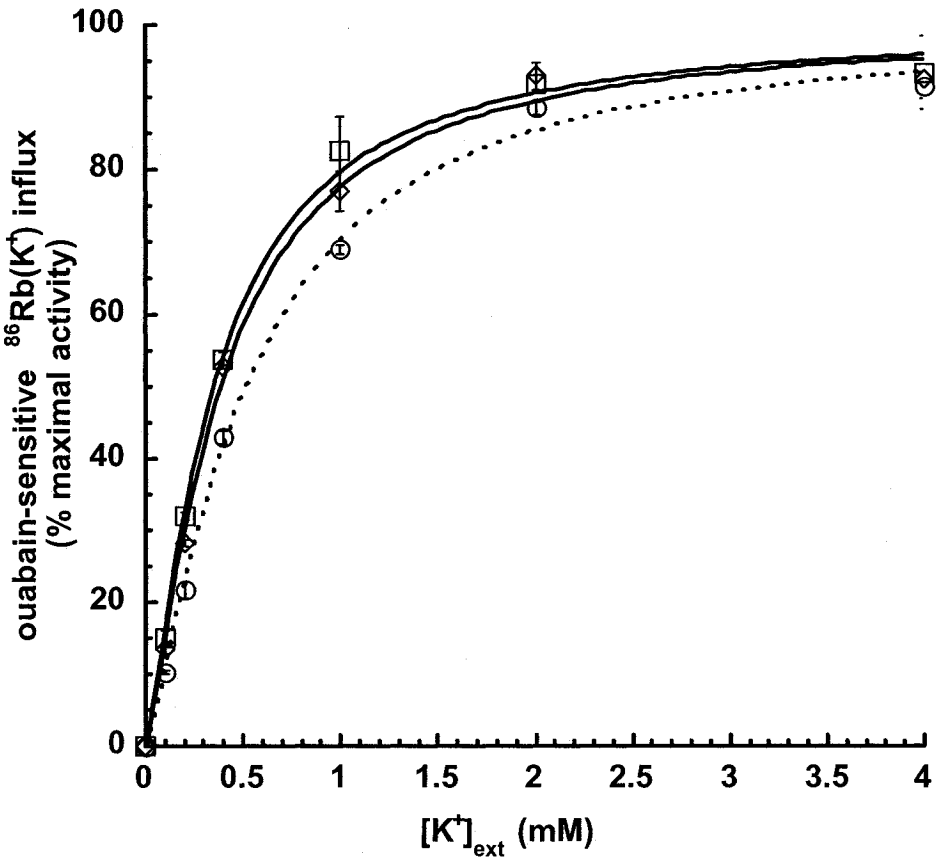

100
80
60
40
20
0
ouabain-sensitive $^{86}Rb(K^+)$ influx
(% maximal activity)
0
0.5
1
1.5
2
2.5
3
3.5
4
$[K^+]_{ext}$ (mM)

Table 2-3: Effects of γ on $K_{0.5(K)}$ as a function of varying extracellular Na^+

	$K_{0.5(K)}$ (mM)	
Na^+ (mM)	mock	γa and γb
10	0.34 ± 0.03 (4)	0.25 ± 0.02** (7)
[a]20	0.62 ± 0.02 (14)	0.40 ± 0.01** (14)
40	1.01 ± 0.12 (7)	0.73 ± 0.04* (6)
80	2.90 ± 0.02 (3)	1.87 ± 0.05** (4)

Numbers in brackets indicate the number of separate experiments.

[a] Data taken from Table 2-1

** $p < 0.01$, * $p < 0.05$ compared to control (unpaired t-test)

Does γ affect the ATP-dependence of Na/K pump activity?

To determine whether the γ-mediated increase in ATP affinity seen in biochemical Na,K-ATPase assays carried out with fragmented membranes is evident in intact cells, the relationship between pump flux and cellular ATP concentration was compared in γ-transfected and mock-transfected HeLa cells. In preliminary experiments, we used various strategies to deplete intracellular ATP and found that preincubation of cells with antimycin A and varying the concentration of glucose prior to the transport assay provided an optimal means of varying intracellular ATP in a graded fashion. Fig. 2-3 depicts a typical range of intracellular ATP concentrations obtained following a 2 h depletion. The data show similar depletion patterns for control and γ cells. It was shown previously (12) that incubation of HeLa cells with metabolic inhibitors causes the cells to gain Na^+ to different extents depending on the intracellular ATP content. To avoid changes in $[Na]_{in}$ as the ATP is altered, cells were equilibrated with monensin and 40 mM Na^+ after ATP depletion as described in Experimental Procedures. Figure 2-4A shows a representative experiment in which pump activity was measured in mock- and γb-transfected cells with varying ATP concentration. In all experiments, the flux showed no saturation up to the highest ATP concentration measured. In each paired experiment, the slope of the relationship between flux and ATP concentration was greater for γ- compared to mock-transfected cells. Thus, for 8 paired experiments (3 with γa- and 5 with γb- versus the mock-transfected control cells) a modest 22.7 ± 4.5% increase in pump rate as a function of ATP concentration in γ-transfected compared to control mock-transfected cells

was seen. When γbG41R-transfected and mock-transfected cells were compared (2 experiments), a difference was not detected (representative experiment shown in Fig. 2-4B), which is consistent with results that this mutant lacks any of the functional effects of wild-type γ (20), thus behaving like mock-transfected cells.

Fig. 2-3: ATP concentration in cells varied by incubation for 2 hours with antimycin A and varying concentrations of glucose. See Experimental Procedures. Values for control- (○), γ- (□).

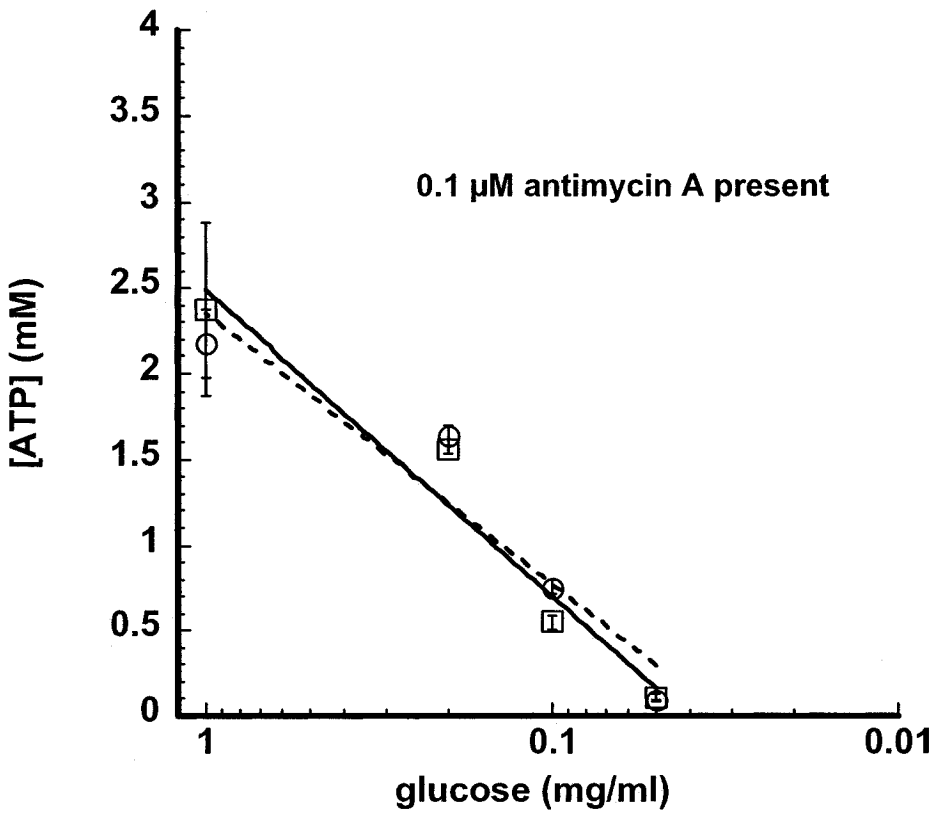
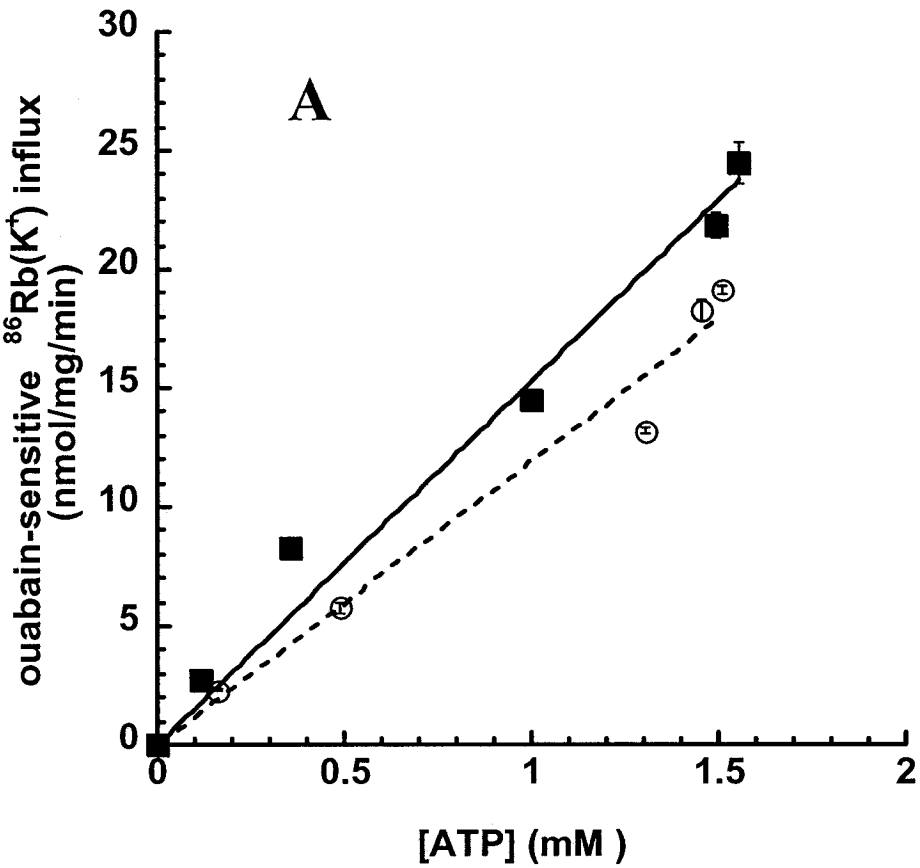
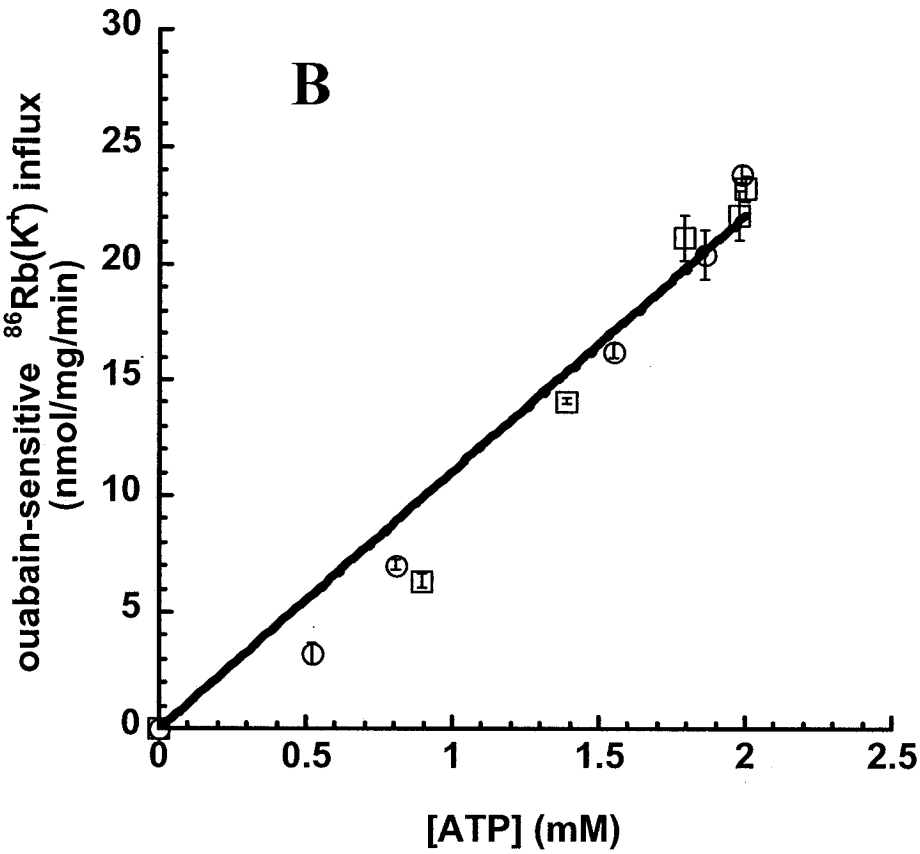

0.1 µM antimycin A present
[ATP] (mM)
4
3.5
3
2.5
2
1.5
1
0.5
0
1
0.1
0.01
glucose (mg/ml)

Fig. 2-4: Effect of ATP depletion on ouabain-sensitive $^{86}Rb(K^+)$ influx. Following ATP depletion, $^{86}Rb(K^+)$ influx was measured as described in Experimental Procedures. (A) Representative experiment of control- (○) vs γb- (■) transfected α1-HeLa cells. (B) Representative experiment of control-(○) vs γbG41R- (□) transfected α1-HeLa cells.


A
ouabain-sensitive $^{86}Rb(K^+)$ influx
(nmol/mg/min)
30
25
20
15
10
5
0
0
0.5
1
1.5
2
[ATP] (mM)


B
ouabain-sensitive $^{86}Rb(K^+)$ influx
(nmol/mg/min)
30
25
20
15
10
5
0
0
0.5
1
1.5
2
2.5
[ATP] (mM)

2.6 DISCUSSION

The present study with intact cells confirms our earlier conclusion from experiments with membrane fragments (Na,K-ATPase assays; see ref. 19), that both variants of the γ modulator of the sodium pump lower the apparent affinity for Na^+. We showed further that the γ-mediated decrease in apparent Na^+ affinity is secondary to an increase in K^+ affinity at intracellular Na^+-activation sites (K^+/Na^+ antagonism) (23). Thus, an earlier extensive kinetic analysis showed that a γ-mediated decrease in apparent Na^+ affinity seen at high K^+ concentrations is due to a higher apparent affinity for K^+ as an antagonist at Na^+ activation sites rather than a difference in Na^+ affinity per se. Consistent with those experiments, the γ-mediated increase in $K_{0.5(Na)app}$ is seen here in intact cells in which the intracellular K^+/Na^+ ratio is high. A similar result was reported by Garty et al. (9), although the variant analyzed was not specified. The present study also shows that the lowered apparent affinity for Na^+ is associated with an ≅ 30% increase in the steady-state Na^+ content of our cultured γb-transfected HeLa cells (Table 2-2). Despite the similar increase in $K_{0.5(Na)app}$ seen with both γ variants, a significant increase in $[Na]_{in}$ is not seen in our γa clones; presumably the effect of a higher $K_{0.5(Na)}$ on $[Na]_{in}$ of those cells is offset by their higher V_{max}. It is noteworthy that in both variants, $[Na]_{in}$ values agree with those predicted from the relationship between $K_{0.5(Na)}$ and V_{max} values assuming that all cells have the same leak constant as control mock-transfected cells (see Eq. 2).

The γ-stimulated increase (≅ 20%) in pump flux as a function of varying intracellular ATP concentration is notably lower than the increase (≅ 50%) in ATP

affinity observed in membrane Na,K-ATPase assays (20). This discrepancy probably reflects the two distinct and opposite effects of γ on apparent Na^+ and ATP affinities. Thus, if one takes into account the lower apparent Na^+ affinity caused by γ as depicted in Fig. 2-1 and Table 2-1, an ≅ 18% decrease in flux rate due to the change in $K_{0.5(Na)app}$ is predicted under the condition used in Fig. 2-4A (cells equilibrated with 40 mM Na^+). Therefore, the expected γ-mediated increase in flux over the range of ATP concentrations of the experiments in Fig. 2-4A should be reduced from 50% (due to the effect on K'_{ATP}) to 23% (due to the additional effect on $K_{0.5(Na)app}$; Fig. 2-1), which is similar to that observed (22.7 ± 4.5%; see Results). It is also clear that the γ modulator decreases the apparent affinity for Na^+ at high K^+ in both cells (this study) and membranes (19, 20), without a significant difference in the effect of γa versus γb. However, the absolute values of $K_{0.5(Na)app}$ and $K_{0.5(Na)}$ (Na,KATPase assays) cannot be quantitatively compared since, despite the presence of monensin, chemical equilibrium is not attained. The physiological relevance of these opposing effects of γ is discussed below.

Na,K-ATPase assays of isolated membrane preparations measured as a function of ATP concentration indicate that the Na/K pump has a $K_{0.5}$ for ATP of approximately 400 μM, whereas experiments with intact cells reveal that the ouabain-sensitive flux does not appear to saturate with ATP up to at least 2 mM. The near linear relationship between $^{86}Rb(K^+)$ influx and ATP seen in this study is consistent with previous results obtained with HeLa cells (12). This behaviour is also reminiscent of the findings with intact renal tubules in which a linear

dependence of the Na/K pump on ATP up to about 3 mM was observed (21). As these authors point out, the products of ATP hydrolysis inhibit the forward pump reaction. Thus, increased formation of products, particularly ADP, as ATP is lowered would inhibit the pump, thereby linearizing the normally hyperbolic curve relating flux to ATP concentration.

Overall, the increase in flux as a function of ATP concentration as well as the increase in steady-state intracellular Na^+ concentration are relatively modest. Nevertheless, considering the major role of the pump in salt and water balance and as we have discussed elsewhere (27, 28), the γ modulator may serve to maintain pump activity under energy-deprived conditions such as may occur in the anoxic mTAL. At the same time, by lowering apparent Na^+ affinity, an appropriate intracellular Na^+ level for Na^+-coupled transport processes is maintained.

The increase in apparent K^+ affinity of Na/K pumps seen in the present study with intact cells was not seen in experiments with permeable membrane preparations (19). We considered the possibility that this difference is a consequence of the high intracellular K^+/Na^+ concentration ratio of intact cells since we showed previously that the γ modulator increases the apparent affinity for K^+ as an antagonist of cytoplasmic Na^+ activation (19, 23). Accordingly, γ may increase the backward rate of the $E_2(K) \leftarrow \rightarrow E_1 + K^+_{cyt}$ reaction step and thus increase the apparent affinity for extracellular K^+ in the preceeding $E_2P + K^+_{ext} \rightarrow E_2(K) + P_i$ step. This is an unlikely explanation since the γ-mediated decrease in $K_{0.5(K)}$ was similar in cells equilibrated over a range of Na^+ concentrations (see

Table 2-3) and hence changing intracellular K^+/Na^+ ratios. Alternatively, it is notable that in experiments with oocytes, the apparent affinity for extracellular K^+ was increased by both γ variants provided that the membrane potential was clamped at values more negative than ≅ -50 mV (4, 15). Therefore it is plausible that in HeLa cells as in oocytes, there is a membrane potential-sensitive γ-mediated increase in the intrinsic affinity of the pump for extracellular K^+.

What is the physiological significance of the increase in apparent K^+ affinity caused by γ seen in experiments with intact cells? Under physiological conditions the normal serum K^+ concentration ranges from 3.7 to 5.2 mM and thus, extracellular K^+ activation sites would not be completely saturated, particularly in cells without γ. Thus, at the highest concentration of Na^+ tested (80 mM), $K_{0.5(K)}$ values were 2.90 mM and 1.87 mM for control and γ-transfected cells, respectively (see Table 2-3). These values would be even higher at physiological concentrations (≅ 140 mM) of extracellular Na^+ due to the well-documented Na^+/K^+ competition at extracellular K^+-activation sites (3). Accordingly, the increase in apparent affinity caused by γ is another means of maintaining pump activity under energy-compromised conditions.

2.7 ACKNOWLEDGMENTS

We thank Dr. J. B. Lingrel for the rat α1-transfected HeLa cells. This work was supported by grant MT-3876 from the Canadian Institutes for Health research (to RB) and from the NIDDK R37 DK-33640 (to PBD).

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CHAPTER 3

Structure/function analysis of the transmembrane segment of γ

3.1 PREFACE

This manuscript has been published in the Journal of Biological Chemistry and is titled "Modulation of Na,K-ATPase by the γ subunit: studies with transfected cells and transmembrane mimetic peptides" (Zouzoulas, A., Therien, A.G., Scanzano, R., Deber, C.M., and Blostein, R. *J. Biol. Chem.* **278**, 40437-40441, 2003). Its aim was to gain insight into (i) regions of the TM helix that are important for trafficking and/or association with αβ pumps, (ii) the structural basis for the increase in K'_{Na} effected by γ.

3.2 ABSTRACT

The enzymatic activity of the Na,K-ATPase, or sodium pump, is modulated by members of the so-called FXYD family of transmembrane proteins. The best characterized member, FXYD2, also referred to as the γ subunit, has been shown to decrease the apparent Na^+ affinity and increase the apparent ATP affinity of the pump. The effect on ATP affinity had been ascribed to the cytoplasmic C-terminal end of the protein, whereas recent observations suggest that the transmembrane (TM) segment of γ mediates the Na^+ affinity effect. Here we use a novel approach involving synthetic transmembrane mimetic peptides to demonstrate unequivocally that the TM domain of γ effects the shift in apparent Na^+ affinity. Specifically, we show that incubation of these peptides with membranes containing αβ pumps modulates Na^+ affinity in a manner similar to transfected full-length γ subunit. Using mutated γ peptides and transfected proteins, we also show that a specific glycine residue, Gly-41, which is associated with a form of familial renal hypomagnesemia when mutated to Arg, is important for this kinetic effect, whereas Gly-35, located on an alternate face of the transmembrane helix, is not. The peptide approach allows for the analysis of mutants that fail to be expressed in a transfected system.

3.3 INTRODUCTION

The Na,K-ATPase or sodium pump is an integral membrane protein found in the cells of virtually all higher eukaryotes and is one of the most important systems involved in cellular energy transduction (8, 19). It catalyzes the electrogenic exchange of three intracellular Na^+ for two extracellular K^+ ions energized by the hydrolysis of one molecule of ATP. The transporter plays a major role in ion homeostasis, and, in epithelia, the sodium gradient created by the pump also plays an important role in secondary active transport mechanisms that are necessary for Na^+-dependent reabsorption of a variety of solutes including sugars and amino acids.

There is an increasing body of evidence that members of a family of membrane proteins, the so-called FXYD family (21), associate with and modulate the kinetic behaviour of the sodium pump (for a recent overview, see ref. 3). Members of this family of proteins are small, single transmembrane (TM) proteins characterized by an N-terminal PFXYD motif that remains invariant in all mammals. There are at least seven known family members, of which several appear to modulate the kinetic behaviour of the pump in a tissue-specific manner (1, 2, 12, 25). To date, the γ "subunit" of the renal Na,K-ATPase is the best characterized member (reviewed in refs. 23 and 28). Gamma, or FXYD 2, exists as two main splice variants, γa and γb, with distinct as well as overlapping localization along the nephron (16). Mass spectrometry of γa and γb indicate that they differ only in the N terminus; in rat γa, TELSANH is replaced by Ac-MDRWYL in γb (9). Previous studies using membrane fragments isolated from γ-

transfected rat α1-HeLa cells have shown that γ serves at least two distinct functions in regulating the pump, and the effects are similar for both variants (16, 26). These distinct effects of γ include the following: (i) an increase in the apparent affinity for ATP; and (ii) an increase in K^+/Na^+ competition at cytoplasmic Na^+ activation sites, as seen by an increase in K'_{Na} at high $[K^+]$. Previous studies have suggested that the effect on ATP affinity is mediated by the cytoplasmic C-terminal domain of the protein (17, 25), whereas effects on K^+/Na^+ antagonism may be associated with the TM domain (10).

In this study, we have used a novel strategy to assess directly the functional effects of the TM region of FXYD 2 whereby peptides corresponding to the TM domain of γ are added directly to membranes derived from cells devoid of the protein. In addition, we describe a mutagenesis approach to structure/function analysis of γ using both transfected cells and peptides. A residue of particular interest is Gly-41, because it is replaced by Arg (G41R) in a familial form of renal magnesium wasting (13) and is invariant throughout the FXYD family. The other residue examined is Gly-35 because of its location at the alternate face of the membrane relative to Gly-41 (24). Effects of peptides with either of these Gly residues replaced by Arg or Leu are compared with those of the full-length transfected γ proteins (wild-type or mutants of the γb variant) expressed in cultured rat α1-transfected HeLa cells. This approach allows for assessment of the role of the TM region alone and provides an opportunity to distinguish the effects of mutations on biogenesis and routing to the plasma

membrane from the direct effects on association and kinetic modulation of αβ pumps.

3.4 EXPERIMENTAL PROCEDURES

Mutagenesis, Transfection, Tissue Culture, and Membrane Preparations- Point mutations were introduced into γb cDNA subcloned into the pIRES expression vector and then transfected into HeLa cells stably expressing the rat α1 subunit of the Na,K-ATPase (α1 HeLa cells were kindly provided by Dr. J. B. Lingrel) as described previously (17). Unless indicated otherwise, cellular membranes from the stably transfected cells were prepared as described by Jewell and Lingrel (7).

Polyacrylamide Gel Electrophoresis and Western Blotting- SDS-PAGE and Western blotting were carried out as described previously (16), and, following transfer to polyvinylidene difluoride membranes, the lower half was analyzed with a polyclonal γ antibody (γC32 raised against the C-terminal KHRQVNEDEL peptide and essentially the same as γC33 described previously in ref. 27), and the upper half was analyzed with anti-α1 subunit A277 (Sigma).

Coimmunoprecipitation- The method used is a modification of Garty et al. (5). Briefly, 250 μg of membranes, prepared from γb_{WT}- and γb_{mutant}-transfected cells as described previously (7), were resuspended and incubated at room temperature for 30 min in immunoprecipitation buffer containing 50 mM imidazole, pH 7.5, 1 mM EDTA, 10 mM RbCl, and 5 mM ouabain in a final volume of 250 μl. Polyoxyethylene 10 lauryl ether ($C_{12}E_{10}$) (250 μl of a 2 mg/ml solution in water) was then added (final concentration, 1 mg/ml), and the suspension was incubated for 30 min at 4 °C with end to-end rotation. Following centrifugation at 4°C for

30 min at 16,000 x *g*, the supernatant was added to 50 µl of bovine serum albumin (1 mg/ml)-treated protein A Sepharose beads and incubated for 30 min at 4°C, and the beads were then centrifuged to remove non-specifically bound proteins. The supernatant was removed and a 10-µl aliquot was taken for SDS-PAGE. To another 450-µl aliquot, 90 µl of RbCl (118 mM final concentration) and 30 µl of γC32 anti-serum (stored at -20°C in 50% glycerol) were added, and the suspension was further incubated for 4 h at 4°C with end-to-end rotation. The suspension was then mixed with protein A-Sepharose beads (100 µl) and incubated overnight at 4°C. Beads were washed six times with immunoprecipitation buffer without ouabain, and the immunoprecipitated proteins retained by the beads were eluted with 100 µl of SDS-PAGE sample buffer containing 5% β-mercaptoethanol at 37°C for 30 min. A 20-µl aliquot of the eluate and the 10-µl sample removed before addition of antiserum were resolved by SDS-PAGE followed by Western blotting.

Cell Surface Biotinylation- Transfected α1-HeLa cells were grown to ≅80% confluence in 6-well plates. The surface biotinylation is a modification of Stephan et al. (20). Briefly, the cell surface biotinylation reaction was carried out on ice for one 20-min period using sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (sulfo-NHS-SS-biotin) (Pierce). After biotinylation, each well was rinsed briefly twice with a phosphate-buffered saline (PBS), 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (CM) solution containing 100 mM glycine and then treated with the same solution for 30 min to ensure complete quenching of unreacted sulfo-

NHS-SS-biotin. The cells were then lysed for 45 min with 500 μl of L1 buffer (1% Triton X-100/0.1% SDS in 150 mM NaCl, 5 mM EDTA, and 50 mM Tris-HCl, pH 7.5, containing 10 μg/ml each leupeptin and pepstatin and 200 μM phenylmethylsulfonyl fluoride). Each well was then scraped, and the sample was collected and centrifuged at 18,000 x *g* for 10 min. One 20-μl aliquot of supernatant (total fraction, *T*) was taken for protein determination (Lowry method) and another (5 μl), for Western blotting. The biotinylated surface-exposed proteins were isolated by incubating a 150-μg (≅85 μl) fraction in L2 buffer (L1 buffer without SDS; final volume, 500 μl) with 100 μl of streptavidin-agarose beads (Pierce) overnight at 4°C with gentle rotation. The samples were then centrifuged to separate beads and supernatant (unbound fraction, *U*), and an aliquot of *U* (15 μl ≅ 2.5 μl of *T*) was taken for Western blotting. The beads were washed three times with L2 buffer, twice with high salt L2 buffer (L2 buffer with 500 mM NaCl and 0.1% Triton X-100), and then once with 50 mM Tris-HCl, pH 7.5. The biotinylated proteins were eluted from the beads (Fraction *B*) by incubation in 100 μl of SDS-PAGE sample buffer containing 5% β-mercaptoethanol at 85°C for 10 min and an aliquot (30 μl ≅ 25 μl of *T*) was taken for Western blotting.

Peptide Synthesis- Synthetic peptides corresponding to residues 23–48 of the TM segment of wild-type γb, mutant TM peptides with replacements G41R, G41L, G35R, G35L, and the scrambled peptide, all containing three lysine residues on the N- and C- termini to aid in synthesis and purification, were prepared as

described previously (14, 24). The sequences of these peptides are shown in Fig. 3-1.

Kinetic Assays and Data Analysis- Kinetic assays of Na,K-ATPase were carried out in triplicate as described previously (16) using membranes isolated from mock-transfected and either mutant or WT γb transfected rat α1-HeLa cells. For studies of the kinetic effects of the various peptides, the permeabilized membranes were incubated in buffered medium (15 mM Tris-HCl, pH 7.4 containing 1 mM EDTA) with peptide such that the peptide concentration was 16.7 μM (3.3 μM in the final assay mixture) and the membrane protein concentration was 0.15–0.2 mg/ml. Following incubation for 10 min at room temperature and then for 1 h on ice, the membranes were preincubated for 10 min at 37°C with 5 μM ouabain and assayed for Na,K-ATPase activity with the data analyzed as described previously (16).

Fig. 3-1: Amino acid sequences of transmembrane mimetic peptides.

TM Peptides	Sequences
γWT	KKKTVRKGGLIFA**G**LAFVV**G**LLILLSKRFKKK
γG41R	----------**R**----------
γG41L	----------**L**----------
γG35R	----------**R**----------
γG35L	----------**L**----------
Scrambled	KKKGLRKLLGLTVRIFVLAIFSGVFLKRAKKK

3.5 RESULTS

Studies with Transfected Cells

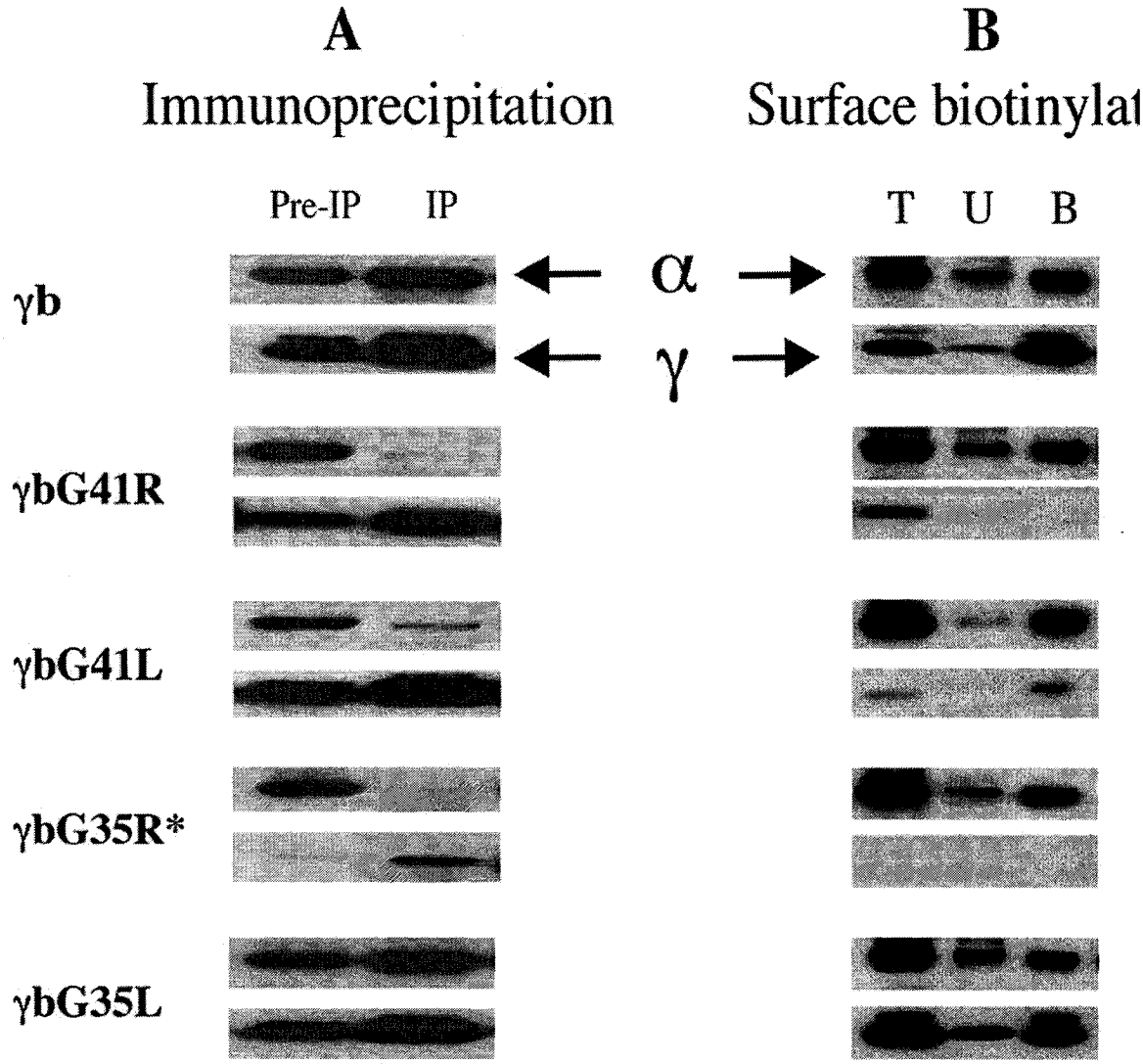
Several approaches were used to gain insight into the role of two glycine residues, Gly-41 and Gly-35, on γ processing, trafficking, and association with the Na,K-ATPase on the one hand and the functional effect on K^+/Na^+ antagonism on the other.

To assess γ association with the catalytic α subunit and γ surface expression, we tested α/γ coimmunoprecipitation and cell surface biotinylation followed by streptavidin bead isolation and Western blotting of α and γ, respectively (see ref. 17). The coimmunoprecipitation experiment (Fig. 3-2A) shows that γb and γb-G35L associate with α, whereas γb-G41L shows weak association, and γb-G41R and γb-G35R show little, if any, association. In additional experiments (not shown), α/γ association seen at lower detergent concentration (0.4 mg/ml) is increased for γb-G41L but not for γb-G41R. The surface biotinylation results (Fig. 3-2B) show that γb and γb-G35L and, to some extent, the γb-G41L mutant, traffic to the surface. As shown earlier (26), little if any γb-G41R appears at the surface. Although the low expression of γb-G35R precluded assessment of its surface expression, these results indicate that the appearance of the WT and mutant γ chains at the cell surface parallels their ability to associate with α. A surprising finding is the low level of γb-G41L relative to α in the Triton X-100-solubilized cells (total fraction, Fig. 3-2B) compared with the relative amounts seen in Western blots of total membranes that were not subjected to this detergent treatment (see Fig. 3-2A). The reason for this apparent

discrepancy is under investigation, although this observation does not detract from the conclusion that this mutant does, in fact, traffic to the cell surface.

Further evidence for altered γ trafficking in the Gly → Arg mutants is the failure to detect post-translational modification of γ. Thus, in Western blots of multiple clones of rat α1-HeLa cells transfected with WT γb and each of the above-mentioned mutants (experiments not shown), the γb chain appeared as a doublet (γb and γb'; *cf.* ref. 16) in WT γb and the conservative mutants γbG41L and γbG35L but not in the γbG41R and γbG35R mutants. We then looked at the functional properties of the various γb transfected cells. Table 3-1 summarizes measurements of apparent Na^+ affinity of Na,K-ATPase measured at high K^+ concentration (100 mM) of membranes isolated from the mock-transfected (control) as well as the WT and mutant γb-transfected cells. The results indicate that γb-G35L behaves like WT γ in that it decreases the apparent affinity for Na^+; K'_{Na} is increased 2.5-fold by γb-G35L and nearly 2-fold by γb. In contrast, γb-G41L, like γb-G41R, does not increase K'_{Na}. Unfortunately, γb-G35R mutants were expressed at levels too low for meaningful kinetic analysis.

Fig. 3-2: Effect of γ-TM mutations on α-γ association and cell surface expression of α and γ. *A*, membranes were solubilized and immunoprecipitated with γC32 as described under "Experimental Procedures." Aliquots of the total solubilized protein (*PreIP*) and the immunoprecipitate (*IP*) were resolved by SDS-PAGE and analyzed by Western blotting using anti-γC32. As described, the aliquots of PreIP and IP represent 5 and 45 μg, respectively, of the original membrane protein. *B*, cell surface biotinylation was carried out as described under "Experimental Procedures." For the unbound (*U*) and bound (*B*) fractions, the amounts analyzed by Western blot analysis were 0.5 and 5 times the amounts of the total fraction (*T*) analyzed, respectively. For the biotinylation analysis in *panel B*, the amount of material analyzed with respect to the original cell number was ≅1/100th that used in the coimmunoprecipitation analysis shown in *panel A*; for chemiluminescent detection, the time of exposure of the polyvinyl difluoride membrane to x-ray film was 60–120 times greater in *panel B* compared with *panel A*. *, expression of γb-G35R was very low as shown by the PreIP (*A*) and the T fraction (*B*).


A
Immunoprecipitation
B
Surface biotinylat
Pre-IP
IP
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B
γb
γbG41R
γbG41L
γbG35R*
γbG35L
α
γ

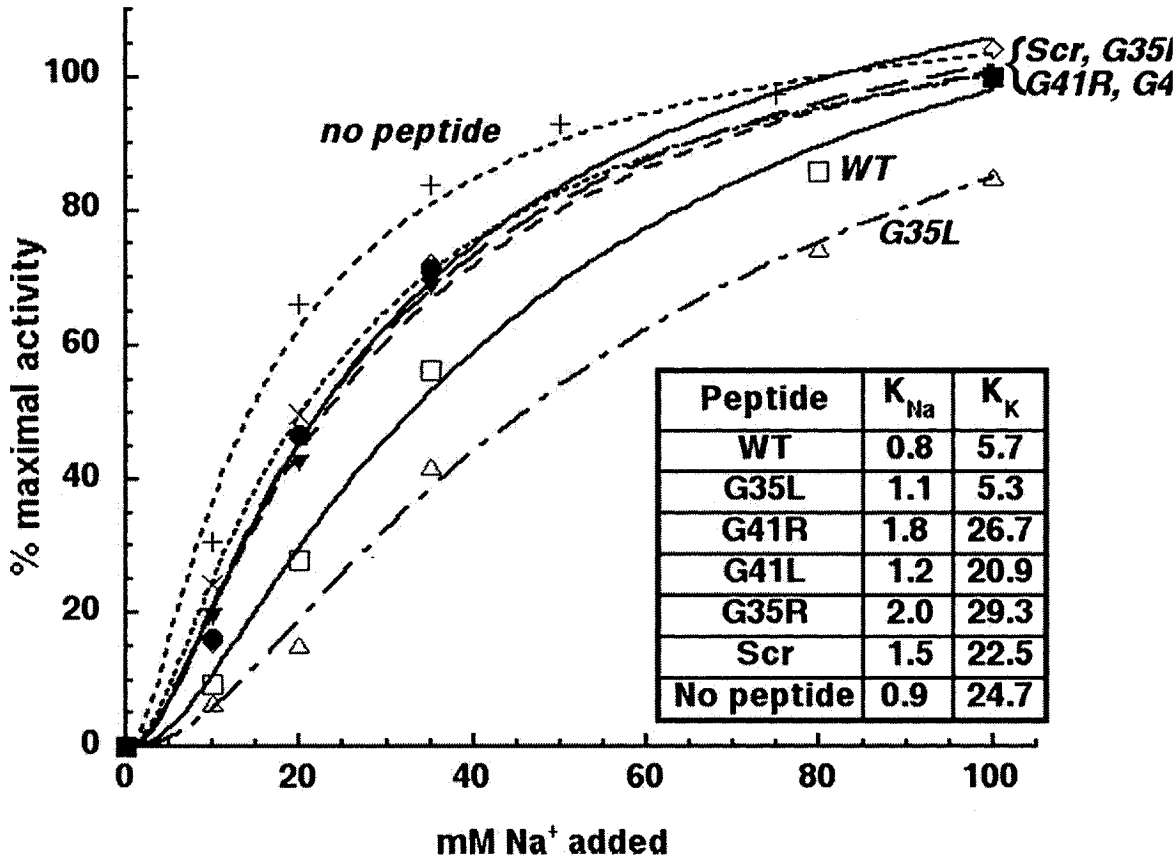
Effects of Transmembrane Mimetic Peptides

Studies of the effects of peptides comprising the TM sequence of γ (γ-TM), G41R (γG41R-TM), G41L (γG41L-TM), G35R (γG35R-TM), and G35L (γG35L-TM) γ as well as a "scrambled" peptide ("γScr-TM") were carried out with synthetic peptides containing "lysine tags" at both the N- and the C- termini. These tags overcome the inherent insolubility of these γ-helical TM peptides, thus aiding in their synthesis and purification, but retain their native TM oligomeric states (14, 24). Fig. 3-3 shows the Na^+ activation profile measured at high (100 mM) K^+ concentration without and with the various TM peptides. Compared with γ-Scr-TM, the addition of either γ-TM or γG35L-TM peptide to γ-devoid membranes isolated from rat α1-HeLa cells increases K'_{Na}. The increase effected by G35L is even greater than seen with the WT peptide, similar to the greater effect of γbG35L compared with γb in γ-expressing cells (described above). In contrast, γG41R-TM, γG41L-TM, and γG35R-TM did not increase K'_{Na} above that observed with the control scrambled peptide (γScr-TM). A nonspecific effect of peptide addition is evident in a small but consistent increase in K'_{Na} seen irrespective of whether γScr-TM or any of the three mutants, γG35R-TM, γG41R-TM and γG41L-TM, is added. The other notable kinetic effect of the full-length γ chain, the decrease in K'_{ATP}, was not seen with γ-TM (experiments not shown), consistent with earlier evidence (17) that this effect is mediated primarily by the cytoplasmic C-terminus.

We showed previously that K'_{Na} is a linear function of K^+ concentration, approximating a simple competitive model of cytoplasmic K^+/Na^+ competition,

i.e. $K'_{Na} = K_{Na} (1 + [K^+]/K_K)$ (4, 18, 27), and that the main effect of either γa or γb was not due to an increase in K_{Na}, but rather to a decrease in K_K. To further assess the specificity of the mimicry of the TM peptides, we determined K'_{Na} as a function of K^+ concentration and, thus, values of K_{Na} (K'_{Na} when $[K^+] = 0$) and K_K (the apparent K^+ affinity for competition with cytoplasmic Na^+). The results shown in the inset to Fig. 3-3 support the conclusion that γ-TM and γG35L-TM peptides, like their full-length counterparts (see table in ref. 16), decrease K_K with little effect on K_{Na}.

Fig. 3-3: Effect of TM peptides on apparent Na^+ affinity determined at 100 mM K^+. Na,K-ATPase assays were carried out as described (16) with or without preincubation in the presence of various TM peptides as described in "Experimental Procedures." Data points are presented as percentages of the maximal activity observed in separate experiments in which the Na^+ concentration was increased to 200 mM (total chloride concentration, 300 mM). Values of K'_{Na} determined at 100 mM K^+ (mean ± S.E. of the number of replicate experiments shown in brackets) following addition of WT, G41R, G41L, G35R, G35L, and Scr peptides were 15.41 ± 0.80 (18), 8.79 ± 0.33 (6), 7.01 ± 0.97 (3), 8.65 ± 0.58 (6), 21.56 ± 2.45 (4), and 8.35 ± 0.39 (3), respectively, and, in the absence of an added peptide, 4.78 ± 0.18 (10). *Inset*, K_{Na}, and K_K were estimated from values of K'_{Na} determined at various concentrations of K^+ using the relationship $K'_{Na} = K_{Na} (1 + [K^+]/K_K)$, where ($K_{Na} = K'_{Na}$ at $[K^+] = 0$) (4).



Peptide	K_{Na}	K_K
WT	0.8	5.7
G35L	1.1	5.3
G41R	1.8	26.7
G41L	1.2	20.9
G35R	2.0	29.3
Scr	1.5	22.5
No peptide	0.9	24.7

Table 3-1: Summary of comparative effects of γ_{wt} and $\gamma_{mutants}$ on apparent affinity for Na^+ at 100 mM K^+.

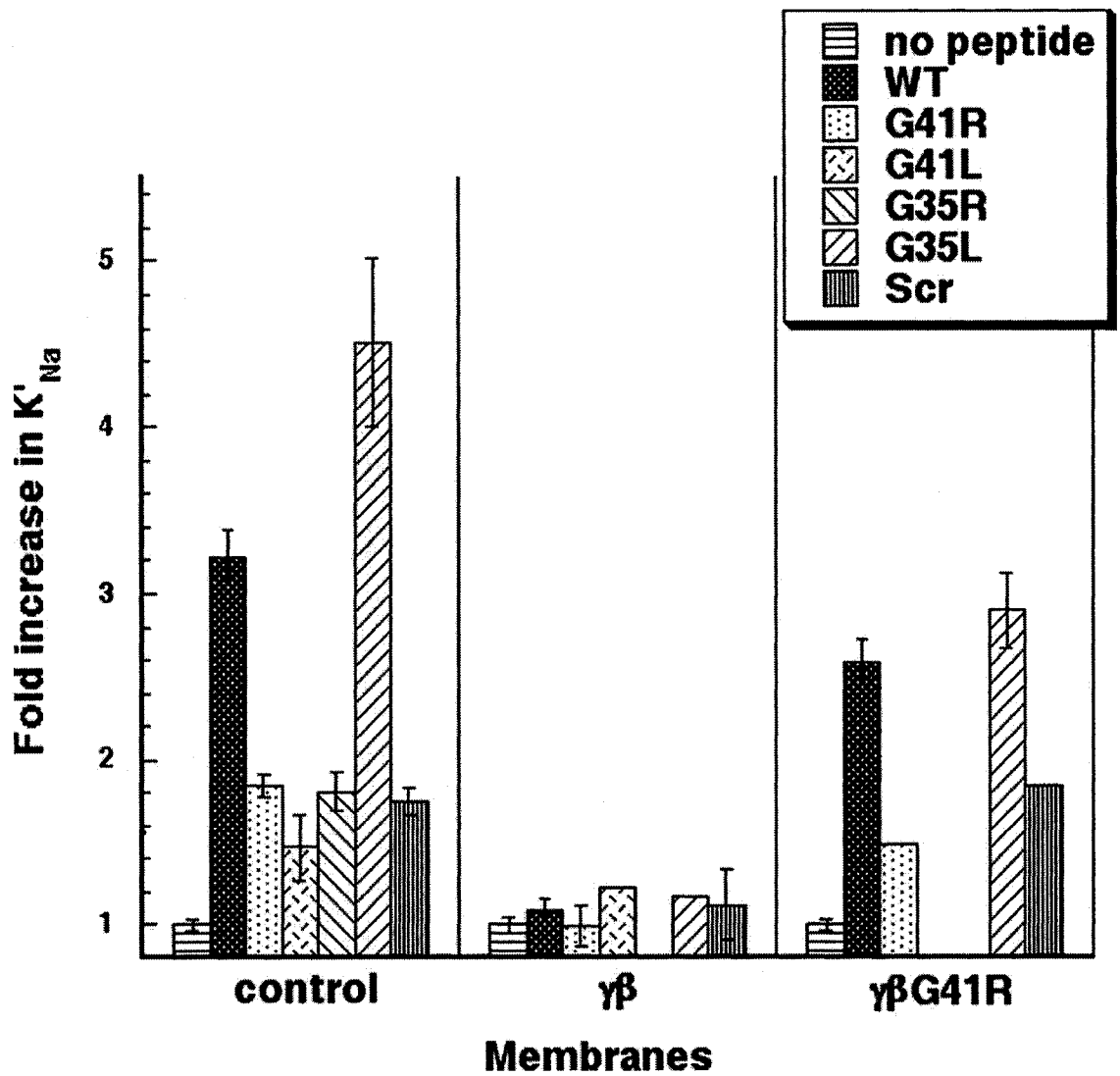
Membranes	K'_{Na} at 100 mM K^+
Control (mock)	4.8 ± 0.2
γb[a]	8.3 ± 0.4
γbG41R	5.3 ± 0.2
γbG41L	4.5 ± 0.02
γbG35R	n.d.[b]
γbG35L[a]	10.7 ± 0.8

[a]$p < 0.01$ for γb and γbG35L compared to control membranes.

[b]n.d., not determined due to low expression

As shown in Fig. 3-4, in control experiments carried out with the γ-TM peptide and/or the γG35L-TM peptide, an increase in K'_{Na} seen with mock-transfected cell membranes is seen also with γbG41R-transfected but not γb-transfected membranes. Thus, the kinetic effects of the mimetic peptides are highly specific in that they are seen only with pumps not already associated (mock transfected) or minimally associated (γb-G41R) with full length γ chains. As expected, γG41L-TM and γG41R-TM peptides had no effect on the K'_{Na} values of pumps in any of the transfected systems.

Fig. 3-4: Effect of TM peptides on membranes isolated from γb and γbG41R transfected rat α1-HeLa cells. K'_{Na} values were determined as in Fig. 3-3. Activity with TM peptides added to either α1-HeLa (*control*), γb-α1-HeLa, or γbG41R-α1-HeLa membranes are presented as the fold change in K'_{Na} compared with membranes with no peptide added, normalized to 1.0. Error bars are S.E. of at least three experiments. In the absence of added peptide, values of K'_{Na} were 4.8, 8.3, and 5.3 mM for control, γb, and γbG41R membranes, respectively (see Table 3-1). Data for control membranes are taken from Fig. 3-3.


no peptide
WT
G41R
G41L
G35R
G35L
Scr
Fold increase in K'Na
5
4
3
2
1
control
γβ
γβG41R
Membranes

3.6 DISCUSSION

The experiments described herein unequivocally show that the effect of γ (FXYD 2) on the K'_{Na} values of Na,K-ATPase is mediated solely by the TM domain of the protein, as suggested recently (10). Thus, peptides comprising only this region of γ simulate functional effects obtained in assays performed on membranes isolated from wild-type and mutant γb-transfected α1-HeLa cells. The mimetic effects of the peptides underscore the feasibility of using TM peptides to evaluate kinetic effects of TM helices, including instances in which adequate expression is problematic, as is the case of the γb-G35R mutant. TM mimetic peptides offer the unique advantage of examining the function of the TM domain without the complexity of long range effects of extramembranous regions. In fact, concern over this complexity was mentioned by Lindzen et al. (10) who raised the possibility that a change in K'_{ATP} effected by the cytoplasmic domain may mask an apparent effect of γ-TM on K^+/Na^+ antagonism, which may account for the larger decrease in K_K effected by γ-TM and γG35L-TM compared with the analogous transfectants (compare *inset* of Fig. 3-3 with Table 3-1).

The results of the present study provide insight into the role of two Gly residues, Gly-35 and Gly-41, which reside on opposite faces of the helical TM domain of γ. The observation that both the γb-G35R (Fig. 3-1) and γb-G41R (16) mutants show either minimal expression or processing and trafficking led us to consider more conservative mutations in our attempts to understand the role of these residues in modulating pump function. We therefore studied G35L and G41L mutants in experiments with both transfected cells and synthesized

peptides. These mutants circumvent the energetic cost associated with inserting a positively charged residue within the hydrophobic core of the membrane and are therefore more relevant in studying the role of the native glycine residues. This consideration may not only explain the low processing/trafficking of Arg mutants but also the lack of effect of the γG35R-TM peptide on sodium pump kinetics (Fig. 3-3).

The studies with both transfected cells and peptides provide evidence that disruption of a Gly residue (Gly-41) on one face of the γ helix but not on the other (Gly-35) abrogates the increased K^+/Na^+ antagonism effected by γ as seen by a notable increase in K'_{Na} at a high K^+ concentration. Thus, the abolition of the kinetic effect is seen not only with the disrupting mutation G41R replacement associated with autosomal dominant renal magnesium wasting (13) but also with the conservative mutation, G41L. Previous results from our laboratory (17) suggested that Gly at position 41 is essential for normal trafficking to the cell surface and association with the pump. However, even when the cellular processing and trafficking problems are circumvented, as is the case with the G41L mutant and the peptide experiments presented herein, mutating this residue fails to increase K'_{Na} above levels seen with a control scrambled peptide. Importantly, γbG41L appears to be expressed at the surface (Fig. 3-2B) and to associate, at least to an extent, with the α subunit (Fig. 3-2A), confirming a role for Gly-41 in mediating one of the kinetic effects of γ but not primarily in αβ-γ association. Such a dissociation between residues important for the functional

effects of γ and its interaction with the sodium pump is consistent with the findings of Lindzen et al. (10).

In contrast to Gly-41, Gly-35 on the opposite face of the helix does not appear to be functionally important, because the G35L mutation does not abrogate kinetic function. Our results also suggest that Gly-35 is not involved in association of γ with the αβ complex, because γb-G35L was able to co-immunoprecipitate with the α subunit (Fig. 3-2A). The latter finding is consistent with recent experiments showing that the residues important for this association are on a different side of the helix (see Fig. 5 in ref. 10).

As described previously (24), γ-TM and γG35R-TM peptides form oligomers in the weak detergent PFO, but mutants of Gly-41 (γG41R-TM and γG41L-TM) do not. Using PFO-PAGE analysis (see ref. 24), we have confirmed that the γG35L-TM peptide can form oligomers in PFO (not shown), revealing a potential correlation between the effects of γ peptides on K'_{Na} and their ability to form oligomers in PFO. The physiological relevance of this observation remains to be investigated.

Mimetic TM peptides reconstituted in detergent micelles have been used previously in structural studies of membrane proteins (11, 14, 29) and have been shown to inhibit membrane protein function (6, 15, 22). However, to our knowledge, the results reported here constitute an unique example of a TM peptide that can display specific kinetic effects attributable to the TM domain of a membrane protein. The remarkable mimetic properties of the TM peptides and the expressed γ mutants have particular significance. The failure of the G41R and

G41L mutants to affect K'_{Na}, whether added as TM peptides to cell membranes or expressed as full-length proteins in transfected cells, underscores the conclusion that Gly-41, which is associated with renal magnesium wasting when mutated to Arg, is important for the effect of the γ subunit on K^+/Na^+ antagonism and possibly for oligomerization of the protein, whereas Gly-35 is not.

3.7 ACKNOWLEDGMENTS

We thank Anthony Partridge and Roman Melnyk for help with peptide synthesis.

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CHAPTER 4

A structure/function analysis of α chain interaction with γ

4.1 PREFACE

This manuscript has been published in the Journal of Biological Chemistry and is titled "Regions of the Catalytic α Subunit of Na,K-ATPase Important for Functional Interactions with FXYD 2" (Zouzoulas, A. and Blostein, R. *J. Biol. Chem.* **281**, 8539-8544, 2006). The goal of this study was to determine the region(s) of α that not only interact(s) with γ, but are also important for the modulatory effects of γ.

4.2 ABSTRACT

The γ modulator (FXYD 2) is a member of the FXYD family of single transmembrane proteins that modulate the kinetic behaviour of Na,K-ATPase. This study concerns the identification of regions in the α subunit that are important for its functional interaction with γ. An important effect of γ is to increase K^+ antagonism of cytoplasmic Na^+ activation apparent as an increase in K'_{Na} at high [K^+]. We show that although γ associates with α1, α2 and α3 isoforms, it increases K'_{Na} of α1 and α3, but not α2. Accordingly, chimeras of α1 and α2 were used to identify regions of α critical for the increased K'_{Na}. As with α1 and α2, all chimeras associate with γ. Kinetic analysis of $\alpha 2_{front}/\alpha 1_{back}$ chimeras indicate that the C-terminal (^{907}K-Y^{1018}) region of α1, which includes TM 9 close to γ, is important for the increase in K'_{Na}. However, similar experiments with $\alpha 1_{front}/\alpha 2_{back}$ chimeras indicate a modulatory role of the loop between TM's 7 and 8. Thus, as long as the α1 L7/8 loop is present, replacement of TM 9 of α1 with that of α2 does not abrogate the γ effect on K'_{Na}. In contrast, as long as TM 9 is that of α1, replacement of L7/8 of α1 with that of α2 does not abolish the effect. It is suggested that structural association of the TM regions of α and FXYD 2 is not the sole determinant of this FXYD's effect on K'_{Na}, but is subject to long range modulation by the extramembranous L7/8 loop of α.

4.3 INTRODUCTION

The Na,K-ATPase or Na^+ pump is an integral membrane protein found in the cells of virtually all higher eukaryotes. It couples the hydrolysis of one molecule of ATP to the electrogenic exchange of three intracellular Na^+ for two extracellular K^+ ions against their electrochemical gradients. The Na^+ pump is an oligomer of two subunits, a large catalytic α subunit and a smaller, highly glycosylated β subunit whose role is to ensure the proper folding, insertion and maturation of α in the plasma membrane. Four isoforms of α and three of β are expressed in a tissue- and development-specific manner (for reviews, see refs. 15, 16).

The Na^+ pump is subject to diverse modes of regulation by substrates, membrane-associated components, hormones and neurotransmitters (30). In recent years, considerable attention has been focused on tissue-specific regulation by small transmembrane proteins referred to as FXYD proteins (for reviews, see refs. 5, 6, 10, 12). These proteins belong to a gene family of small, single transmembrane proteins, with the exception of Mat-8 which has two TM's. There are at least seven members of which several (FXYD's 1-4 and 7) appear to regulate the kinetic behaviour of the Na^+ pump in distinct and specific ways, in particular the apparent affinity of the enzyme for ligands (Na^+, K^+ and ATP). Members possess a 35 amino acid signature sequence that includes 6 invariant amino acids before, in and after the transmembrane (TM) domain. The N-terminal FXYD (Phe-X-Tyr-Asp) motif is invariant, along with two glycines in the TM domain and a serine in the C-terminal domain. The first FXYD member

identified and characterized was γ (FXYD 2), originally called the γ "subunit" and present predominantly in the kidney. Other members include phospholemman (PLM or FXYD 1) Mat-8 (FXYD 3), CHIF (FXYD 4), RIC or Dysadherin (FXYD 5), Phosphohippolin (FXYD 6) and FXYD 7. A phospholemman-like protein, PLMS, is present in the shark rectal gland.

The γ modulator exists as two main variants, γa and γb, with distinct as well as overlapping localization along the nephron (26, 35). Mass spectrometry indicates that they differ only in the N-terminus (17). In rat γa, TELSANH is replaced by Ac-MDRWYL in γb. Previous studies in our laboratory (26, 27, 29, 31, 32) using membrane fragments isolated from γ-transfected rat α1-HeLa cells have shown that γ serves at least two distinct regulatory effects on pump kinetics and that these effects are the same for both variants. Thus, (i) γ increases the apparent affinity for ATP, (ii) increases K^+/Na^+ competition at cytoplasmic Na^+ activation sites, as seen by an increase in K'_{Na} at high $[K^+]$ concentration. In addition, in intact cells, γ increases the apparent K^+ affinity (3, 36).

Although the functional effects of γ on Na,K-ATPase have been extensively characterized, the structural basis of these effects is largely unknown. Earlier cryoelectron microscopy of the purified renal (α1β1) pump (13) suggested that the γ subunit is located in a pocket comprising TM 9, TM 6, TM 2 and possibly TM 4. Furthermore, recent homology modeling of the Na,K-ATPase based on the high resolution structure of the Ca-ATPase as well as cross-linking experiments have shown further that γ makes contacts with TM 2, TM 6 and TM 9 of the α subunit (8, 20).

An important issue concerns the region(s) of α that interact(s) with the γ modulator, focusing on the interactions that are critical for producing the modulatory effects of γ. Mutagenesis of the α1 subunit (Ala replacement of Phe956 and Glu960) indicate the importance of TM 9 in affecting the apparent affinity for extracellular K^+ (20). However, neither of these replacements abrogated the increase in K'_{Na} effected of γ.

The present study focuses on regions of the α subunit with which γ interacts to effect the increase in K'_{Na} at high $[K^+]$ (K^+/Na^+ antagonism). Kinetic studies using γ mutants (21, 27) and mimetic peptides comprising the transmembrane domain of γ, γ-TM (37), have clearly shown that the increase in K^+/Na^+ antagonism is mediated by the TM region of γ. Here we show that while γ interacts with α1, α2 and α3 and increases K'_{Na} of α1 and α3, a significant change in K'_{Na} of α2 could not be detected. Accordingly, an α1/α2 chimera approach was used to gain insight into regions of α which are relevant to its functional interaction with γ resulting in increased K^+/Na^+ antagonism.

4.4 EXPERIMENTAL PROCEDURES

Mutagenesis, Transfection, and Cell Culture- All of the chimeric α's used in this study were derived from the ouabain-insensitive rat α1 and α2[2] cDNAs (kindly provided by Jewell and Lingrel) introduced into the HindIII site of a modified pIBI shuttle vector. Using Stratagene's QuikChange Site-Directed Mutagenesis kit, silent mutations encoding for novel restriction sites were introduced into identical positions of rat α1 and α2 cDNAs to create interchangeable restriction cassettes as described below. Chimeras $\alpha1_{(1\text{-}311)}/\alpha2$ and $\alpha2_{(1\text{-}309)}/\alpha1$ were prepared as described previously (28). Chimeras $\alpha1_{(1\text{-}786)}/\alpha2$ and $\alpha2_{(1\text{-}783)}/\alpha1$ were prepared by introducing a MunI site which splits the codons for Ser-752 of α1 and the corresponding Ser-749 of α2 (the amino acid sequence is identical up to Leu-786 of α1 and Leu-783 of α2) and by exchanging the 5' $SphI_{(334)}$-$MunI_{(2491)}$ restriction cassettes of α1 with the 5' $SphI_{(334)}$-$MunI_{(2343)}$ cassette of α2. Chimeras $\alpha1_{(1\text{-}875)}/\alpha2$ and $\alpha2_{(1\text{-}872)}/\alpha1$ and $\alpha1_{(1\text{-}907)}/\alpha2$ and $\alpha2_{(1\text{-}904)}/\alpha1$ were prepared as above, by introducing a NdeI site which splits the codon for Ala-848 of α1, corresponding to Ala-845 of α2. The amino acid sequence is identical up to Pro-875 of α1 (Pro-872 of α2). BsiWI was introduced as well, by splitting codons Thr-902 of α1 and the corresponding Thr-899 of α2 (the amino acid sequence is identical up to Lys-907 of α1 (Lys-904 of α2)). The 5' $SphI_{(334)}$-$NdeI_{(2780)}$ cassette of α1 was exchanged with the 5' $SphI_{(334)}$-$NdeI_{(2630)}$ cassette of

[2] For simplicity, asterisks normally used to denote the ouabain-resistant forms of α2 and α3, i.e. α2* and α3* constructed by Jewell and Lingrel (14) are omitted.

α2 to create $\alpha 1_{(1\text{-}875)}/\alpha 2$ and $\alpha 2_{(1\text{-}872)}/\alpha 1$. Similarly, the 5' $SphI_{(334)}$-$BsiWI_{(2944)}$ cassette of α1 and the 5' $SphI_{(334)}$-$BsiWI_{(2792)}$ cassette of α2 were exchanged to generate $\alpha 1_{(1\text{-}907)}/\alpha 2$ and $\alpha 2_{(1\text{-}904)}/\alpha 1$. The full-length cDNAs for all chimeric constructs were then excised from the shuttle vector using HindIII and ligated into pCDNA3.1. Orientation was confirmed by restriction analysis and full length cDNA sequences were verified. HeLa cells containing either the empty pIRES vector (HeLa-mock) or expressing γb protein (HeLa-γb-pIRES) (17) were transfected with the pCDNA chimeric α constructs using the LipofectAMINE technique (LipofectAMINE, Invitrogen). Cells expressing the relatively ouabain-resistant rat α enzymes were then selected and maintained in culture as previously described (27).

Membrane Preparations- NaI- and non NaI-treated microsomal membranes were prepared from the chimeric and WT cells as described earlier (14, 18). Protein concentration was determined using a detergent-containing modification (23) of the Lowry method (22).

Polyacrylamide Gel Electrophoresis and Western Blotting- SDS-PAGE and Western blotting were carried out as described previously (26). Following transfer of the SDS-PAGE gels to polyvinylidene fluoride (PVDF) membranes, a section of the membrane comprising the lower molecular size proteins (≤ 28 kDa) was analyzed with a polyclonal γ antibody (γC32) raised against the C-terminal KHRQVNEDEL peptide that is essentially the same as γC33 used previously

(26). The remaining membrane was blotted with monoclonal antibodies to detect the α subunit as described in the figure legends.

Coimmunoprecipitation- The method used was a modification of Garty et al. (11) as described elsewhere (37). Band densities were quantified using Molecular Dynamics ImageQuant software.

Enzyme assays- Na^+ dependent activation of Na,K-ATPase activity was measured as described previously (27). Briefly, Na,K-ATPase activity was determined at 100 mM KCl and varying concentrations of NaCl and with 10 μM ouabain present to inhibit endogenous pumps. Baseline ATPase activity was determined at 100 mM KCl and absence of NaCl, and with 100 mM choline chloride added to maintain a constant (200 mM) chloride concentration. For determination of the effects of the γ-TM peptide on α2, the permeabilized membranes were assayed as previously described (37), except that the peptide concentration in the final assay mixture was 1.67 μM. All experiments were carried out at least three times on two clones each of mock- and γ-transfected cells, with assays performed in triplicates.

Data analysis- The data were analyzed using the Kaleidagraph computer program (Synergy Software) with the noninteractive model of cation binding described by Garay and Garrahan (9), i.e.

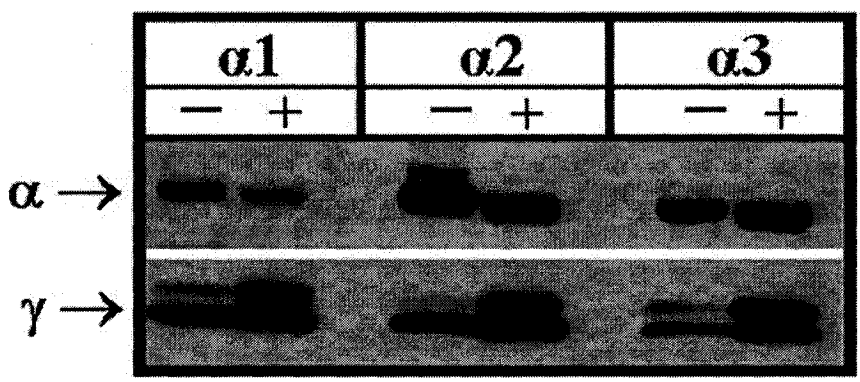
$$v = V_{max} / (1 + K'_{Na} / [Na^+])^3 \qquad (1)$$

where v represents the rate of the reaction, V_{max} is the maximal rate, and K'_{Na} is the apparent affinity for Na^+. Superscript 3 denotes the number of Na^+ binding sites. Values of V_{max} and K'_{Na} were obtained from this fitting procedure.

4.5 RESULTS

Earlier studies with cRNA injected Xenopus oocytes showed that the γ subunit can associate with the three major isoforms of the α subunit (3). In the present study, this finding was confirmed with mammalian cells. For these experiments rat α1-, α2- and α3- HeLa cells were transfected with γ (γb variant) and following isolation of stable transfectants, membranes were isolated, solubilized with detergent and subjected to immunoprecipitation with anti-γ antibodies using the conditions developed by Garty et al. (11) as outlined in Experimental Procedures. These authors showed that coimmunoprecipitation is efficient only in conditions (solublization in $C_{12}E_{10}$ in the presence of either Rb^+ + ouabain as used here, or Na^+ + oligomycin) that preserve native pump structure (11). Samples taken before and after immunoprecipitation were subjected to SDS-PAGE and Western blotting with anti-α-specific antibodies as indicated. The results (Fig. 4-1) show that α1, α2 and α3 associate with γ since all are immunoprecipitated by anti-γ antibodies. As shown by Lindzen et al. (21) a relatively small fraction (≈5%) of the total protein is precipitated under these same conditions. As indicated in the legend to Fig. 4-1, quantitation of the bands indicates that the efficiency of immunoprecipitation is similar (≈6%) for all three isoforms.

Fig. 4-1: Coimmunoprecipitation of α isoforms with γ. Membranes were solubilized and immunoprecipitated with anti-C-terminal antibody γC32 as described in Experimental Procedures. Aliquots corresponding to 5μg and 45 μg of the original solubilized protein taken before (-) and after immunoprecipitation (+), respectively, were resolved by SDS-PAGE and analyzed by Western blotting using the following monoclonal antibodies: for the upper section of the blot, A277 (Sigma) for α1, McB2 (gift of Dr. K. Sweadner) for α2 and A273 (Sigma) for α3; for the lower section, γC32 to detect γ. Efficiencies of coimmunoprecipitation as determined by densitometry of the bands (ratio of the α/γ density post-immunoprecipitation/pre-immunoprecipitation) were 6.2% for α1γb, 5.9% for α2γb and 6.2% for α3γb.


α1
α2
α3
− + − + − +
α →
γ →

Effects of γ on the Na^+ activation kinetics of the α isoforms

Although γ associates with all three isoforms of α, the question remains whether the γ-mediated increase in K^+/Na^+ antagonism seen previously for α1 also holds true for α2 and α3. In the experiments described below, Na,K-ATPase activity for α1 and α2 was measured with NaCl varied and KCl kept constant at 100 mM. α3 was assayed at 50 mM KCl since K'_{Na} of WT α3 is high (16.5 mM at 100 mM K^+; see ref. 24) and even higher with α3-γb membranes, precluding satisfactory extrapolation to V_{max}. The results presented in Table 4-1 show that whereas γ increases K'_{Na} of α1 and α3 by almost ≈1.6-fold, a significant change in K'_{Na} of α2 could not be detected. It is noteworthy that like the effect of full length γ, the transmembrane mimetic peptide of WT γ (γ-TM) increases K'_{Na} of α1 but not α2.

The lack of K'_{Na} modulation of α2 pumps by γ, in spite of the high sequence identity between α1 and α2, provided a unique opportunity to use an α1/α2 chimera approach to identify regions of α interaction with γ which are functionally important for the K'_{Na} effect. Fig. 4-2 depicts a linear representation of the α1-front half/α2-back half ($\alpha1_f/\alpha2_b$) and the reverse α2-front half/α1-back half ($\alpha2_f/\alpha1_b$) chimeras used for these experiments. The high degree of amino acid identity (86%) among the isoforms notwithstanding, the regions of most diversity between α1 and α2 are within residues 1-311 and 429-565 (α1 numbering), which span the cytoplasmic N-terminus and part of the large cytoplasmic loop between TM 4 and TM 5 (see top of Fig. 4-2).

Table 4-1: Isoform specificity of the γ-mediated modulation of K^+/Na^+ antagonism

K'_{Na} (mM)			
Membranes	mock-transfected	γb-transfected	Fold increase (γb/control)
α1**	5.3 ± 0.3	8.3 ± 0.1	1.6*
α2	9.3 ± 0.7	9.5 ± 1.2	~ 1.0
α3	9.3 ± 1.4	16.6 ± 1.1	1.8*
	no addition	plus γ-TM	
α1†	4.8 ± 0.2	15.4 ± 0.8	3.2*
α2	8.1 ± 0.7	6.9 ± 0.7	~ 1.0

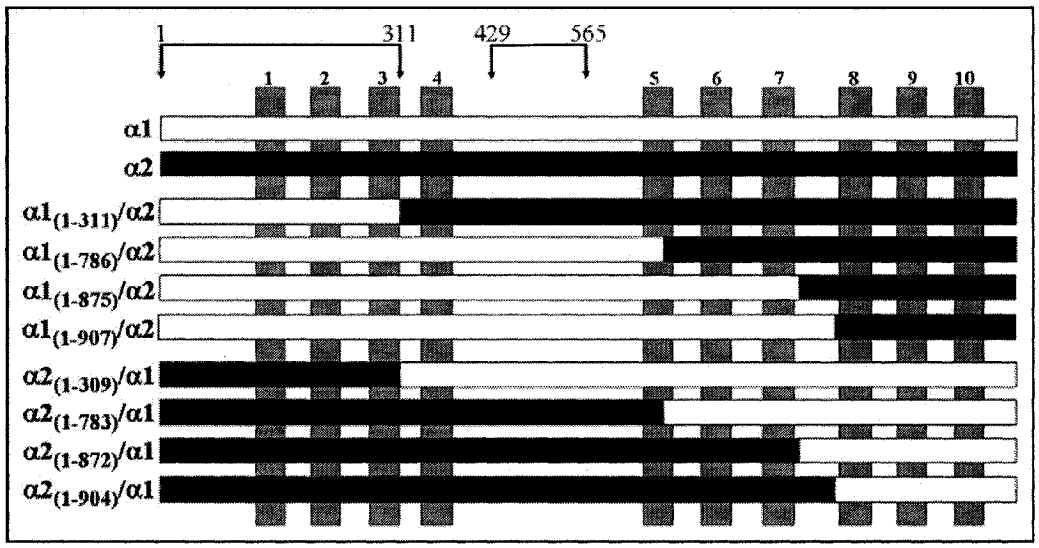
K'_{Na} was measured at either 100 mM K^+ (α1, α2) or 50 mM K^+ (α3) and represents the average of at least three experiments (average ± SEM)

* $p \leq 0.01$ compared to control

** data taken from Pu et al. 2001 (26)

† data taken from Zouzoulas et al. (37)

Fig. 4-2: Schematic illustration of the α1 and α2 chimeras. Regions of greatest divergence are indicated by the brackets above α1 (α1 numbering beginning with the mature N-terminus, GRDKY).


1
311
429
565
1
2
3
4
5
6
7
8
9
10
α1
α2
α1(1-311)/α2
α1(1-786)/α2
α1(1-875)/α2
α1(1-907)/α2
α2(1-309)/α1
α2(1-783)/α1
α2(1-872)/α1
α2(1-904)/α1

Association of γ with $\alpha 1_f/\alpha 2_b$ and $\alpha 2_f/\alpha 1_b$ chimeras and its effects on their Na^+ activation kinetics

The coimmunoprecipitation experiment shown in Fig. 4-3 indicates that similar to its association with α1 and α2 (see Fig. 4-1), γb associates with all $\alpha 1_f/\alpha 2_b$ and $\alpha 2_f/\alpha 1_b$ chimeras.

In the experiments depicted in Fig. 4-4, the effects of γ on the apparent K'_{Na} of $\alpha 1_f/\alpha 2_b$ and $\alpha 2_f/\alpha 1_b$ chimeras were measured at 100 mM K^+ and compared with the effects of γ on WT α1 and α2 isoforms. Fig. 4-4 (top panel) shows representative graphs of α2 and $\alpha 2_f/\alpha 1_b$ chimeras and Fig. 4-4 (bottom panel) shows chimeras in the reverse $\alpha 1_f/\alpha 2_b$ configuration. Fig. 4-5 summarizes the results of the averages of replicate experiments carried out as shown for the representative experiments in Fig. 4-4 (see absolute values presented in Table 4-2).

Fig. 4-4 (top panel), Fig. 4-5 and Table 4-2 indicate that γ causes a significant increase in the K'_{Na} of all chimeras in the $\alpha 2_f/\alpha 1_b$ configuration, similar to that reported for WT α1. It is notable that this holds true for chimera ($\alpha 2_{(1\text{-}904)}/\alpha 1$) comprising only TM's 8-10 of α1 and thus encompassing TM 9 which has been shown to be close to (13), and to interact with γ (8, 20).

As shown in Fig. 4-4 (bottom panel), Fig. 4-5 and Table 4-2, γ did not increase K'_{Na} of chimeras $\alpha 1_f/\alpha 2_b$ up to $\alpha 1_{(1\text{-}875)}/\alpha 2$, similar to its lack of effect on the WT α2 enzyme. However, a notable γ-mediated increase in K'_{Na} of $\alpha 1_{(1\text{-}907)}/\alpha 2$ was observed. This latter effect of γ was not seen at low (10 mM) K^+

concentration (experiment not shown) indicating that like WT α1 (27), the γ-mediated increase in K'_{Na} of the $\alpha1_{(1-907)}/\alpha2$ chimera reflects increased cytoplasmic K^+/Na^+ antagonism. Taken together and as discussed below, these observations suggest that the extracellular loop between TM 7 and TM 8 (L7/8) has a role in modulating the effect of γ on K'_{Na}.

Although Table 4-2 shows that there are notable differences in K'_{Na} values of mock-transfected α1, α2 and chimeras thereof, it is clear that the γ-mediated increases in K'_{Na} are independent of these intrinsic differences.

Fig. 4-3: Coimmunoprecipitation of α1 and α2 chimeras with γ. Experiments were carried out as described in Fig. 4-1. Each lane was loaded with protein corresponding to an activity (V_{max}) of ≈25 nmoles/min of the original solubilized sample. Top panel represents γ-α association of α2 wild type and $\alpha 2_f/\alpha 1_b$ chimeras while the bottom panel represents α1 wild type and $\alpha 1_f/\alpha 2_b$ chimeras. For Western blots, the following monoclonal antibodies were used to detect the α subunit chimeras: for the $\alpha 2_f/\alpha 1_b$ chimeras, McB2; for the $\alpha 1_f/\alpha 2_b$ chimeras, McK1 for $\alpha 1_{(1\text{-}311)}/\alpha 2$ and all others, with A277 (Sigma).

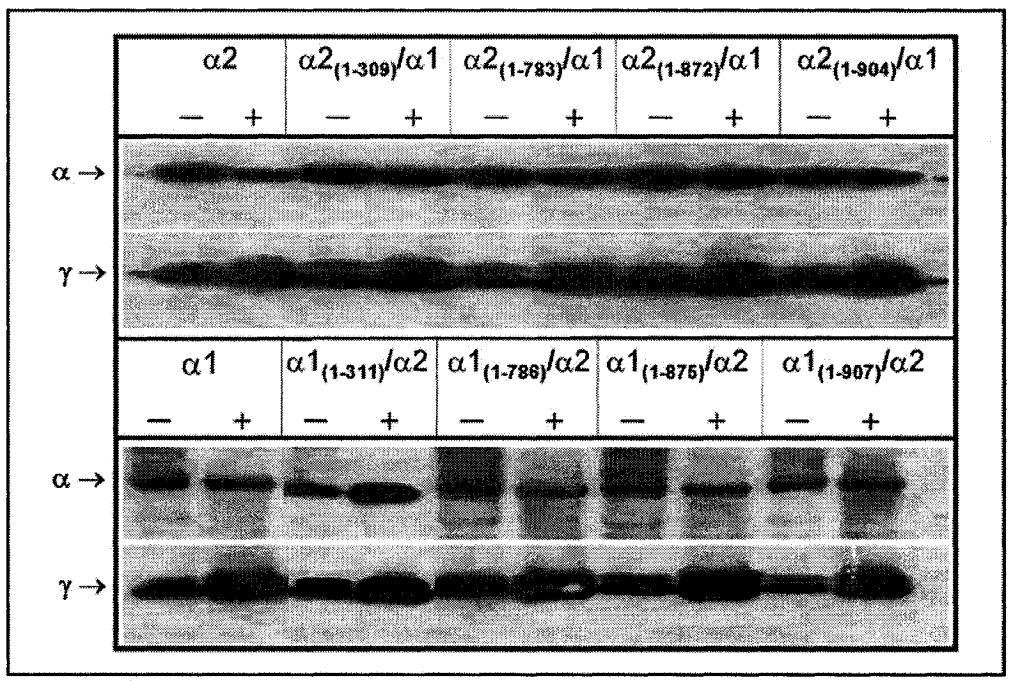

α2
α2(1-309)/α1
α2(1-783)/α1
α2(1-872)/α1
α2(1-904)/α1
— + — + — + — + — +
α →
γ →
α1
α1(1-311)/α2
α1(1-786)/α2
α1(1-875)/α2
α1(1-907)/α2
— + — + — + — + — +
α →
γ →

Fig. 4-4: Effects of γ on Na^+ activation of Na,K-ATPase of forward and reverse α1/α2 chimeras. Membranes were isolated and assays were carried out at 100 mM KCl and varying concentrations of NaCl as indicated in Experimental Procedures. Data points shown are means ± S.D. (triplicate determinations) of representative experiments and are expressed as percentages of V_{max}. Top panel, α2 and $\alpha 2_f/\alpha 1_b$ chimeras. Bottom panel, α1 and $\alpha 1_f/\alpha 2_b$ chimeras. *Dashed line*, mock-transfected cells. *Solid line*, γ-transfected cells.

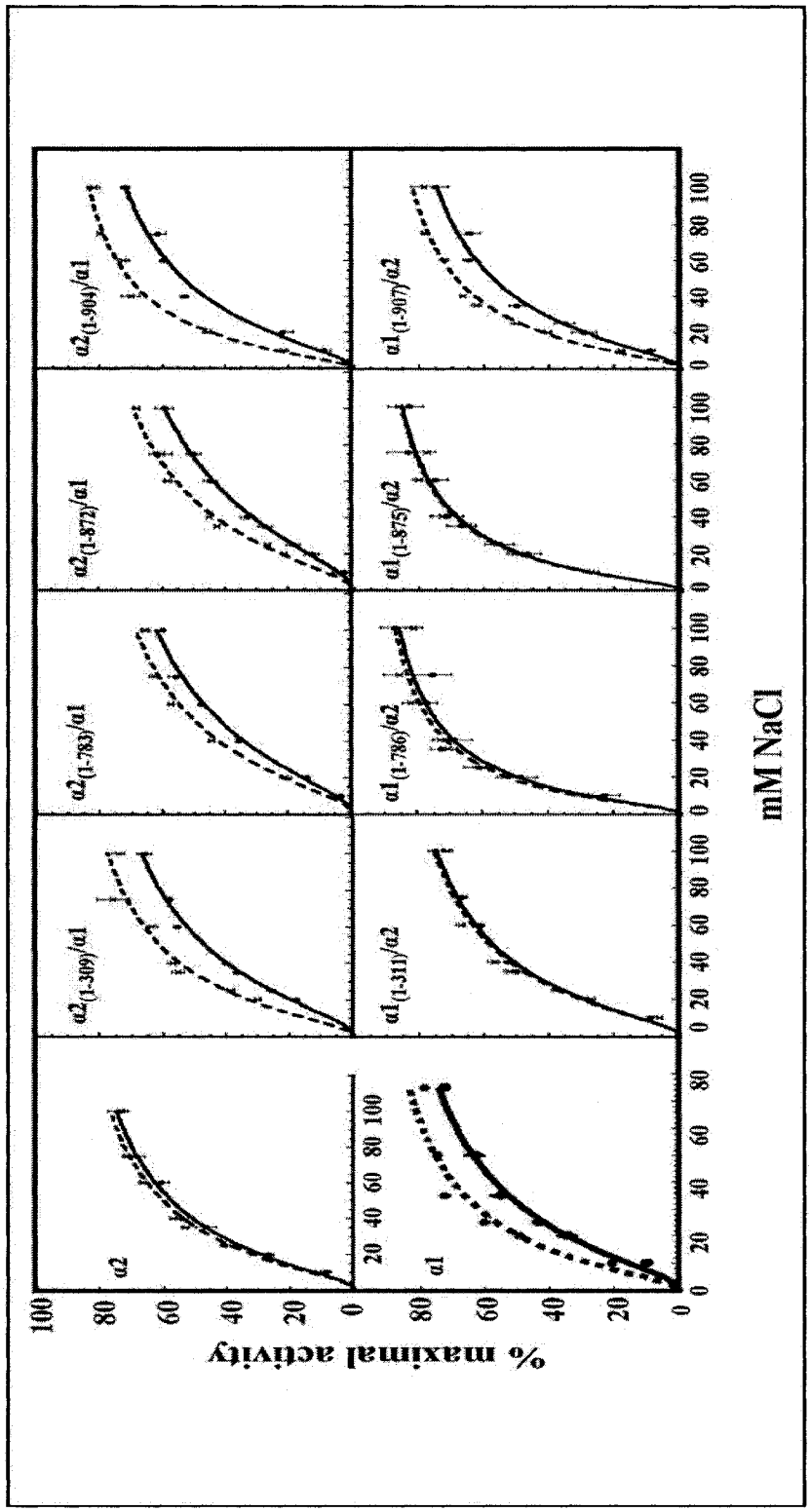

% maximal activity
mM NaCl
α2
α1
α2(1-309)/α1
α2(1-783)/α1
α2(1-872)/α1
α2(1-904)/α1
α1(1-311)/α2
α1(1-786)/α2
α1(1-875)/α2
α1(1-907)/α2

Fig. 4-5: Summary of effects of γ on K'_{Na} of α1, α2, $\alpha1_f/\alpha2_b$ and $\alpha2_f/\alpha1_b$ chimeras. Data are taken from Table 4-2 and are expressed as the percent increase in K'_{Na} caused by γ.

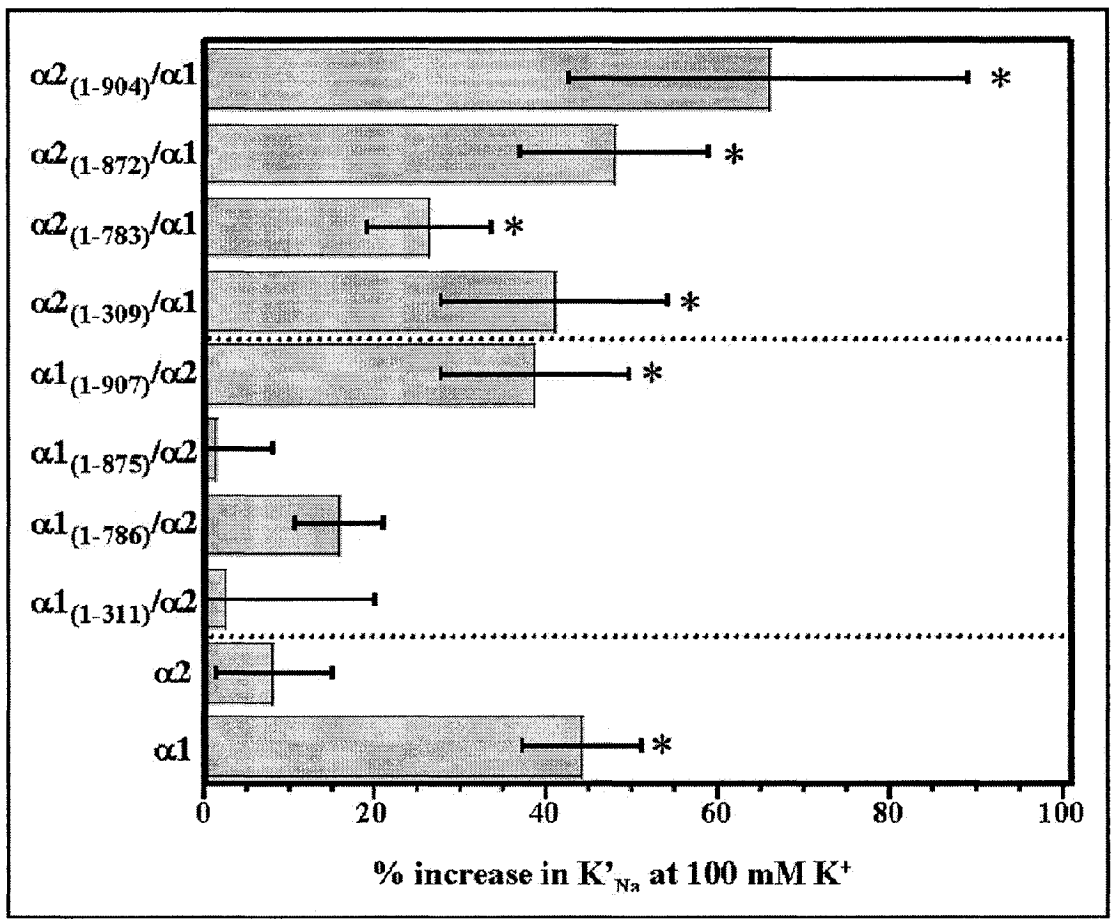

α2(1-904)/α1
α2(1-872)/α1
α2(1-783)/α1
α2(1-309)/α1
α1(1-907)/α2
α1(1-875)/α2
α1(1-786)/α2
α1(1-311)/α2
α2
α1
*
0
20
40
60
80
100
% increase in K'Na at 100 mM K+

Table 4-2: Summary of the effects of γ on K'_{Na}

	K'_{Na} (mM)				
CELLS	α2	$\alpha1_{(1-311)}/\alpha2$	$\alpha1_{(1-786)}/\alpha2$	$\alpha1_{(1-875)}/\alpha2$	$\alpha1_{(1-907)}/\alpha$
mock	8.9 ± 0.4 (7)	9.3 ± 0.7 (4)	4.7 ± 0.2 (7)	5.2 ± 0.3 (5)	6.8 ± 0.4
γb-tf	9.7 ± 0.4 (7)	9.5 ± 1.2 (4)	5.4 ± 0.2 (9)	5.3 ± 0.1 (5)	9.4 ± 0.6*
	α1**	$\alpha2_{(1-309)}/\alpha1$	$\alpha2_{(1-783)}/\alpha1$	$\alpha2_{(1-872)}/\alpha1$	$\alpha2_{(1-904)}/\alpha$
mock	5.3 ± 0.3	8.2 ± 0.4 (4)	13.9 ± 0.4 (6)	12.7 ± 1.2 (5)	7.3 ± 0.4
γb-tf	8.3 ± 0.1*	11.6 ± 0.9* (4)	17.5 ± 0.8* (7)	18.8 ± 0.4* (5)	12.2 ± 1.4*

Na,K-ATPase assays were carried out as described in Fig. 4-4. Each value is the mean ± SEM of the number of separate experiments shown in brackets.

V_{max} values (nmol/mg/min) for α2, $\alpha1_{(1-311)}/\alpha2$, $\alpha1_{(1-786)}/\alpha2$, $\alpha1_{(1-875)}/\alpha2$ and $\alpha1_{(1-907)}/\alpha2$ were 300±20, 81±7, 212±48, 270±22 and 224±16, respectively, for mock-transfected cells and 277±17, 120±9, 121±6, 298±30 and 257±46, respectively, for γb-transfected cells.

V_{max} values (nmol/mg/min) for α1, $\alpha2_{(1-309)}/\alpha1$, $\alpha2_{(1-783)}/\alpha1$, $\alpha2_{(1-872)}/\alpha1$ and $\alpha2_{(1-904)}/\alpha1$ were 375±25, 177±11, 253±78, 250±12 and 168±6, respectively, for mock-transfected cells and 520±196, 244±26, 330±26, 405±17 and 192±2, respectively, for γb-transfected cells.

* $p \leq 0.01$ compared to mock-transfected

** data taken from Pu et al. 2001 (26)

4.6 DISCUSSION

Earlier studies showed that the γ modulator has distinct kinetic effects on the Na,K-ATPase of cultured mammalian cells such as rat α1-transfected Hela cells transfected with either γa or γb. One effect is a γ-mediated increase in the apparent ATP affinity (31, 32). Another effect is a γ-mediated increase in K'_{Na} at high K^+ concentration (26, 27) due to an increase in K^+ antagonism of cytoplasmic Na^+ activation, which can have a significant effect under physiological conditions in which the intracellular Na^+ concentration limits the rate of pump activity. (For further discussion of the physiological importance of this γ effect, see ref. 10). It is noteworthy that these differences in apparent affinities are consistent with the differences in affinities seen with α1β1 pumps in γ-rich kidney preparations compared to γ-free tissues (29). An additional distinct effect, observed only in intact HeLa cells, is an increase in apparent affinity for extracellular K^+ (36). This effect may be similar to a membrane potential-dependent decrease in $K_{(0.5)K}$ reported with Xenopus oocytes bathed in Na^+-containing medium (1, 3).

Regions of γ important for modulation of ligand affinities

Distinct regions of γ are important for its aforementioned distinct effects. The cytoplasmic C-terminus is responsible for the γ -mediated decrease in K'_{ATP}. This effect has been localized to its penultimate four residues (27) since their deletion abrogates the decrease in K'_{ATP}. However, this γ effect can be modified by long range perturbations as seen in experiments in which the effect was

abrogated by deletion of the first seven residues from the extramembranous N-terminus (mutant γbNΔ7; ref. 27). The γ-mediated increase in K'_{Na} has been attributed to the γ-TM domain alone (37) since a γ-TM peptide that is devoid of extramembranous regions elicits this property (also see Table 4-1) and a disruptive mutation in the TM helix (γbG41R) abrogates the γ effect of both full length γ (27) as well as γ-TM (37).

Regions of α involved in functionally important γ-αβ associations

Several earlier studies have provided insight into regions of α with which γ associates. Thermal denaturation experiments suggested a region between TM's 8-10 (7). Cryoelectron miscroscopy (13) as well as homology modeling (20) based on SERCA-Phospholamban interaction (33) have pointed to the importance of TM 2, TM 6 and TM 9.

Regions of γ-αβ pump interaction relevant to the kinetic effects of γ are only beginning to be identified. Recent mutagenesis studies have indicated the importance of TM 9 in abolishing the functional effect of γ on the voltage dependence of K^+ affinity seen in γ cRNA-injected oocytes (20). The present study focuses on regions of the α subunit which interact with γ to effect its modulation of K'_{Na}. The results with $\alpha 2_f/\alpha 1_b$ chimeras are consistent with a key role of TM 9 in mediating the K^+ antagonism of cytoplasmic Na^+ activation. Thus, the γ-mediated increase in K'_{Na} at high K^+ concentration, characteristic of its effect on α1, is seen with all $\alpha 2_f/\alpha 1_b$ chimeras including chimera $\alpha 2_{(1\text{-}904)}/\alpha 1$. In the C-terminal portion (residues 907-1018 of α1), only TM 9 is proximal to γ (8,

13, 20). Even if one considers all α TM's near γ, namely TM 2, TM 6 and TM 9 as in the model of Fusezi et al. (8), and possibly TM 4 seen by cryo-electron microscopy (13), it is notable that amino acid sequences of TM 4 and TM 6 of α1 and α2 are identical. In TM 2, only one of the three conservative differences between α2 and α1 is also different between α2 and α3, yet γ increases K'_{Na} of both α1 and α3, but not α2. In TM 9, there is one non-conservative residue difference in α2, namely Leu951 in α2 is Phe in both α1 and α3. This difference may be key to the functional importance of TM 9. The putative importance of TM 9 notwithstanding, the effects of γ on K'_{Na} of the reverse $\alpha1_f/\alpha2_b$ suggest that its role is modified by extramembranous region(s) as discussed below.

Role of the L7/8 loop

Similar to the lack of its effect on WT α2, γ does not increase K'_{Na} of the $\alpha1_f/\alpha2_b$ chimeras up to $\alpha1_{(1\text{-}875)}/\alpha2$ whereas like its effect on WT α1, γ increase K'_{Na} of $\alpha1_{(1\text{-}907)}/\alpha2$. Thus, replacement of residues ^{873}SRLLGIRLDWDDRTT^{887} in the extracellular L7/8 loop of α2 with the analogous residues of α1 (^{876}FHLLGIRETWDDRWI^{890})[3], points to an important but complex role of the L7/8 loop. Thus, as long as the α1 L7/8 sequence is present, replacement of TM 9 of α1 with that of α2 does not abrogate the γ-dependent increase in K'_{Na}. On the other hand, as long as TM 9 is that of α1, replacement of the α1 L7/8 sequence

[3] α2 versus α1 isoform-distinct residues in the aforementioned peptides are underlined.

with the analogous sequence of α2 does not abolish the K'_{Na} effect. Interestingly, there are six non-conservative residue differences between α1 and α2, some of which presumably underlie the isoform-distinct effect of γ on K'_{Na}.

The aforementioned role of L7/8 loop may not be surprising. This loop appears to be the focal region for γ-α-β interactions. Accordingly, at the extramembranous extracellular region, the β subunit interacts with L7/8 of α1 as shown in several earlier studies (4, 8, 19, 25, 34) and γ can also be cross-linked to β (8), and as already suggested (8), the site of γ-β interaction is probably close to the site of α-β interaction.

Although the α1 versus α2 difference in L7/8 is probably key to the loss of the γ effect in α2, the effect must be long range, quite likely affecting associations within the membranous regions since the isoform-distinct effects of γ are seen in the present study in which addition of only the transmembrane region of γ increased K'_{Na} of α1 but not α2 pumps (see Table 4-1). It is noteworthy that, like γ, the functional effects of FXYD7 are also isoform specific (2). Like γ, FXYD 7 associates with α1, α2 and α3 but its effect on affinity for $K_{(0.5)K}$ (decrease in apparent affinity) is seen with α1 and α2, but not α3. Similarly to γ, FXYD 7 resides in a groove made up of TM's 2, 6, and 9 of the α subunit (20).

Conclusions

Our findings support the view that structural associations of transmembrane regions of the catalytic α subunit with FXYD proteins are not

necessarily the sole determinants of the kinetic effects of these regulators on cation affinities. In the case of γ, the extramembranous L7/8 loop of α appears to modulate intramembranous α-γ interactions to effect the γ-mediated increase in K^+ antagonism of cytoplasmic Na^+ activation.

4.7 ACKNOWLEDGEMENTS

We are grateful to Laura Segall for helpful discussions, Rosemarie Scanzano for technical assistance and Delia Susan-Resiga for critical reading of the manuscript. We thank Drs. Jerry B Lingrel for the α1, α2- and α3-transfected HeLa cells, Lois Lane for the α1 and ouabain-resistant α2 cDNA's used for the construction of the chimeras, and Kathleen Sweadner for the gifts of McK1 and McB2 monoclonal antibodies.

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CHAPTER 5

GENERAL DISCUSSION

Na,K-ATPase activity is subjected to both acute and long-term regulation by a variety of physiological factors. The mechanisms of Na,K-ATPase regulation are diverse (16, 38, 134) and include the modulation by its association with other intrinsic membrane proteins. FXYD proteins comprise a gene family of small, single transmembrane proteins that are abundant and widespread in mammalian tissues and represent a novel mechanism for tissue specific regulation of the Na,K-ATPase. They are distributed in a variety of cells including epithelial cells and cells of the neuromuscular system.

The last decade has provided a significant amount of insight into the functional role of FXYD proteins interactions with the Na,K-ATPase. Coimmunoprecipitation of FXYD proteins with the Na,K-ATPase and their distinct modulation of ligand affinities, seen in membrane preparations of various FXYD-bearing cells, expression systems, and to some extent, studies with knock out (KO) mice have provided insight into the function of FXYD proteins. Recently results with three FXYD proteins obtained from studies in KO mice confirm, at least to some extent, FXYD modulation of Na,K-ATPase seen in *in vitro* studies.

5.1 FXYD interactions with αβ subunits

While γ, CHIF and FXYD 7 appear to be isoform-specific regulators of the Na,K-ATPase exclusively modulating α1 *in vivo*, two other FXYD members have been shown to associate with α2 and α3 as well. For instance, PLM has been shown to associate with and modulate more than one α isoform in native

tissue. In native cardiac and skeletal muscle, PLM interacts predominantly with α1β (31, 43, 127) and to a lesser extent α2β isoforms (31). Functional studies showed that with both α1β1 and α2β1, PLM decreases the affinities for both intracellular Na^+ and extracellular K^+ when expressed in Xenopus oocytes (31). Association of PLM to all three α isoforms was also detected in cerebellum in neurons, astrocytes and glial cells (40). The physiological relevance of the association of PLM with more than one catalytic isoform at a time in either cardiac or brain tissue is unclear. It has been shown that PLM KO mice exhibit an increased ejection fraction, an increase in cardiac mass without hypertension and a decrease in maximal enzymatic activity (59). Although PLM had been detected in the CNS (40), the KO phenotype described seems to suggest that PLM's primary role may be in regulation of Na,K-ATPase in cardiac muscle. Interestingly FXYD 7 is also found in neurons and glial cells of the brain (12) and may play a more important role in the brain than PLM. PLMS associates with and regulates the α3 isoform in native tissue (see Table 1-2).

5.2 Regulation of the apparent affinity for ligands by the γ modulator

The γ modulator of the Na,K-ATPase is a primary example of a small single transmembrane protein with a key regulatory role. First to be discovered and subsequently characterized, the γ subunit can be considered as a 'model' of a FXYD protein with a tissue-specific (mainly renal) modulatory function. As summarized in Table 1-2 and from the present studies it may be concluded that γ serves at least three distinct functions in regulating the α1β1 pumps, namely (i) an

increase in K'_{Na}, (ii) a decrease in K'_{ATP} and (iii) decrease in $K_{0.5(K)}$ seen only in hyperpolarized Xenopus oocytes (11, 13) and intact γ-transfected rat-α1 HeLa cells (Chapter 2).

While the functional effects of γ have been well established, the physiological relevance of γ is still unknown. Gamma KO mice are viable and show no phenotype (62), despite γ's association with renal magnesium wasting disease in humans (100, 101) and γ's absence during embryonic development which was shown to be essential for blastocoel formation in mice (60). In membranes prepared from KO mice, γ's effect on apparent Na^+ affinity (i.e. increase in K'_{Na}) was observed while the apparent increase in ATP and K^+ affinity was not. Not surprisingly, no effect on $K_{0.5(K)}$ was seen since these authors only tested fragmented membrane preparations. The absence of the K'_{ATP} effect is more puzzling. It would be interesting to test whether differences in membrane preparation protocols can account for this discrepancy. Membranes prepared from KO mice were purified using the Jorgensen method (63) which requires incubation with SDS in the presence of ATP whereas membrane preparations used in my experiments as well as earlier ones from our laboratory (114, 115) used the (ATP- free) NaI method described in refs. (58, 74). Intact γ transfected rat α1-HeLa cells show a greater increase in K^+ influx as a function of [ATP] compared to mock transfected cells (see Chapter 2).

That the decrease in apparent Na^+ affinity seen at high K^+ concentrations is physiologically relevant was supported by studies with intact cells showing an increase in steady-state intracellular Na^+ concentration ($[Na^+]_{in}$). This effect was

only seen in rat α1-HeLa cells transfected with γb compared to control; presumably an effect on γa was not detected due to the higher V_{max} of γa clones compared to control mock transfected cells which would obscure a change in $[Na^+]_{in}$ due to the kinetic effect (increase) on K'_{Na} (see Results and Discussion in Chapter 2 for details). Consistent with our findings in intact γb-transfected cells, $[Na^+]_{in}$ levels in renal cells measured by electron microprobe analysis, were noted to be higher in nephron segments with highest expression of γ (mTAL) and lowest $[Na^+]_{in}$ levels in segments that are devoid of γ (CCD principal cells) (7, 8). Since a notably higher intracellular Na^+ is seen in cells under the ATP-replete conditions used in studies in Chapter 2, a decrease in K'_{ATP} could presumably oppose an increase in K'_{Na}. It may be of value to determine whether the relative increase in intracellular Na^+ is less apparent when the intact cells are energy-deprived.

5.3 Does γ have a role other than kinetic modulation of the Na,K-ATPase at the plasma membrane?

When γ alone was expressed in Xenopus oocytes, it was able to induce ion currents. The magnitude of these ion currents remained unaffected upon coexpression with exogenous α and β subunits (103). Sha et al. (122) also reported that human γ in oocytes can activate Ca^{2+} and voltage-gated, non-selective large diameter pores that are intrinsically present on the oocyte membrane.

The physiological implication of the presence of the two variants with distinct as well as overlapping expression along the nephron remains to be determined. While no functional differences have been detected in assays using either permeabilized membranes (114) or intact cells (see Chapter 2), it has been shown that γa and not γb is upregulated when exposed to NaCl, heat shock and stress, suggesting a role for at least one of the variants in the cell's response to stress (145). It has also been suggested that γa may differentially target the Na,K-ATPase to caveolae, microdomains of the plasma membrane where signaling cascades are initiated (39).

To-date, the effect of γ on cell surface expression has only been addressed cursorily (115). For example, the question of whether γ association to the Na,K-ATPase affects the rate of pump trafficking and, if so, whether the two variants have a distinct role in pump protein turnover at the cell surface has not been addressed. Although early studies showed that γ leaves the ER with αβ to be delivered to the plasma membrane (13), those experiments were performed with only one variant, γa, with an erroneously extended N-terminus, as discussed in (11).

5.4 Role of the transmembrane segment of γ in modulating Na,K-ATPase function, with emphasis on the effect of mutation G41R

In 2000, Meij et al. reported that a dominant negative point mutation in the TM region of the human γ subunit is associated with renal magnesium wasting.

Their immunofluorescence studies showed that γ does not appear at the surface of either transfected Sf-9 cells or COS cells and suggested that α trafficking to the cell surface was also affected. In a later study, using rat γ- and γG41R-transfected rat α1-HeLa cells Pu et al. (115) showed that whereas the G41R mutation prevented appearance of γ at the plasma membrane, surface expression of α was unaffected. Results with peptides provide direct evidence of the primary effect of G41R to be failure to interact with α (see Chapter 3). From these aforementioned studies, whether or not the misrouted γG41R mutant, alters αβ pump rates of trafficking/turnover is unclear.

In view of the dominant negative effect of the G41R mutation and the evidence that γ- but not G41R- TM peptides form dimers (135), it would be important to determine whether the dominant negative effect of γG41R can be explained by an abrogation of dimerization. Although our studies failed to reveal γ oligomers in PFO-PAGE, it remains plausible that γ-γ association is relevant to γ-αβ interaction at the subcellular level (i.e. ER, Golgi) in which case an effect of γG41R on γ trafficking (abrogation) to the cell surface may occur. This might be approached by experiments with cells transiently transfected with γ and γG41R, separately and together, with subsequent determination of α-γ association (coimmunoprecipitation) as well as α and γ expression at the cell surface (surface biotinylation). This may provide information relevant to the question of whether dimerization has a function in normal γ processing or possibly stabilization of αβγ oligomers at the plasma membrane.

5.5 Sites of α-γ interaction

While interaction of γ's TM domain alone with the α1 subunit can elicit the K'_{Na} effect (result in Chapter 3), results in Chapter 4 indicate that the interaction of α with γ is complex and long range. Replacement of residues ^{873}SRLLGIRLDWDDRTT^{887} in the extracellular loop of L7/8 of α2 with the analogous residues of α1 (^{876}FHLLGIRETWDDRWI^{890}), provides evidence of a role of the L7/8 loop in long range modulation of K'_{Na}. These 'non-conservative' residue differences (underlined above) may be responsible for this long range effect of the isoform-specific modulation of the γ-mediated increase in K'_{Na}. The L7/8 loop is probably the 'structural crossroad' of γ-αβ interactions. At the extramembranous extracellular region, the β subunit interacts with L7/8 of α1 as shown in several earlier studies (29, 77, 110, 144) and γ can also be cross-linked to β (44), and as already suggested (44), the site of γ-β interaction is probably close to the site of α-β interaction. Although these studies with α1/α2 chimeras indicate the importance of the C-terminus of α1 in K^+/Na^+ antagonism effected by γ, it would be of interest to specifically pin-point which residue(s) in L7/8 is responsible for long range modulation of K'_{Na}.

5.6 Future Perspectives

In the future, it is likely that additional families of transport modulator proteins will be identified among the many "orphan" genes present in many genomes. Recently, one such member has been defined. A yeast two-hybrid screen identified the β1 and β3 subunits of the Na,K-ATPase as MONaKA

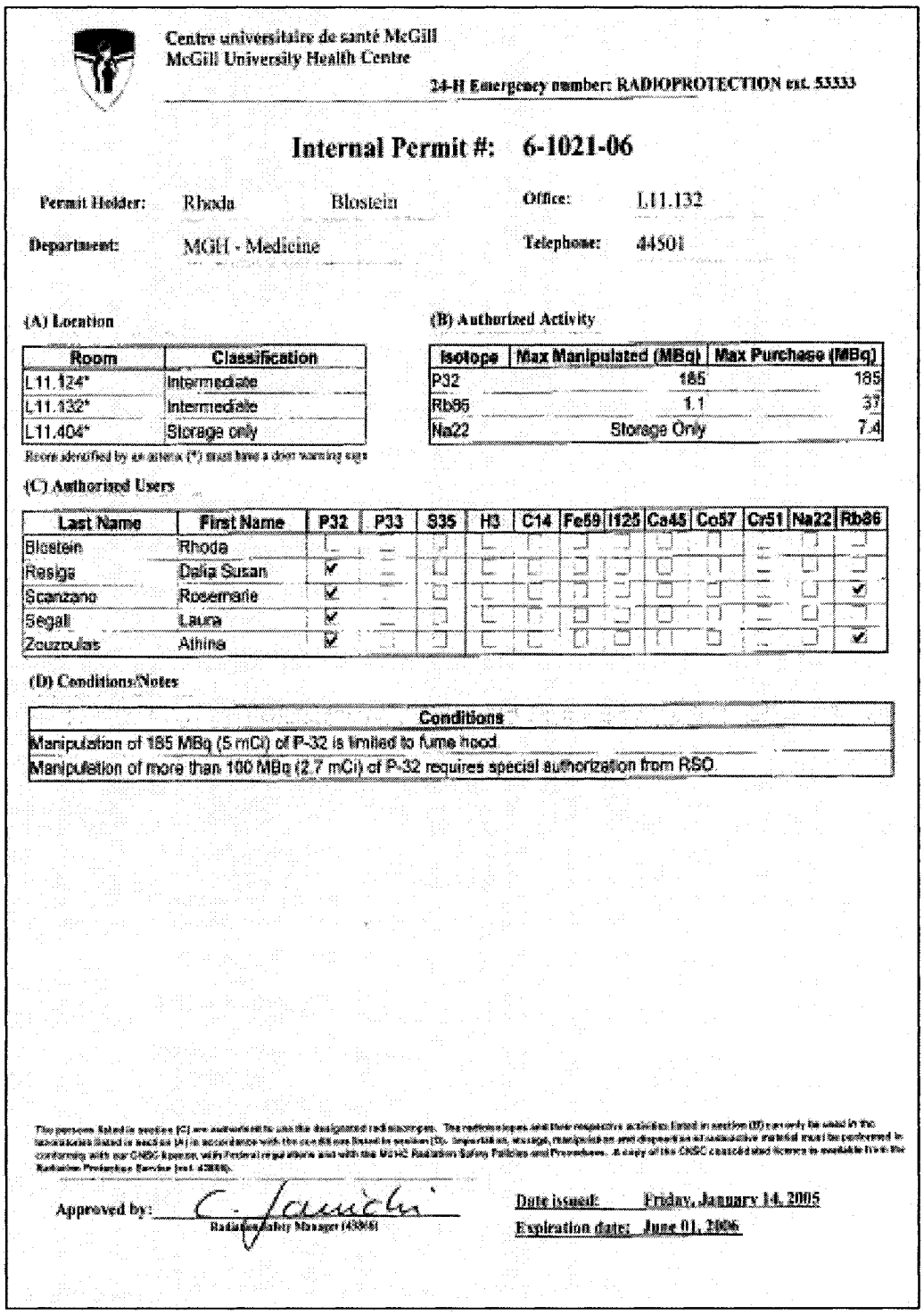
(modulator of Na, K-ATPase) binding partners (96). The interaction between MONaKA and Na,K-ATPase β subunits was detected in transfected tsA201 cells derived from HEK293 cells, mouse brain, and cultured mouse astrocytes. These studies indicated a decrease in Rb^+ influx following transfection.

The experimental evidence gathered over the years has established the FXYD family of small single TM proteins as tissue specific modulators of the Na,K-ATPase. More studies are needed to better understand their physiological relevance and the implications of the potential loss of FXYD modulation of the Na,K-ATPase in disease states. Likewise, although the mechanistic basis for renal magnesium wasting by the γG41R mutation remains unknown, it may indicate an indirect/secondary role of the Na,K-ATPase in causing this disease and further underscores the fundamental importance of the Na,K-ATPase in the maintenance of secondary transport processes.

CHAPTER 6

APPENDIX

6.1 Radioisotope Permit and Certificate



Centre universitaire de santé McGill
McGill University Health Centre

24-H Emergency number: RADIOPROTECTION ext. 53333

Internal Permit #: 6-1021-06

Permit Holder: Rhoda Blostein Office: L11.132

Department: MGH - Medicine Telephone: 44501

(A) Location

Room	Classification
L11.124*	Intermediate
L11.132*	Intermediate
L11.404*	Storage only

Room identified by an asterix (*) must have a door warning sign

(B) Authorized Activity

Isotope	Max Manipulated (MBq)	Max Purchase (MBq)
P32	185	185
Rb86	1.1	37
Na22	Storage Only	7.4

(C) Authorized Users

Last Name	First Name	P32	P33	S35	H3	C14	Fe59	I125	Ca45	Co57	Cr51	Na22	Rb86
Blostein	Rhoda												
Resiga	Dalia Susan	✔											
Scanzano	Rosemarie	✔											✔
Segall	Laura	✔											
Zouzoulas	Athina	✔											✔

(D) Conditions/Notes

Conditions
Manipulation of 185 MBq (5 mCi) of P-32 is limited to fume hood.
Manipulation of more than 100 MBq (2.7 mCi) of P-32 requires special authorization from RSO.

The persons listed in section (C) are authorized to use the designated radioisotopes. The radioisotopes and their respective activities listed in section (B) are only for use in the laboratories listed in section (A) in accordance with the conditions listed in section (D). Importation, storage, manipulation and disposal of radioactive material must be performed in conformity with our CNSC licence, with Federal regulations and with the MUHC Radiation Safety Policies and Procedures. A copy of the CNSC associated licence is available from the Radiation Protection Service (ext. 42888).

Approved by: C. Jaworski
Radiation Safety Manager (43868)

Date issued: Friday, January 14, 2005
Expiration date: June 01, 2006

Le centre universitaire de santé McGill (CUSM)
McGill University Health Center (MUHC)

Service de radioprotection
Radiation Protection Service

Ceci certifie que:
This is to certify that:

Athina Zouzoulas

A réussi avec succès le cours théorique de base de radioprotection en laboratoires
Has successfully completed a basic theoretical radiation safety course for laboratories

26 March, 2002

Maureen McQueen
Officier de radioprotection
Radiation Safety Officer

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CHAPTER 7

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