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COMPARATIVE KINETIC PROPERTIES OF TISSUE-SPECIFIC NA,K-PUMPS

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ABSTRACT

The catalytic α subunit of the heterodimeric Na,K-ATPase comprises three distinct isoforms which are expressed in a tissue-specific manner. For example, the α_1 isoform can be detected in virtually all mammalian tissues, whereas the appearance of the α_2 and α_3 isoforms is more restricted to particular tissues such as muscle and nervous tissue. Previous comparative functional studies of Na,K-ATPases isolated from various tissues indicated that there are differences, including apparent cation affinities, among these enzymes. While these differences often appear to correlate with the presence of distinct isozymes, their precise molecular bases remain to be determined. Moreover, certain studies suggest that the behavior of the same isoform can vary from tissue to tissue (*e.g.*, the erythrocyte *versus* the kidney, both of which contain only the α_1 isoform). An hypothesis that may explain these observations is that the cell-specific membrane environment influences Na,K-ATPase activity. To investigate this possibility, polyethylene glycol-mediated membrane fusion was used to deliver pumps from high-specific-activity microsomes derived from various tissues into mammalian erythrocyte membranes. The success of this methodology was verified using two distinct experimental systems. In the first system, rabbit sarcoplasmic reticulum Ca-ATPase was delivered into human erythrocyte membranes. Cellular Ca^{2+} uptake fueled by extracellular ATP was used as a measure of the functional delivery of the Ca-ATPase into these membranes. In the second system, ATP- and cardiac glycoside-dependent rubidium fluxes verified the functional delivery of axolemma or kidney Na,K-ATPases into mammalian erythrocytes. Among these studies was a series of experiments demonstrating that the L_p-antigen of sheep erythrocyte membranes is a distinct membrane component that interacts with and alters the behavior of rat kidney pumps fused into LK sheep erythrocytes. In order to compare the behavior of the α_1 isoform derived from two different tissues in the same membrane environment, rat kidney pumps were transferred into human erythrocytes. The apparent

cation affinities of these pumps, as judged either by sodium or rubidium flux assays, were nearly identical. In contrast, differences in apparent cation affinities were observed between rat kidney and rat axolemma pumps delivered into dog erythrocytes. Quantitative immunoblot analyses as well as phosphoenzyme inhibition profiles showed that the latter tissue contains mainly the α_3 catalytic subunit isoform. Supported by this conclusion, the apparent affinity values obtained from the flux assays agree qualitatively with those reported for ATPase measurements on membranes of HeLa cells individually transfected with the three rat α subunit isoforms (Jewell, E. A., and Lingrel, J. B. (1991) *J. Biol. Chem.* 266, 16925-16930). Subsequent cation transport assays on these HeLa cell transfectants also agreed with the results from the ATPase measurements and were quantitatively very similar to those from the microsome-fused erythrocytes - *i.e.* the α_3 isoform is more sensitive to K^+ and less sensitive to Na^+ than either the α_1 or α_2 isoforms. Taken together, these studies have demonstrated that there are isoform-specific differences in the sided cation responsiveness of the Na,K-ATPase. In addition, these findings are compatible with the hypothesis that the cellular membrane environment modulates the activity of the Na,K-ATPase, possibly including the differences in apparent cation affinities that are observed between rat kidney and rat axolemma pumps.

RÈSUMÈ

La sous-unité catalytique (α) de l'Na,K-ATPase est composée de trois isoformes qui présentent une distribution tissulaire spécifique. Par exemple, l'isoforme α_1 peut être détectée dans tous les tissus mammaliens tandis que les isoformes α_2 et α_3 sont exprimées majoritairement dans les tissus musculaires et nerveux. Des expériences antérieures ont également montré des divergences dans l'affinité apparente des Na,K-ATPase vis-à-vis des cations selon le tissu où elles s'expriment. Bien que ces différences semblent s'expliquer par la présence d'isoformes distinctes, la base moleculaire de ces variations n'est pas encore élucidée. De plus, certaines études indiquent qu'une même isoforme (α_1) peut se comporter différemment d'un tissu à l'autre (ex: la cellule rouge vis-à-vis du rein, qui contiennent tous deux l'isoforme α_1). Une hypothèse pouvant expliquer ces observations est que l'environnement de la membrane dans une cellule donnée pourrait influencer l'activité de l'Na,K-ATPase. Afin d'examiner cette possibilité, nous avons employé la technique de fusion de la membrane par polyethyleneglycol afin de transférer les pompes d'ion de microsomes enrichies à des pompes de membranes d'érythrocytes de mammifères. Le succès de cette méthodologie a été verifié par l'utilisation de deux systèmes expérimentaux distincts. Dans le premier système, la Ca-ATPase du réticulum sarcoplasmique de lapin a été transférée dans des membranes d'érythrocytes humains. La capture du calcium cellulaire sous l'influence de l'ATP extracellulaire a servi de mesure du transfert de la Ca-ATPase dans ces membranes. Dans le deuxième système, les flux de rubidium dépendant de l'ATP et les glycosides cardiaques permis d'évaluer le transfert fonctionnel de l'Na,KATPase d'axolemme et de rein dans des érythrocytes mammifères. Une serie d'éxpériences nous a permis de démontrer que l'antigène-L_p de membranes d'érythrocytes de mouton est un composant modifiant le fonctionnement de pompes de rein de rat incorporées dans ces cellules. Afin de comparer le fonctionnement de l'isoforme α_1 provenant de deux tissus distincts mais dans le même environnement membranaire, nous avons transferé des pompes de rein de rat aux érythrocytes humains. Les affinités apparentes vis-à-vis des cations, estimées par les essais de flux de sodium ou de rubidium, ont été presque identiques. En revanche nous avons observé des différences dans les affinités apparentes des cations entre les pompes de rein ou de l'axolemme de rat incorporées dans des érythrocytes de chien. L'analyse quantitative d'immunoblots ainsi que de courbes d'inhibition de phosphoenzyme a montré que l'axolemme de rat contient surtout l'isoforme α_3 de la sous-unité catalytique. Ces résultats sont en accord avec une étude antérieure dans laquelle les isoformes des trois sous-unités furent individuellement transfectées dans des cellules HeLa (Jewell, E. A., et Lingrel, J. B. (1991) *J. Biol. Chem.*

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I would like to begin by extending my heartfelt thanks and deep appreciation to my thesis director, Dr. Rhoda Blostein. She has provided me with a great deal of support, both moral and financial, during the course of this work. The high standards and integrity which she demands of herself and those who work with her will always remain with me. In addition, I would like to express my appreciation to her for the hours spent discussing and reviewing drafts of this thesis.

I extend special thanks to Dr. John Silvius for his invaluable advice and contributions regarding the development of the polyethylene glycol mediated membrane fusion methodology. Other researchers who I would like to thank for their contributions to this work include Drs. Jerry Lingrel and Elizabeth Jewell-Motz, University of Cincinnati, for their HeLa cell transfectants; Professor Michael A. Kaplan, Institute National de la Recherche Scientifique Télécommunications, Université du Québec, for assistance with the probabilistic modeling of vesicle-cell fusions; Dr. Amira Klip, Hospital for Sick Children, Toronto, for membrane preparations from rat muscles; Dr. Michael Caplan, Yale University School of Medicine, for antibodies to the α_1- and α_3-isoforms of the Na,K-ATPase; and the late Dr. John Parker, for very helpful advice on working with dog erythrocytes.

I acknowledge and thank the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR), Medical Research Council of Canada (MRC), and the McGill University Faculty of Medicine for studentship funding over the course of this degree. In addition, this work was funded in part by operating grants from the MRC and the Heart and Stroke Foundation of Canada.

To my thesis committee, Drs. Joyce Rauch, Pierre Drapeau, Rob Dunn, and John Silvius, thank you for your critiques and insightful suggestions regarding this project.

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To my family, I express my love and appreciation for their continued support of and enthusiasm for my endeavors.

Above all, I extend my deepest love and gratitude to my wife, Wendy. Thanks to her faithful support, saintly patience, and love, this journey has been all the more rewarding. In addition, her tireless work in the editing and preparation of this manuscript is very much appreciated.

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PREFACE

Section B.2 (Manuscripts and Authorship) of the "Guidelines Concerning Thesis Preparation" of McGill University Faculty of Graduate Studies and Research states that: "Candidates have the option, subject to the approval of their Department, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis. If this option is chosen, concerning texts, providing logical bridges between the different papers are mandatory.

The thesis must still conform to all other requirements of the 'Guidelines Concerning Thesis Preparation' and should be in literary form that is more than a mere collection of manuscripts published.

The thesis must include, as separate chapters or sections: (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary.

Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (eg. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent; supervisors must attest to the accuracy of such claims at the Ph.D. Oral Defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers."

In accordance with these guidelines, the results of this thesis work are presented in the form of three manuscripts, which have been published or are in press, as well as an appendix which presents the abstract of a manuscript of which I am a co-author.

Chapter 2 deals with the development and characterization of the polyethylene glycol-mediated membrane fusion methodology. I received assistance from Dr. J.Silvius with the preparation of rabbit sarcoplasmic reticulum membranes. In addition, he provided valuable advice about the membrane fusion aspects of this project and assisted in the preparation of this manuscript.

Chapter 3 describes the comparative cation activation kinetics of rat kidney and human erythrocyte Na,K-pumps in the human erythrocyte.

Chapter 4 comprises a comparative study of cation activation kinetics of Na,K-pump isoforms using both the microsome-fused erythrocyte system and the HeLa transfectant system. Assistance with the experimental procedures involving the HeLa cells was provided as follows: Drs. J. Lingrel and E. Jewell-Motz of the University of Cincinnati provided the isoform-transfected cells and Dr. S. Daly in our laboratory carried out the tissue culture procedures. R.-P. Zhang carried out the ion flux assays in our laboratory on the HeLa cells (Fig.'s 4 and 6). In addition, she carried out the ouabain-sensitivity profiles of the phosphoenzyme in axolemma membranes (Fig. 3).

Appendix I, which describes the modulation of exogenous rat kidney pumps by the L-antigen of LK sheep erythrocytes, was the first publication in the Ph. D. thesis of X.-C. Xu in our laboratory. In addition to developing the microsome-erythrocyte fusion method, I carried out preliminary experiments demonstrating the delivery of dog kidney pumps into LK erythrocytes and suggesting that they may be affected by the L-antigen.

ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

The author considers the the following to be original contributions to knowledge:

1. Polyethylene glycol-mediated membrane fusion was used as the basis for the development of a novel system to deliver ion-motive ATPases from high-specific-activity microsomes into mammalian erythrocyte membranes. The functional incorporation of transport-competent Ca- and Na,K-ATPases into red cells was demonstrated by the presence of ATP-dependent cation fluxes which were not observed in unfused control cells. ATP-dependent Na^+ and Rb^+ fluxes of inside-out vesicles derived from dog erythrocytes fused with dog kidney microsomes further verified the functional and permanent incorporation of exogenous dog kidney pumps into dog erythrocyte membranes. This study also indicated that the Na/O flux of dog kidney microsomes delivered into Na,K-ATPase-deficient dog erythrocytes is much lower than that expected for erythrocyte pumps in general.

2. The cation activation kinetics of rat kidney Na,K-ATPases delivered into human erythrocyte membranes were compared with those of endogenous human erythrocyte pumps. This is the first study in which the behavior of the kidney and red cell pumps has been compared with both enzymes in the same membrane environment. Their individual activities were distinguished by taking advantage of the nearly 1000-fold difference in the sensitivities of these two kinds of pumps to cardiac glycosides. The responses of both human and rat pumps to extracellular K^+ and intracellular Na^+ were nearly identical.

3. A comparative study of the cation activation kinetics of rat kidney and axolemma Na,K-ATPases delivered into dog erythrocyte membranes was carried out. This is also the first report in which the behavior of these tissue-specific pumps has been compared with both of them in the same membrane environment. Quantitative immunoblotting and ouabain-sensitive EP assays revealed that axolemma membranes comprise mainly the α_3 isoform of the Na,K-ATPase catalytic subunit. Recently-developed HeLa cell transfectants that individually express the three isoforms of the catalytic α subunit were examined for their cation activation kinetics using Rb^+ flux assays on intact cells. The results of both assay systems indicated that the α_3 isoform has a three-fold higher apparent affinity for extracellular K^+ and at least a three-fold lower apparent affinity for intracellular Na^+.

PUBLICATIONS

CHAPTER 2

Delivery of Ion Pumps from Exogenous Membrane-Rich Sources into Mammalian Red Blood Cells

Munzer, J. S., Silvius, J. R., and Blostein, R.
J. Biol. Chem. (1992) 267: 5202-5210.

CHAPTER 3

Functional Diversity of Tissue-Specific Na^+/K^+-Pumps Delivered from Exogenous Sources into Erythrocytes

Munzer, J. S., and Blostein, R.
Proceedings of the VIIth International Conference on the Sodium Pump (in press).

CHAPTER 4

Tissue- and isoform-specific kinetic behavior of the Na,K-ATPase

Munzer, J. S., Daly, S. E., Jewell-Motz, E. A., Lingrel, J. B., and Blostein, R.
J. Biol. Chem. (1994) 269: 16668-16676.

APPENDIX I

Rat kidney Na-K pumps incorporated into low-K^+ sheep red blood cell membranes are stimulated by anti-L_p antibody

Xu, J.-C., Dunham, P. B., Munzer, J. S., Silvius, J. R., and Blostein, R.
Am. J. Physiol. (1992) 263: C1007-C1014.

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LIST OF ABBREVIATIONS

AMOG	adhesion molecule on glial cells
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
C_3	chymotryptic cleavage site on the Na,K-ATPase catalytic subunit
$[Ca]_{in}$	intracellular calcium ion concentration
Ca-ATPase	calcium-stimulated adenosine triphosphatase
cAMP	cyclic adenosine monophosphate
CCD	cortical collecting duct of kidney
ClR-ATP	*g*-(4-*N*-2-chloroethyl-*N*-methylamino)benzolamide-ATP
DA	dopamine
DA_1	first category of dopaminergic receptor
DA_2	second category of dopaminergic receptor
DARPP-32	dopamine-activated regulator of protein phosphatase
DCCD	N,N'-dicyclohexylcarbodiimide
E^*P	Na,K-ATPase phosphoenzyme in the third conformation
E_1	Na,K-ATPase in the first conformation
E_1P	Na,K-ATPase phosphoenzyme in the first conformation
E_2	Na,K-ATPase in the second conformation
E_2P	Na,K-ATPase phosphoenzyme in the second conformation
EP	phosphoenzyme
ER	endoplasmic reticulum
F-type ATPase	F_0F_1- adenosine triphosphatase
FITC	fluorescein isothiocyanate
H,K-ATPase	proton plus potassium-stimulated adenosine triphosphatase
H-ATPase	proton-stimulated adenosine triphosphatase
H_3O^+	hydronium ions
HK	high-potassium
IOV's	inside-out vesicles from erythrocyte membranes
IP_3	phosphatidylinositol
kDa	kilodaltons

K_{ext}	extracellular potassium ions
$[K]_{ext}$	extracellular potassium ion concentration
K_{in}	intracellular potassium ions
$[K]_{in}$	intracellular potassium ion concentration
$K_{0.5(Kext)}$	apparent affinity for K_{ext} calculated by equation 2 (see below)
$K_{0.5(Na)}$	apparent affinity for Na^+ calculated by equation 2
$K_{0.5(Nain)}$	apparent affinity for Na_{in} calculated by equation 2
K_K	apparent affinity for K^+ calculated by equation 1 (see below)
$K_{K(ext)}$	apparent affinity for K_{ext} calculated by equation 1
K_m	Michaelis constant
$K_{m(ATP)}$	apparent affinity for ATP calculated by equation 3 (see below)
$K_{m(K)}$	apparent affinity for K^+ calculated by equation 3
$K_{m(Na)}$	apparent affinity for Na^+ calculated by equation 3
K_{Na}	apparent affinity for Na^+ calculated by equation 1
$K_{Na(in)}$	apparent affinity for Na_{in} calculated by equation 1
LK	low-potassium
MTAL	medullary thick ascending limb of kidney
Na_{ext}	extracellular sodium ions
$[Na]_{ext}$	extracellular sodium ion concentration
Na_{in}	intracellular sodium ions
$[Na]_{in}$	intracellular sodium ion concentration
Na,K-ATPase	sodium plus potassium-stimulated adenosine triphosphatase
Na,K-pump	Na,K-ATPase (with regard to ion transport activity)
Na/H exchange (flux)	sodium-proton exchange
Na/K exchange (flux)	sodium-potassium exchange
Na/O exchange (flux)	uncoupled sodium efflux
NE	norepinephrine
NEM	N-ethylmaleimide
P-type ATPase	phosphoenzyme-forming adenosine triphosphatase
PCT	proximal convoluted tubules of kidney
PEG	polyethylene glycol

P_i	inorganic phosphate
PKA	protein kinase A
PKC	protein kinase C
pNPP	*p*-nitrophenyl phosphate
pNPPase	*p*-nitrophenyl phosphatase activity
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SR	sarcoplasmic reticulum
T_1	first tryptic cleavage site on the Na,K-ATPase catalytic subunit
T_2	second tryptic cleavage site on the Na,K-ATPase catalytic subunit
T_3	third tryptic cleavage site on the Na,K-ATPase catalytic subunit
V-type ATPase	vacuolar adenosine triphosphatase
V_{max}	apparent maximum velocity

EQUATIONS

Equation 1
Non-cooperative sites
(Garay and Garrahan, 1973)

$$v = \frac{V_{max}}{1 + \frac{K_s}{[S]^n}}$$

Equation 2
Cooperative sites
(Lytton, 1985)

$$v = \frac{V_{max}[S]^n}{K_{n0.5_{(s)}} + [S]^n}$$

Equation 3
Single site
(Michaelis-Menten)

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

The symbols in the equations are as follows (see also Chapter 4, p. 147):

v is the rate of the reaction
[S] is the substrate concentration
n is the number of ions (substrate molecules) transported
K is the apparent affinity for the substrate
V_{max} is the maximum velocity

CHAPTER 1

INTRODUCTION

1.1. ION-MOTIVE PUMPS

Transmembrane ionic gradients are essential to many cellular functions including pH and volume regulation, ion-coupled nutrient uptake, and the maintenance of the resting membrane potential. Biological membranes, serving as the barriers between intracellular compartments and between cells and their external environments, play an essential role in the regulation of ionic homeostasis. Due to their high charge density and degree of hydration, ions rarely traverse the lipid portion of the membrane bilayer (see Stein, 1986). Instead, ion fluxes across membranes are mainly associated with the proteins embedded in the bilayer. In some cases, exemplified by ion channels, these events represent passive ion "leaks" driven by the transmembrane electrochemical gradient. In other cases, such as with ion exchangers, the energy of the transmembrane gradient of one ion can be used to drive the transport of another ion against its concentration gradient. However, these processes ultimately lead to the dissipation of the transmembrane ionic gradients and thus the loss of cellular cation homeostasis.

In response to this challenge, cells have developed ion-transporting membrane proteins which function as primary active transporters. Known collectively as ion pumps, these proteins use the energy from light, redox reactions, or chemical bond cleavage to transport ions against their concentration gradients. By continuously restoring the transmembrane electrochemical gradients, ion pumps are the ultimate effectors of the ionic homeostasis which is essential to cellular survival.

1.1.1. Light-driven and redox-linked ion pumps

Two examples of light-driven ion pumps are bacteriorhodopsin, an H^+ transporter, and halorhodopsin, a Cl^- transporter, both of which exist in halophilic archaebacteria (reviewed by Stoeckenius and Bogomolni, 1982; Läuger, 1991). Among the most ancient of the known ion pumps, they use the energy of light trapped by retinal groups in the protein to drive ion transport (Oesterhelt and Tittor, 1989). Most other photosynthetic

organisms carry out H^+ transport by coupling lipid-soluble proton carriers, such as quinones or cytochromes, with the electron transport chains of various chlorophylls (for review, see Clayton, 1980).

Redox-linked ion pumps, such as cytochrome oxidase, involve the transfer of electrons from reduced coenzymes to molecular oxygen via an electron-transport chain (see Läuger, 1991). It is not yet clear exactly how these pumps couple electron transfer with the H^+ transport that generates the transmembrane electrochemical potential gradient. This scenario is further complicated by observations that certain bacteria, such as *Vibrio alginolyticus*, can use their electron transport chains to generate a transmembrane Na^+ gradient (Tokuda and Unemoto, 1982; Dibrov *et al.*, 1986; Dimroth, 1987).

1.1.2. ATP-driven ion pumps

1.1.2.a. F- and V-type ATPases

Adenosine triphosphate- (ATP-) driven pumps constitute a diverse group of ion pumps that are nearly ubiquitously distributed among living organisms (see reviews by Pedersen and Carafoli, 1987; Pedersen and Amzel, 1993). Although they share many structural features, these pumps may be classified according to their reaction mechanisms, susceptibility to inhibitors, and subunit composition.

F- (F_0F_1-) type adenosine triphosphatases (ATPases) exist in mitochondria, chloroplasts and bacteria (reviewed by Senior, 1988; Penefsky and Cross, 1991). These pumps have relatively complex structures involving at least eight distinct subunits. The F_1 catalytic domain, also called the "head", is composed of five different subunits (α, β, γ, δ, ε), while the F_0 transmembrane portion, or "stalk", contains three subunits (a, b, c). During respiration in mitochondria or photosynthesis in chloroplasts, the F-type ATPases of these organelles use the energy of the transmembrane electrochemical gradient generated by the aforementioned redox- or light-driven pumps to synthesize ATP. Bacterial F-type ATPases, located in the plasma membrane, carry out a similar synthesis of ATP. In certain

halophilic bacteria, such as *Propionigenium modestum*, pumps of the this family use the energy from Na^+ gradients to synthesize ATP (Hilpert *et al.*, 1984; Laubinger and Dimroth, 1988).

V- (vacuolar-) type ATPases are found in most intracellular organelles including lysosomes, endosomes, Golgi vesicles, synaptic vesicles, secretory granules, and plant and fungal vacuoles (for reviews, see Nelson, 1991, 1992). Like F-type ATPases, these pumps contain multiple subunits — five (A, B, C, D, E) in the "head" domain but only two (a, c) in the "stalk" domain. Although there are structural homologies among the main catalytic subunits (α and A, β and B) of the F- and V-type ATPases, the auxiliary catalytic subunits (C, D, E, and γ, δ, ε) are not related to each other. Organellar V-type ATPases use the energy of ATP hydrolysis to acidify the lumina of their respective organelles. Alternatively, when in the plasma membrane, these pumps can mediate H^+ excretion to acidify the extracellular environment; bone dissolution by osteoclasts and urinary acidification are two examples. This family of pumps is very specifically inhibited by bafilomycin A_1.

P-type pumps, named for the formation of a phosphorylated enzyme intermediate during their reaction cycle, constitute the principle plasma membrane ion pump in higher organisms.

1.1.2.b. P-type ATPases

Individual family members. P-type ATPases exist in all eukaryotes as well as in some bacteria (reviewed by Sachs and Munson, 1991). They are designated "P-type" because they are transiently phosphorylated at their active site by the γ-phosphate group of ATP during the course of their reaction cycle. Structurally, the P-type ATPases consist mainly of a single, large catalytic subunit (α). Smaller, auxiliary subunits (β and γ) may be present in some cases. As summarized by Green & Stokes (1992), the members of this

highly diverse family of pumps can be divided into five major groups according to the deduced amino acid sequences of their catalytic subunits.

The smallest two groups include the bacterial ATPases that transport K^+, Mg^{2+} or Cd^{2+} and the H-ATPases from various plants, fungi and bacteria. They range in size from 680-908 amino acids and 920-974 amino acids, respectively.

The sarcoplasmic reticulum (SR) and endoplasmic reticulum (ER) Ca-ATPases, referred to as SERCA, comprise the next group (≈1000 residues), and are found in yeast as well as in animal cells. These pumps play an important role in maintaining intracellular Ca^{2+} homeostasis. Subdivisions of this group include: (i) SERCA1 of fast-twitch skeletal muscle; (ii) the four isoforms of SERCA2, found in slow-twitch skeletal muscle, cardiac muscle, smooth muscle, and certain non-muscle tissues; and (iii) SERCA3, also found in non-muscle cells.

The animal H,K- and Na,K-ATPases (≈1020 residues) represent a fourth group of P-type ATPases. They are very closely related to each other, sharing 63% homology of their deduced amino acid sequences. The H,K-ATPase effects an electroneutral H/K exchange which is responsible for stomach acidification. This pump generates the largest ion gradient of any P-type pump (four million-fold). The Na,K-ATPase, of which there are three known isoforms of the α catalytic subunit, is the major plasma membrane ion pump in animal cells. By mediating an electrogenic exchange of three intracellular Na^+ ions (Na_{in}) for two extracellular K^+ ions (K_{ext}), it maintains the transmembrane electrochemical gradient. Whereas the Na,K-ATPase is directly involved in Na^+ and K^+ homeostasis, it participates indirectly, with the assistance of cotransporters and exchangers, in the maintenance of other cellular gradients that are driven by the energy stored in the transmembrane Na^+ gradient (see Lechene, 1988). Some of the cellular processes which depend on this gradient include volume and pH regulation, nerve impulse propagation, muscle contraction, and Na^+-coupled solute transport.

The largest of the P-type ATPases are the plasma membrane Ca-ATPases (≈1020 amino acids) of animal cells. Their function is similar to that discussed above for SERCA pumps, except that they pump Ca^{2+} ions out of the cell instead of sequestering them in organelles.

Structural features of P-type ATPases. With the exception of the H- and Na,K-ATPases (see above), the overall deduced amino acid sequence homology of the catalytic α subunits of the P-type ATPases is approximately 20% (Jørgensen & Andersen, 1988). There are about ten regions, comprising less than one third of the total amino acid residues, whose sequences are well conserved among the various members of this ion pump family (Green, 1989; Green & Stokes, 1992). These conserved regions appear to be sufficient to account for the remarkably similar tertiary structures of these different pumps. For example, all of the enzymes in this family appear to have 8-10 membrane-spanning domains (Green & Stokes, 1992). While the N-terminal regions are intracellular, the location of the C-termini, and therefore the number of transmembrane domains, has been established for only some of the P-type ATPases. Recent studies of the Ca-ATPase (Matthews *et al.*, 1989; Matthews *et al.*, 1990; Clarke *et al.*, 1990b), the H,K-ATPase (Scott *et al.*, 1991; Munson *et al.*, 1991; Mercier *et al.*, 1991), and the Na,K-ATPase (see Section 1.2.2.a.) support structural models with ten transmembrane domains and a cytoplasmic C-terminus.

The overall tertiary structure of the P-type ATPases as deduced from high-resolution electron micrographs of the Ca-ATPase (Stokes and Green, 1990; Toyoshima *et al.*, 1993) is somewhat analogous to that of the F- and V-type-ATPases. There is a "head" region, which contains the ATP hydrolysis domain, and a "stalk-like" structure which consists mainly of the transmembrane domains. The ATP-binding and hydrolysis domain appears to be located near the membrane-spanning regions (Green and Stokes, 1992), thus suggesting a structural link between the events of phosphorylation-induced conformational changes and ion transport (see Section 1.2.3.).

At least two of the P-type ATPases, the H,K- and Na,K-ATPases, contain smaller peptides called the β subunit (Sachs and Munson, 1991; Section 1.2.2.b.) and the γ subunit (Mercer *et al.*, 1993; Section 1.2.2.c.). Tightly bound to the large catalytic (α) subunit of the these pumps, the β subunit appears to stabilize the α subunit and facilitate its intracellular transport to the plasma membrane (see Section 1.2.2.e.). The role of the much smaller γ subunit is not yet known.

Functional properties of P-type ATPases. The P-type ATPases have several common functional or mechanistic features (Jørgensen & Andersen, 1988). As mentioned above, one of the events which distinguishes this family of pumps is the formation of a phosphorylated enzyme intermediate during its reaction cycle. The highly conserved structure of the regions near this phosphorylation site and the nearby nucleotide binding domain suggests that all the P-type ATPases operate via a similar mechanism (Mercer, 1993). Another feature common to this family of pumps is the "occlusion" of transported ions such that they have no access to either side of the membrane (see Section 1.2.3.e.). Although this phenomenon may occur with other types of ATPases, it seems likely that the molecular mechanisms involved are considerably different from those used by the P-type ATPases (Glynn and Karlish, 1990).

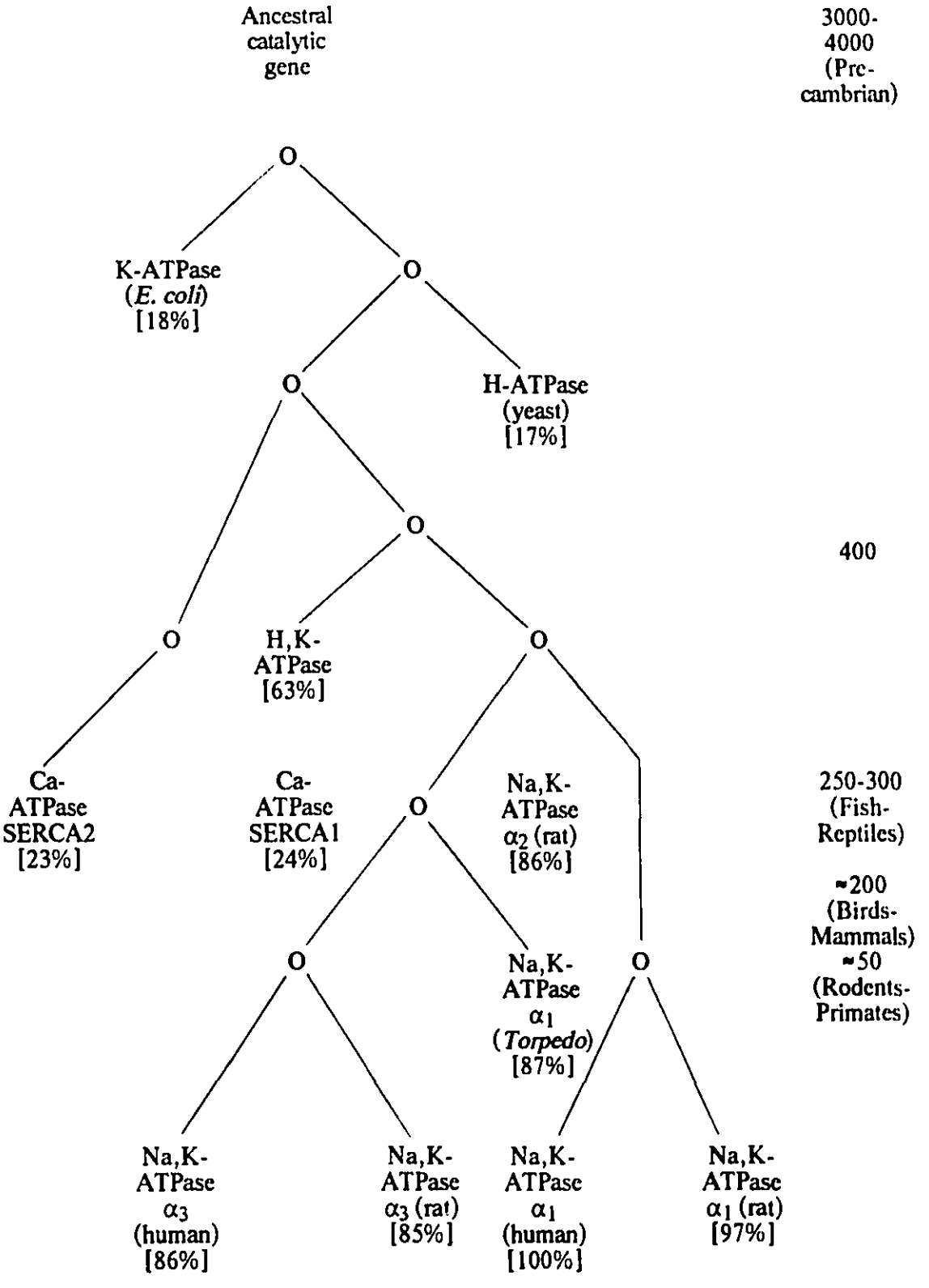
1.1.3. Evolution of ion pumps

By combining information derived from nucleotide sequence analysis with estimates of phyletic divergence times and mutation rates, Jørgensen and Andersen (1988) have constructed an evolutionary tree of the P-type ATPases. Their scheme, presented with a slightly modified time scale, is reproduced in Fig. 1. Clearly, most of the divergence in this family of pumps occurred well over 200 million years ago.

The earliest forms of pumps probably evolved to meet the demands of osmoregulation (Wilson and Lin, 1980; Maloney and Wilson, 1985). While plant cell swelling is limited by the rigid cell wall, animal cells require other means to regulate their

FIG. 1. **Evolution of the P-Type ATPases.**

Redrawn and slightly modified from Jørgensen and Andersen (1988). Numbers at right show approximate time of divergence (millions of years ago). Numbers in brackets [ ] show the percent homology of the deduced amino acid sequence relative to the human α_1 isoform.


Ancestral catalytic gene
3000-4000 (Pre-cambrian)
O
K-ATPase (*E. coli*) [18%]
O
O
H-ATPase (yeast) [17%]
O
400
O
H,K-ATPase [63%]
O
Ca-ATPase SERCA2 [23%]
Ca-ATPase SERCA1 [24%]
O
Na,K-ATPase α_2 (rat) [86%]
250-300 (Fish-Reptiles)
≈200 (Birds-Mammals)
≈50 (Rodents-Primates)
O
Na,K-ATPase α_1 (*Torpedo*) [87%]
O
Na,K-ATPase α_3 (human) [86%]
Na,K-ATPase α_3 (rat) [85%]
Na,K-ATPase α_1 (human) [100%]
Na,K-ATPase α_1 (rat) [97%]

volume. A plausible scheme would entail a combination of a H^+-extruding pump, an Na/H exchanger, and an anion channel to allow passive Cl^- fluxes, resulting in a net loss of NaCl. In addition to volume regulation, Glynn (1993) describes how pH regulation may also have served as a determinant of pump evolution. In this scenario the earliest pumps, driven by ATP or a similar compound, removed the H^+ generated during cellular metabolism. Light-driven, and later respiration-driven H^+ pumps probably evolved independently of these ancestral pumps and may have rendered them obsolete. It is plausible that the respiration-driven H^+ pumps responded to the new environment by operating in reverse. Using the energy of the electrochemical H^+ gradient to drive ATP synthesis, they would have functioned much like "modern" mitochondria and chloroplasts. This concept, like the scheme proposed in Fig. 1, argues in favor of a very early evolutionary divergence of the P-type ATPases from a common ancestor. Also, as mentioned above, these ATPases contain highly homologous regions of amino acids and are found in many species of bacteria, which supports suggestions of their primitive origins (see also Läuger, 1991). While the non-phosphorylating F- and V-type pumps of cellular organelles bear little resemblance to the P-type ATPases, the high degree of sequence homology between the main catalytic subunits of V- type (B, A) and F-type (α, β) ATPases (Nelson, 1992) suggests that they too evolved from a common ancestor.

During the evolution of ion transporters, certain events represented significant advancements in cells' abilities to regulate their ionic homeostases. One of these was the development of redox-coupled H^+ pumps, which led to the generation of large electrochemical potential differences (Wilson and Lin, 1980). Another, as explained by Läuger (1991), was the evolution of electrogenic ion transport. Acceleration of the charge-translocating steps of an electrogenic pump's reaction cycle by the transmembrane potential is kinetically more efficient than using a simple concentration gradient as the driving force. In addition, a low membrane conductance of the ion species involved permits the electrical gradient to build up much faster than the chemical gradient. The resulting electrochemical

gradient also provides the driving force for secondary active transport. A third major evolutionary advancement in ion pumping was the appearance of coupled Na^+ and K^+ transport, which paralleled the appearance of excitable cells approximately 600 million years ago (Rossier *et al.*, 1987).

1.2. THE NA,K-ATPASE

1.2.1. Discovery of the Na,K-ATPase

The existence of a mechanism fulfilling the function of the Na,K-ATPase (EC 3.6.1.37) had been anticipated many years in advance of its discovery in 1957 by Skou (for historical perspectives, see Post, 1989; Skou, 1989; Glynn, 1993). In 1902, Overton (1902) recognized that heart muscle maintains the same levels of Na^+ and K^+ during a person's lifetime, even though Na^+ enters and K^+ leaves with each contraction. Thus, he hypothesized that there must be some mechanism to restore the ionic equilibrium. Later, Steinbach (1940) observed that a thermodynamically uphill loss of Na^+ and gain of K^+ occurred when frog muscles that had been stored cold were warmed to 37 °C. He also noted that Na^+ extrusion required K_{ext}. Shortly thereafter Dean (1941) proposed the hypothesis that "pumps" specific for Na^+, K^+, or both ions must exist. Using erythrocytes, Danowski (1941) and Harris (1941) demonstrated the important role of glucose in similar uphill ion transport processes.

The first experiments identifying this pumping mechanism as an Na^+, K^+-dependent ATPase were published by Skou (1957). He demonstrated that a substantial portion of ATP hydrolysis in crab nerve homogenates required Na^+, K^+, and Mg^{2+} for optimal activity. Furthermore, he ascribed the location of this activity to the sheath or membrane of the nerves. Post *et al.* (1960) and Dunham and Glynn (1961), working with similar ATPase activity in the membranes of human erythrocytes, recognized the relationship between this activity and that of ion transport in intact erythrocytes. These experiments also demonstrated that this activity could be inhibited by cardiac glycosides,

which correlated nicely with a previous report by Schatzmann (1953) describing the specific inhibition of Na^+ and K^+ active transport in red blood cells by cardiac glycosides. The definitive identification of the Na,K-ATPase as the ouabain-sensitive molecular entity responsible for active Na^+, K^+ transport occurred more than a decade later when the techniques for reconstitution of detergent-solubilized enzymes into proteoliposomes became available. Using this methodology on Na,K-ATPase purified from canine kidney renal medulla, Goldin and Tong (1974) demonstrated ATP-dependent Na^+ transport, and later (Goldin, 1977), coupled Na^+ and K^+ transport in proteoliposomes. Hilden *et al.* (1974) and Hilden and Hokin (1975), using the enzyme purified from dogfish (*Squalus acanthias*) rectal glands, also characterized alkali cation pumping in reconstituted vesicles.

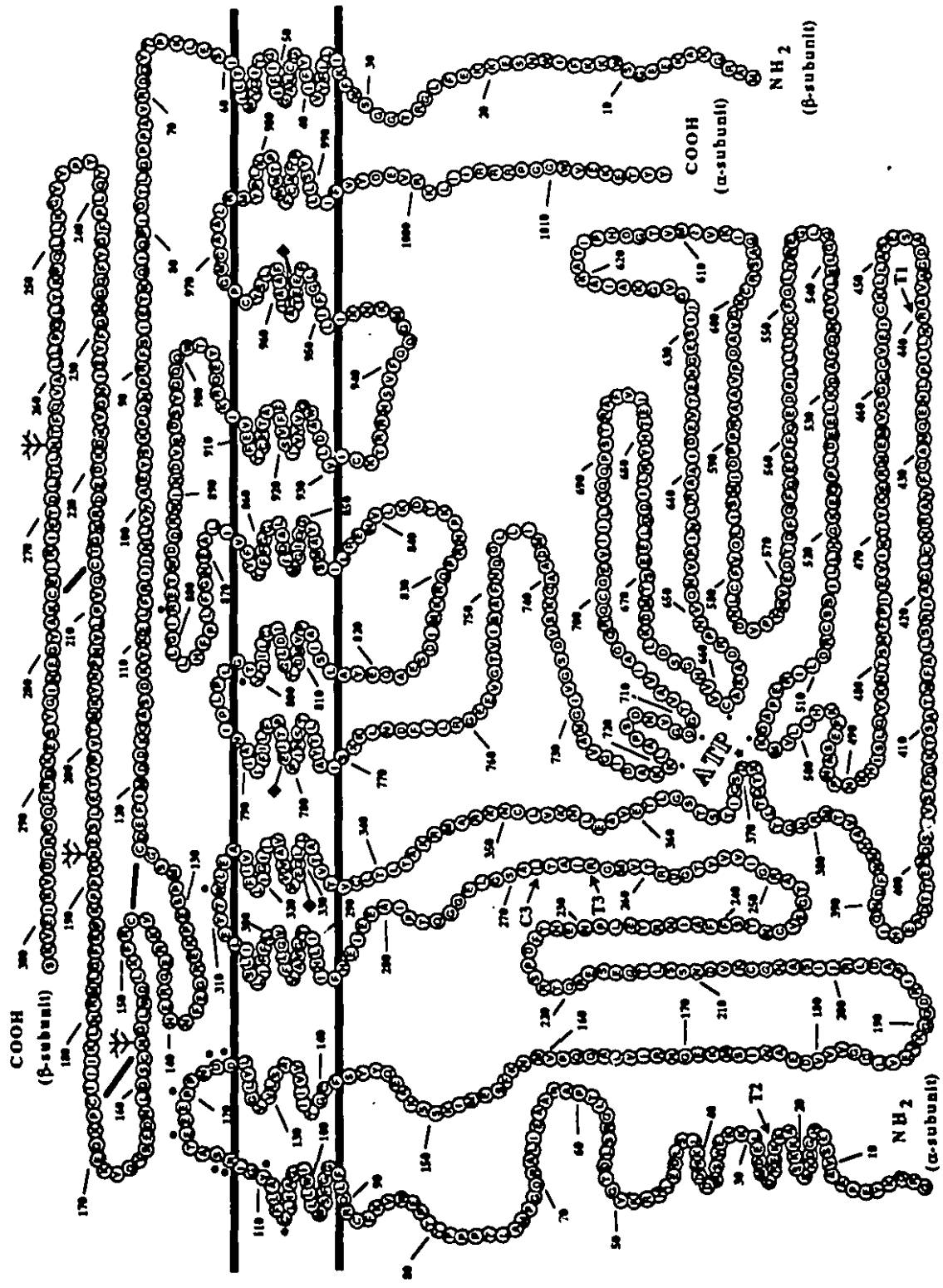
1.2.2. Structural features of the Na,K-ATPase

1.2.2.a. Alpha subunit structure and topology

The Na,K-ATPase, shown in Fig. 2, is a heterodimeric protein consisting of α catalytic subunits and β subunits in a 1:1 stoichiometry (for reviews, see Jørgensen and Andersen, 1988; Lingrel *et al.*, 1990; Horisberger *et al.*, 1991c; Vasilets and Schwarz, 1993; Mercer, 1993). The α subunit, with multiple membrane-spanning domains, can be categorized as a class IV protein according to the criteria of von Heijne and Gavel (1988). It has an approximate molecular weight of 80-120 kiloDaltons (kDa) as determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Kyte, 1971; Craig and Kyte, 1980). In spite of the presence of several intracellular N-glycosylation consensus sequences (Vasilets and Schwarz, 1993) and evidence that they may, in fact, be associated with small carbohydrate moieties (Pedemonte and Kaplan, 1992), the α subunit is not considered to be a glycoprotein. All of the enzymatic functions, as well as important substrate and inhibitor binding sites, have been assigned to the α subunit (see Glynn, 1985 and Sections 1.2.2.d. and 1.2.3.g.).

FIG. 2. **Proposed transmembrane structure of the rat Na,K-ATPase α_1 isoform.**

Based on Lingrel et al. (1990, 1994), but modified to correspond to the deduced rat amino acid sequences for the α and β subunits. The lipid membrane is represented by the two solid bars drawn across the top of the figure, with the uppermost region corresponding to the extracellular domains. Also shown are the ATP binding region (▪), the catalytic phosphorylation site (*), four tryptic cleavage sites (arrows), residues implicated in ouabain or cardiac glycoside binding (•), and glutamate residues implicated in cation binding (◊). Three branched structures on the β subunit represent N-linked glycosylation sites.


COOH
(β-subunit)
COOH
(α-subunit)
NH2
(β-subunit)
NH2
(α-subunit)
ATP
T1
T2
T3
C3

The first reported amino acid sequences of the α subunit were deduced from sheep kidney (Shull *et al.*, 1985) and *Torpedo californica* electroplax (Kawakami *et al.*, 1985) cDNA. Amino acid sequences of many species have subsequently been published (for specific references, see Lingrel *et al.*, 1990; Vasilets and Schwarz, 1993). On average, the deduced amino acid sequence homology among the α subunits of most species is approximately 90% (see Horisberger *et al.*, 1991c). Among the Na,K-ATPase genes known to code for pump isoforms (see Section 1.2.4.c.), sequence homologies vary considerably (see Vasilets and Schwarz, 1993). For example, each of the corresponding rat and chicken α subunit isoforms is 93-96% homologous (Mercer, 1993), whereas the homology between α subunit isoforms within each species is only 80-86% (Takeyasu *et al.*, 1990; Horisberger *et al.*, 1991c).

As mentioned in Section 1.1.2.b., the α subunit of the Na,K-ATPase has an intracellular N-terminus (see, for example, Jørgensen, 1975). Contrary to an earlier report that suggested an extracellular location for the C-terminus (Ovchinnikov *et al.*, 1987), more recent findings support an intracellular location (Krämer *et al.*, 1990; Antolovic *et al.*, 1991; Modyanov *et al.*, 1992; Karlish *et al.*, 1993; Ning *et al.*, 1993). In addition, a fusion protein, consisting of the Na,K-ATPase α subunit joined through its C-terminus to the corresponding N-terminus of the β subunit, formed properly oriented and functional pumps. This finding strongly supports structural models assigning a cytoplasmic location to the C-terminus of the α subunit (Emerick and Fambrough, 1993).

Assuming that both termini of the pump are intracellular, the number of transmembrane domains must be even. Hydropathy plots suggest the presence of 6-10 such regions, but ambiguities in their interpretation preclude a definitive assessment (see Crimi and Esposti, 1991). There is, however, mounting evidence favoring models with ten transmembrane domains. As discussed in Section 1.1.2.b., the high level of sequence homology between the Na,K-ATPase and the H,K- and Ca-ATPases, especially within the predicted transmembrane domains, implies that they share similar structures (Green and

Stokes, 1992). This view is supported by similarities observed in the two-dimensional structural analyses of crystalline arrays of the Na,K- and Ca-ATPases (Jørgensen and Andersen, 1988; Taylor and Varga, 1994). Other evidence in favor of a 10-helix model is the localization of a domain in the C-terminus of the Na,K-ATPase that is phosphorylated by protein kinase A (PKA), an intracellular protein (Fisone *et al.*, 1994). According to 8-helix models, this region would have an extracellular location (see Fig. 3 in Lingrel *et al.*, 1990). Similarly, ouabain binding (Schultheis *et al.*, 1993) and α/β subunit assembly (Lemas *et al.*, 1994), both of which occur at the extracellular face of the pump, are associated with C-terminal regions, which, according to 8-helix models, would be intracellularly located. Finally, information obtained from "19-kDa" tryptic digestion fragments of the α subunit of Na,K-ATPase (Karlish *et al.*, 1993) supports a 10-transmembrane domain model of this peptide.

1.2.2.b. Beta subunit structure and topology

The β subunit of the Na,K-ATPase, which has the characteristics of a class II protein (von Heijne and Gavel, 1988), has a single predicted transmembrane domain and is considerably smaller than the α subunit peptide (see Fig. 2). The molecular weight of this protein as analyzed by SDS-PAGE varies among tissues and species largely due to differential glycosylation at various N-linked sites. The size of the unglycosylated protein is approximately 35 kDa (for further discussion and references, see Sweadner, 1989; Geering, 1990). Apart from the transmembrane region and a small, cytoplasmic N-terminus, the bulk of the peptide is extracellular. This latter region contains several disulfide bridges (Esmann, 1982), the disruption of which abolishes ATPase activity (Kawamura and Nagano, 1984; Kawamura and Noguchi, 1991) and substantially reduces Rb^+ occlusion (Lutsenko and Kaplan, 1993).

The earliest reported amino acid sequences of the β subunit were deduced from the cDNAs of sheep (Shull *et al.*, 1986b), rats (Mercer *et al.*, 1986), humans (Kawakami *et*

al., 1986), electric eels (Noguchi *et al.*, 1986), and pigs (Ovchinnikov *et al.*, 1986). Among mammals, the degree of amino acid sequence homology is at least 90%, but it decreases to as little as 30% when comparing mammals with insects (Vasilets and Schwarz, 1993). There is a major group of residues in one of the putative transmembrane domains, as well as numerous smaller regions containing up to ten residues, that are conserved among different species (Mercer, 1993). Some of these regions may contain recognition signals for endocytosis (Gottardi and Caplan, 1993) or the assembly of β subunits with α subunits (Geering *et al.*, 1993).

Until recently, the only known role of the β subunit was that of chaperone to the α subunit during the delivery of functional pumps to the plasma membrane (Geering *et al.*, 1989; McDonough *et al.*, 1990; see also Section 1.2.2.e.). However, there is now evidence that the β subunit is involved in the binding (Lutsenko and Kaplan, 1992, 1993) and transport (Jaisser *et al.*, 1992; Eakle *et al.*, 1992, 1994) of K^+ (or Rb^+). In addition, the carbohydrate groups of the β subunit may be important to the function of the Na,K-ATPase. Although not required for the expression of active enzymes at the cell surface, as demonstrated in cells treated with the glycosylation inhibitor tunicamycin (Tamkun and Fambrough, 1986; Takeda *et al.*, 1988; Zamofing *et al.*, 1988, 1989), their structural similarities with the glycan moieties of cell adhesion molecules (Treuheit *et al.*, 1993) suggest that they may function as intermolecular or intercellular recognition signals. The adhesion molecule on glial cells (AMOG), for example, is identical to the β_2 subunit isoform (Gloor *et al.*, 1990). In addition, Müller-Husmann *et al.* (1993) have recently demonstrated that the β_2 isoform can induce neurite outgrowth *in vitro*, implying that it participates in the coordination of organ development during ontogeny.

1.2.2.c. Gamma subunit

There appears to be a third subunit, designated the γ subunit, which associates with active Na,K-ATPases *in vivo*. Several reports involving the use of photoactivatable

ouabain analogues suggested that a small peptide is included in the ouabain binding site (Forbush *et al.*, 1978; Lowndes *et al.*, 1984). Collins and Leszyk (1987) later demonstrated that this peptide was distinct from the α or β subunits. Mercer *et al.* (1993) recently cloned the γ subunit of sheep, rats, mice, and cows. According to the amino acid sequence, it is a small, amphipathic peptide (≈6 kDa) with a single predicted transmembrane domain. Two mRNAs are expressed in a tissue-specific fashion from the rat γ subunit gene, most likely representing altered gene transcription or mRNA polyadenylation sites (Mercer *et al.*, 1993). This peptide appears to be expressed in most tissues, with the highest levels found in kidney and spleen. Using dog or sheep kidney, Mercer *et al.* (1993) demonstrated that this peptide co-immunoprecipitates with the α and β subunits and co-localizes with the α subunit in immunofluorescent micrographs.

Currently, the function of the γ peptide is unknown. Selective removal of this component using non-ionic detergents did not alter the Na,K-dependent ATP hydrolysis of avian salt gland enzyme preparations (Hardwicke and Freytag, 1981). Moreover, its presence is not required for enzyme activity in a variety of exogenous protein expression systems (see, for example, De Tomaso *et al.*, 1991; Eakle *et al.*, 1992).

Small, hydrophobic peptides are associated with other ATPases such as the H-ATPase (Blondin, 1979; Navarre *et al.*, 1992) and the Ca-ATPase (MacLennan *et al.*, 1972). One of these peptides, the 6-kDa phospholamban, can be reversibly phosphorylated by cyclic adenosine monophosphate- (cAMP-) dependent protein kinases (James *et al.*, 1989). In its phosphorylated state, it binds to and inhibits the SR Ca-ATPase. Another small, peptidic regulator of the Ca-ATPase, which can also be phosphorylated by protein kinases, is the recently discovered phospholemman (Palmer *et al.*, 1991). Apparently acting as a chloride channel (Moorman *et al.*, 1992), it has some sequence homology with the γ subunit near the transmembrane region (Mercer *et al.*, 1993).

1.2.2.d. Locations of important binding sites

The catalytic phosphorylation of the Na,K-ATPase occurs at a specific aspartyl residue (Post and Kume, 1973; Bastide *et al.*, 1973) located within a highly conserved region of the N-terminal half of the catalytic α subunit (see Vasilets and Schwarz, 1993). Site-specific mutagenesis experiments have confirmed that this aspartyl residue is essential for Na,K-ATPase activity (Ohtsubo *et al.*, 1990). The structure and location of the rest of the ATP-binding site have been deduced by combining results from proteolytic cleavage studies with those of labeling experiments using various fluorescent or covalently modifying ATP analogues such as fluorescein isothiocyanate (FITC), 5'-(*p*-fluorsulfonyl)benzoyladenosine (FSBA), pyridoxal 5'-phosphate (PLP), and *g*-(4-*N*-2-chloroethyl-*N*-methylamino)benzolamide-ATP (ClR-ATP) (for reviews and further references, see Jørgensen and Andersen, 1988; Lingrel *et al.*, 1990; Pedemonte and Kaplan, 1990; Mercer, 1993).

The long-standing controversy regarding the issue of distinct high- and low-affinity ATP binding sites seems to have been resolved in favor of one site per α subunit. Research from Kaplan's laboratory (Ellis-Davies and Kaplan, 1990; Kaplan, 1991) showed that the same lysine residue governs both high- and low-affinity ATP binding. However, it is still possible that simultaneous high- and low-affinity sites may arise through the complex interactions of α/β protomers (see Section 1.2.2.f.) or the effects of various ligands on the Na,K-ATPase (Ward and Cavieres, 1993).

As shown in Fig. 2, several regions of the large intracellular loop of the α subunit peptide comprise the nucleotide binding site, suggesting that it arises through a complex, tertiary-folding process. The presence of a hydrophobic "pocket" into which the adenine and ribose groups of the nucleotide can insert appears to be a general feature of most ATPases (Taylor and Green, 1989). Lane *et al.* (1993) recently mutated several of the residues of the rat α_1 catalytic subunit that, on the basis of the aforementioned chemical labeling experiments, have been identified as part of the ATP binding site. The only

mutants that resulted in a loss of ATPase activity were those involving a pair of nearly adjacent aspartate residues within the C-terminal half of peptide. They were Asp^{712} and Asp^{716}, one or both of which is also labeled by the ATP analogue ClR-ATP. The authors suggested that these were the only amino acids of those examined that were involved in the E_1P-E_2P conformational transition (see Section 1.2.3.d.).

The cation binding sites, since they are intimately involved in ion translocation, are presumably located near or within the transmembrane portion of the Na,K-ATPase. Glynn first demonstrated that cations can be occluded within the enzyme (Beaugé and Glynn, 1979; Glynn *et al.*, 1984). To more precisely define the region(s) involved in cation occlusion, studies were carried out using selective proteolysis and site-specific probes such as organic cations (amines), fluorescent and nuclear magnetic resonance (NMR) probes, and group-specific chemical modifiers (for reviews, see Glynn and Karlish, 1990; Pedemonte and Kaplan, 1990). The results of these findings are discussed further in Section 1.2.3.e.

The α subunit of the Na,K-ATPase also contains distinct proteolytic sites which vary according to the conformation of the enzyme (see Jørgensen and Andersen, 1988). There are three conformation-specific sites for trypsin at residues Arg^{438} (T_1), Lys^{30} (T_2), and Arg^{262} (T_3), as well as one for chymotrypsin at Leu^{266} (C_3). The functional significance of these sites is explained in Section 1.2.3.d.

Cardiac glycosides such as ouabain bind specifically to the extracellular and perhaps intramembrane portion of the Na,K-ATPase. Although these compounds clearly bind to the α subunit (see, for examples, Ruoho and Kyte, 1974; Forbush *et al.*, 1978; Price and Lingrel, 1988), they may also associate with the β and γ subunits (Forbush *et al.*, 1978; Hall and Ruoho, 1980). Based on the structure of cardiac glycosides, the ouabain binding site can be divided into three domains: a steroid region, a lactone ring region, and a sugar residue-binding area (see Thomas, 1981). One of the major determinants of ouabain binding affinity, which varies considerably among tissues and species (see Section

1.2.4.a.), is the amino acid composition of the first extracellular domain of the α subunit. Using site-specific mutagenesis of the sheep α_1 isoform, Price and Lingrel (1988) showed that converting certain membrane-proximal neutral amino acids of this segment (see Fig. 2) to charged residues greatly decreased the ouabain sensitivity of this enzyme. Other residues predicted to lie within the first membrane-spanning region also affect ouabain binding (see O'Brien *et al.*, 1993). The involvement of these residues supports the notion that steroid binding could occur in a hydrophobic pocket which is, presumably, near or partly within the membrane (Fortes, 1977). Furthermore, the finding that cardiac glycoside sugar moiety binding does not occur at this region of the α subunit supports the notion that it participates in steroid ring binding (O'Brien *et al.*, 1993). Additional portions of the α subunit which appear to participate in ouabain binding (see Fig. 2) include the second (Lingrel *et al.*, 1990; Canessa et al., 1993), third (Burns and Price, 1993), and fourth (Schultheis *et al.*, 1993) extracellular domains. Also consistent with these results is evidence that low-affinity ouabain binding is mediated by the C-terminal region of an H,K/Na,K-ATPase chimera (Blostein *et al.*, 1993).

1.2.2.e. Biosynthesis, assembly, and degradation of the Na,K-ATPase

As with most membrane proteins, synthesis of the subunits of the Na,K-ATPase begins in the cytoplasm on free ribosomes. Instead of a cleavable N-terminal signal sequence, the α subunit relies on the use of alternating insertion sequences and "stop transfer" signals to account for its multiple membrane crossings (see Sabatini *et al.*, 1982). The β subunit has one uncleaved signal sequence, resulting in a peptide with a single transmembrane domain and a cytoplasmic N-terminus. Deletion mutants of this subunit retain the ability to become inserted into the membrane, demonstrating that the actual signal sequence resides in the membrane-spanning domain (Kawakami & Nagano, 1988; Fambrough *et al.*, 1991).

There are several posttranslational modifications of pump subunits which occur as these peptides are synthesized, including β subunit glycosylation (reviewed by Geering, 1990, 1991) and, in some species, the removal of the first five N-terminal amino acids from the α subunit (Shull *et al.*, 1985; Ovchinnikov *et al.*, 1986). Another, less clearly defined posttranslational event appears to occur during the association or assembly of the α and β subunits. In contrast to the transit time of the mature pump to the cell surface, which requires about 45 minutes from the beginning of its synthesis (Tamkun and Fambrough, 1986; Caplan *et al.*, 1990), this event occurs within 10-20 minutes after peptide synthesis when the subunits are still in the ER or trans-Golgi compartments. It is characterized by an increased resistance of the α subunit to trypsinolysis as well as by the acquisition of cation-dependent conformational states (Geering *et al.*, 1987). Other evidence for early posttranslational processing was reported by Caplan *et al.* (1990), who showed that (Na^+, Mg^{2+}, ATP)-dependent binding of a fluorescent analogue of ouabain appeared only 10-20 minutes after the start of peptide synthesis. According to the authors, this type of binding suggests that the pump, still within the ER or Golgi network, is already capable of cycling between E_1P and E_2P, and can probably effect ATP hydrolysis (see Section 1.2.3.a.).

The locations of the determinants of Na,K-ATPase subunit assembly have recently been revealed. Based on information obtained from H,K/Na,K-ATPase (Gottardi and Caplan, 1993) and Ca/Na,K-ATPase chimeras (Lemas *et al.*, 1992), the assembly signal domain of the Na,K-ATPase α subunit was localized to the C-terminal region. Lemas *et al.* (1994) further restricted the assembly domain of this subunit to the C-terminal half of the fourth extracellular region. With respect to the β subunit, Renaud *et al.* (1991) found that deleting the N-terminus and even a small portion of the transmembrane region did not prevent the association of avian subunits expressed in murine L-cells. This finding suggests that the important assembly determinants of this subunit are also contained in the extracellular domain. In contrast to these findings, however, Jaunin *et al.* (1993) reported that the transmembrane region is important for the assembly efficiency of amphibian pump

subunits expressed in *Xenopus laevis* oocytes. Hence, there are probably species-specific differences in subunit assembly.

Hamrick *et al.* (1993), further exploring the nature of the Na,K-ATPase subunit pairing domains, demonstrated that while the cytoplasmic and transmembrane domains alone were not able to support the assembly of chicken pump subunits expressed in HeLa cells, β subunit chimeras containing the first 100 or so N-terminal amino acids of the nearly 250 extracellular residues could assemble with α subunits. In addition, these authors found that C-terminal deletions of as few as four amino acids prevented pumps from being expressed at the plasma membrane, suggesting that these residues play an important role in subunit folding or maturation. According to recent results from Geering's laboratory, a single proline residue (Geering *et al.*, 1993) and several hydrophobic amino acids (Beggah *et al.*, 1993) in the C-terminal region of the *Xenopus laevis* Na,K-ATPase β subunit significantly influence pump assembly and function as assessed by ouabain binding and Rb^+ uptake assays in oocytes.

In exogenous protein expression systems (see Section 1.2.4.e.), it appears that a variety of α/β subunit combinations produce functional pumps at the cell surface. For example, the HeLa cell transfectants of Jewell and Lingrel (1991) show that all three of the rat α subunit isoforms can assemble with the endogenous HeLa β_1 isoform to form functional pumps. Similarly, using insect cells, the three rat α subunit isoforms have been expressed with the β_1 isoform, resulting in ouabain sensitive ATPase and phosphoenzyme (EP) activity in these normally Na,K-ATPase-deficient cells (Blanco *et al.*, 1993; DeTomaso *et al.*, 1993). Very recent results indicate that β_2 subunits can also pair with each of the α subunit isoforms (Blanco *et al.*, 1994), although full functional activity has not yet been verified. Subunit isoform combinations in oocytes showed that the *Xenopus* α_1 subunit can support Rb^+ uptake when paired with either the *Xenopus* β_1 or β_3 subunits (Horisberger *et al.*, 1991a), the mouse β_2 subunit (Schmalzing *et al.*, 1992), or the H,K-ATPase β subunit (Horisberger *et al.*, 1991b; Noguchi *et al.*, 1992). Pairing of the β

subunit of the H,K-ATPase with the sheep α_1 or rat α_3 subunits was also demonstrated in yeast (Eakle *et al.*, 1992). However, the converse pairing of the α subunit of the rat H,K-ATPase plus the β subunit of the Na,K-ATPase was not successful in COS-1 cells (Gottardi and Caplan, 1993).

The α/β assembly required for the completion of the posttranslational processing and cell surface expression of pumps is a rapid event, occurring either during or immediately after synthesis (Fambrough *et al.*, 1991). Unassembled or improperly folded subunits remain for a period in the ER (Takeyasu *et al.*, 1988; Jaunin *et al.*, 1992), after which they are degraded (Ackermann and Geering, 1990). These subunits may be complexed with a resident ER binding protein (BiP), which is known to keep improperly folded proteins from leaving this organelle (see Pelham, 1989).Working with *Xenopus laevis* oocytes, Jaunin *et al.* (1993) demonstrated that the extracellular domain of the Na,K-ATPase β subunit contains a signal for ER retention. However, β subunits of the H,K-ATPase can reach the plasma membrane (Jaunin *et al.*, 1993; Gottardi and Caplan, 1993) as can monomeric Na,K-ATPase β subunits in some expression systems (Takeyasu *et al.*, 1987; Blanco *et al.* 1993).

In most protein expression systems, the expression of α subunits at the plasma membrane requires pairing with β subunits (see, for examples, Noguchi *et al.*, 1987; Horowitz *et al.*, 1990a). Similarly, in some cultured cell lines, there is a correlation between the levels of β subunit expression and plasma membrane Na,K-ATPase activity (see, for example, Lescale-Matys *et al.*, 1990). An exception, however, is the insect Sf-9 cell, which targets immunologically reactive but non-functional α subunits to the plasma membrane in the absence of β subunit expression (Blanco *et al.*, 1993; DeTomaso *et al.*, 1993). Presumably, this event reflects slight differences in the intracellular transport and sorting machinery of mammalian and insect cells. Other aspects of Na,K-ATPase targeting to membranes are discussed in Section 1.2.5.f.

The degradation of the Na,K-ATPase can proceed via several pathways. The removal of mature heterodimers from the plasma membrane is most likely mediated by endocytosis (Pollack *et al.*, 1981) and occurs coordinately and equally for the two subunits (Fambrough *et al.*, 1991; Lescale-Matys *et al.*, 1993). Although the molecular signals for endocytosis of the Na,K-ATPase have not yet been identified, Gottardi and Caplan (1993) have suggested that there may be one in the cytoplasmic domain of the β subunit, at least for the H,K-ATPase. The possibility that cytoskeletal interactions may affect endocytosis must also be considered (see Section 1.2.5.d.). Immature, unassembled subunits in the ER are also degraded. In a pig kidney cell line, Lescale-Matys *et al.* (1993) found a biphasic turnover rate of β subunits compared to a monophasic rate for α subunits. The authors proposed that the rapid phase represents the degradation of nascent β subunits (still in the ER), while the slow phase, similar to that of the α subunit, represents the degradation of mature α/β heterodimers. Some cells, such as muscle and oocytes, have a rapid mechanism for removing pumps from membranes (see, for example, Takeyasu *et al.*, 1989; Schmalzing *et al.*, 1991). This process, possibly reversible, may represent one way in which cells regulate their Na,K-ATPase activity (see Section 1.2.5.e.).

1.2.2.f. Tertiary and quaternary structure of the Na,K-ATPase

The secondary and tertiary structure of the Na,K-ATPase has been reviewed in detail by Jørgensen and Andersen (1988). Low-resolution diffractional analyses of the crystallized Na,K-ATPase indicate that the bulk of the protein is located in the cytoplasm (see, for example, Hebert *et al.*, 1985; Skriver *et al.*, 1992; Taylor and Varga, 1994) These findings are similar to those reported for the Ca-ATPase (see, for example, Taylor *et al.*, 1986). The membrane-embedded region ranges from 15-40% of the total mass of the protein, depending on the conditions of crystallization (Hebert *et al.*, 1990). The relatively small extracellular portion of the crystallized Na,K-ATPase (<30% of the total mass) probably represents the β subunit (Taylor and Varga, 1994).

Due to uncertainties regarding the algorithms used for the prediction of secondary structure (see Wallace *et al.*, 1986), it is not yet possible to determine whether the transmembrane segments consist of α helices, β sheets, or a mixture of both. The structure of these regions may be stabilized by disulfide bridges and through lipid-protein interactions at some of the charged residues (Jørgensen and Andersen, 1988). There is also a portion of the ATP binding region which contains a conserved glycine-rich motif, possibly representing a flexible segment involved in conformational transitions (Jørgensen and Andersen, 1988). Green and Stokes (1992) have recently proposed a general structural model for P-type ATPases which attempts to show the relationships between various domains (*e.g.*, nucleotide binding, phosphorylation, cation binding) and suggest how conformational changes (see Section 1.2.3.d.) may be transmitted from one region of these enzymes to another. A related model, specific for the Na,K-ATPase and including a role for the β subunit, has been suggested by Lutsenko and Kaplan (1994).

The issue of pump subunit oligomerization (or quaternary structure) remains unresolved (for discussion and references, see Glynn, 1985; Nørby, 1987; Jørgensen and Andersen, 1988; Mercer, 1993). Evidence obtained from diffractional analyses of crystalline arrays (see, for example, Jørgensen *et al.*, 1982; Skriver *et al.*, 1992) have suggested an $(\alpha\beta)_2$ structure. Other studies, involving electron microscopy (Skriver et al., 1980), chemical cross-linking reagents (Craig and Kyte, 1980; Huang and Askari, 1981), neutron scattering (Pachence *et al.*, 1983), radiation inactivation (Ellory *et al.*, 1979; Nørby and Jensen, 1989) and analytical ultracentrifugation (Esmann *et al.*, 1979), support the notion that this enzyme may exist and function as $(\alpha\beta)_2$ dimers. On the other hand, however, Martin and Sachs (1992) recently reported that α subunit dimers could not be observed following chemical crosslinking experiments with human erythrocytes. This may reflect the association of the Na,K-ATPase in these cells with components of the membrane cytoskeleton (see Section 1.2.5.d.). Evidence for Na,K-ATPase dimerization based on functional studies is considered in the following section.

1.2.3. Functional aspects of the Na,K-ATPase

1.2.3.a. Reaction cycle

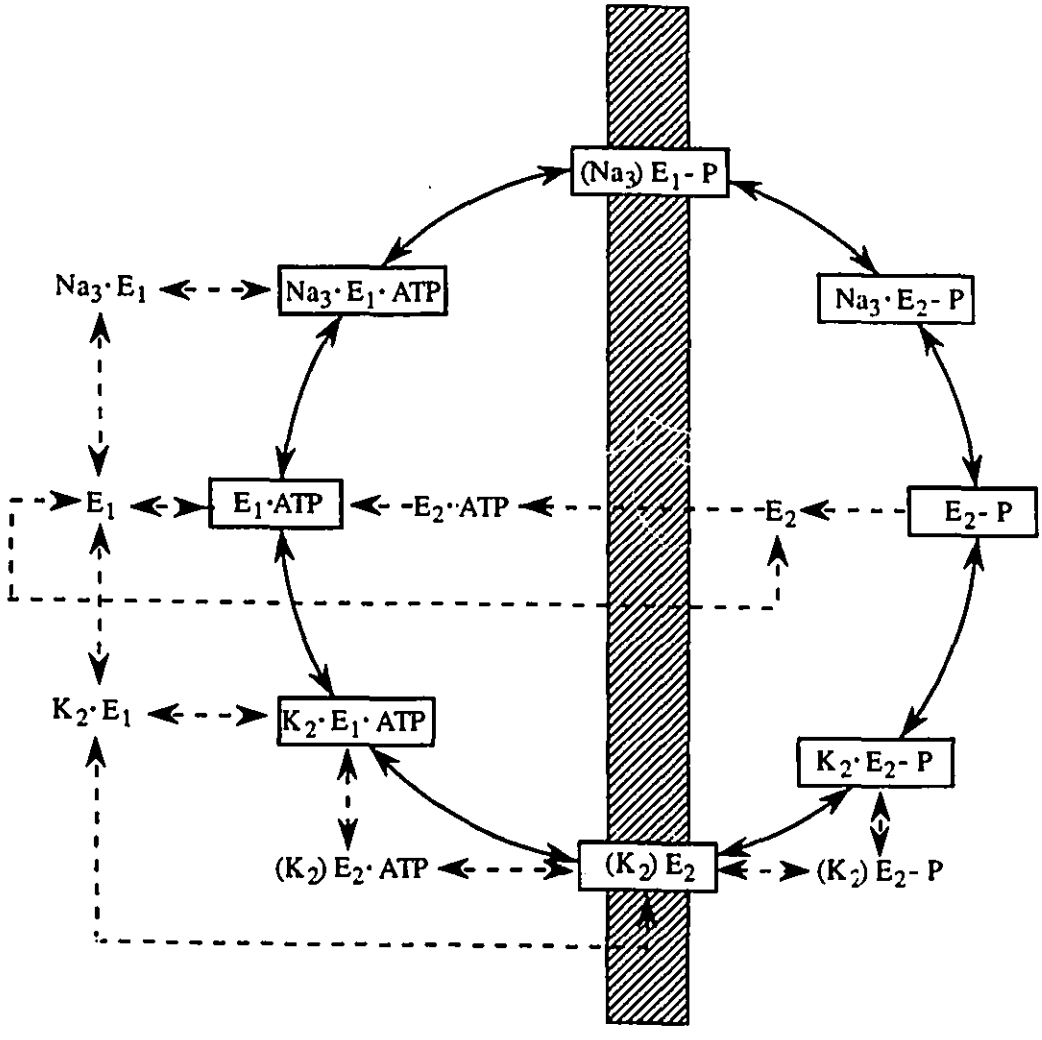
The Albers-Post scheme, named after the two researchers from whose laboratories it originated (Albers, 1967; Post *et al.*, 1972), depicts the mechanism by which the Na,K-ATPase carries out Na^+ and K^+ translocation and ATP hydrolysis. While the basic elements of this model, namely the existence of two conformations of both the phospho- and dephosphoenzymes, are still valid, the scheme has been modified to account for the various partial reactions which the enzyme is capable of mediating (see Section 1.2.3.b.). Also, the model has been changed to accommodate the existence of at least one more EP form, E^*P (see, for example, Fukushima and Nakao, 1981; Yoda and Yoda, 1982; Nørby *et al.*, 1983), as well as enzyme conformations containing occluded cations (see Section 1.2.3.e. and Glynn, 1985, for a detailed review of this model).

As shown in the modified scheme depicted in Fig. 3, the Na,K-ATPase can exist in one of two distinct conformations, E_1 or E_2, which is either phosphorylated or dephosphorylated at the active site aspartyl residue. In the classical first step of the cycle, the substrates ATP and Mg^{2+}, along with three Na_{in}, bind with high affinity to the cytoplasmic surface of the enzyme in the E_1 conformation. Phosphorylation of a β-aspartyl carboxyl group by the γ phosphate of ATP causes Na^+ to be occluded within the transmembrane interior region of the pump. The subsequent release of ADP is followed by the extracellular release of Na^+ and a conformational change of E_1P to E_2P. Next, 2 K^+ bind to E_2P, leading to the rapid hydrolysis of the aspartyl phosphate group and K^+ occlusion. A spontaneous conformational change to E_1K then occurs, releasing K^+ to the intracellular side. This last step is significantly accelerated by ATP binding to a low-affinity site on E_2P.

Under optimal substrate conditions (particularly at high ATP levels), the rate limiting step of the cycle, Na^+ release to the extracellular milieu, is two to three times

FIG. 3. **Reaction mechanism of the Na,K-ATPase.**

Modified Albers-Post reaction mechanism redrawn from Läuger (1991). The normal, physiological pathway of the enzyme is shown as a circle. The hatched bar represents the cell membrane. Events occuring to the right of the bar depict reactions in which the enzyme's cation binding sites are oriented toward the extracellular surface, and those to the left of the bar reactions in which the cation binding sites are oriented toward the cytoplasmic surface. Enzyme forms with parentheses represent those in which the transported cations are occluded.


(Na3) E1 - P
Na3 · E1
Na3 · E1 · ATP
Na3 · E2 - P
E1
E1 · ATP
E2 · ATP
E2
E2 - P
K2 · E1
K2 · E1 · ATP
K2 · E2 - P
(K2) E2 · ATP
(K2) E2
(K2) E2 - P

slower than the rate of K^+ deocclusion at the intracellular surface (Läuger, 1991), thereby favoring a buildup of the E_1P form of the phosphoenzyme. Consequently, the Na,K-ATPase is more likely to be found in the E_1P conformation than the E_2P conformation. The steady-state equilibrium distribution of the enzyme is also driven "forward" through the cycle to E_1P by the stimulatory effect of ATP (acting at the low-affinity site) on K^+ deocclusion. However, when the ATP concentration is low, K^+ deocclusion becomes the rate-limiting step, shifting the steady-state equilibrium distribution of the enzyme toward E_2P.

The nature of the kinetic mechanism which best describes the ion transport sequence of the Na,K-ATPase has been debated for many years. The two major candidates are "simultaneous" and "sequential" ion binding and transport. In the "simultaneous" model, ion transport would only proceed following the simultaneous binding of Na^+ and K^+ to the enzyme (see Sachs, 1979). Logically, this implies that there must be two distinct cation binding sites: one intracellular, with a high affinity for Na^+; the other extracellular, with a high affinity for K^+. In the second model, sometimes referred to as a "ping-pong" mechanism (see Cleland, 1963), ions would be transported in an alternating fashion, possibly using the same binding sites. Due to the existence of uncoupled cation transport (see Section 1.2.3.b.), where either Na^+ or K^+ can be transported apparently without an exchanging cation (at least near pH 7.4), it has not been possible to discriminate between the two models using steady-state kinetic analyses (see Sachs, 1986a). However, by examining the competition of Na^+ with K^+ during K/K exchange (see Section 1.2.3.b.) in erythrocytes, Sachs (1986a, 1986b) has demonstrated that the reaction mechanism is consistent with ping-pong rather than simultaneous kinetics. In addition, the evidence from N,N'-dicyclohexylcarbodiimide (DCCD) labeling experiments (Shani-Sekler *et al.*, 1988) favors the existence of a single cation binding site, which again is more consistent with a ping-pong mechanism.

An unresolved aspect of Na,K-ATPase behavior which may complicate kinetic analyses concerns its subunit oligomerization status (see Section 1.2.2.f. for physical evidence supporting the existence of pump dimers). That the relative stoichiometry of the α and β subunits is 1:1 (see, for example, Peterson and Hokin, 1981) and that $\alpha\beta$ monomers of the Na,K-ATPase can support both ATPase activity and cation occlusion has been confirmed (Vilsen *et al.*, 1987). These findings are similar to those which show that the Ca-ATPase can also function as a monomer (Andersen *et al.*, 1982). However, evidence from a phenomenon known as "half-of-the-sites" behavior implies that, *in situ*, the pump behaves as an $(\alpha\beta)_2$ heterodimer (for further discussion, see Glynn, 1985; Plesner, 1987a, 1987b). In such studies, the enzyme appears to function normally, except that only half of the expected activity is observed given the number of subunits present. For example, the binding of two molecules of ouabain per molecule of EP suggests that the pump may operate such that only one of the two $\alpha\beta$ heterodimers is functional (Askari and Huang, 1981; Askari, 1987). In addition, evidence for negative cooperativity of ATP binding implies that interactions can occur between α subunit protomers (Ottolehghi and Jensen, 1983). Although a recent report has clearly shown that apparent negative cooperativity of ATP binding can occur in $\alpha\beta$ protomers (Ward and Cavieres, 1993), it still does not rule out the possibility that diprotomers may exist.

1.2.3.b. Catalytic activities and flux modes

Apart from the physiological mode of pumping three Na^+ out for two K^+ in as described above, the Na,K-ATPase is capable of a variety of other pumping and catalytic behaviors (for reviews see Anner, 1985; Glynn, 1985; Cornelius, 1991). As with the F_0F_1 ATPase family, the Na,K-ATPase can be made to operate in reverse, synthesizing ATP at the expense of downhill cation movements. This process, which requires high concentrations of intracellular K^+ ($[K]_{in}$) and high concentrations of extracellular Na^+ ($[Na]_{ext}$), was first demonstrated in erythrocytes (Garrahan and Glynn, 1966). Pump

reversal has also been recently reported in cardiac myocytes (Bahinski *et al.*, 1988). One of the most significant aspects of this finding, as stated previously for the F_0F_1-ATPases, is that none of the steps of the reaction cycle is poised very far from equilibrium.

In the presence of Na^+-containing, K^+-free media, two types of Na/Na exchange have been documented. In the absence of ADP, ATP hydrolysis is coupled to the exchange of one Na_{in} for one extracellular Na^+ (Na_{ext}), representing forward and reverse cycling of the Na^+ translocation pathway of the reaction sequence (Lee and Blostein, 1980). Extracellular Na^+ probably acts like K_{ext}, stimulating the K^+ translocation pathway of the reaction cycle, albeit with a much lower affinity. This process is electrogenic as shown by fluxes in reconstituted proteoliposomes (Apell *et al.*, 1990). When ADP is present, a different kind of Na/Na exchange occurs in which there is little or no ATP hydrolysis (Garrahan and Glynn, 1967a; Glynn and Hoffman, 1971; Cavieres and Glynn, 1979). This exchange displays a high affinity for Na_{in} and a low affinity for Na_{ext} and is sensitive to inhibition by oligomycin, which prevents the E_1P-E_2P conversion (see Section 1.2.3.g.). These data are consistent with the idea that this flux represents a simple reversal of the steps in the Na^+ translocation pathway of the reaction cycle. Associated with this latter type of Na/Na exchange is an ADP-ATP exchange which is, unexpectedly, accelerated by oligomycin (Blostein, 1970). This suggests that enzyme phosphorylation alone is not sufficient to transport Na^+, and that the E_1P-E_2P conformational change that is inhibited by oligomycin is also required.

In the presence of K^+-containing, Na^+-free media, the pump may engage in a K/K exchange. This flux mode, which has been studied in human red cells (see, for example, Glynn *et al.*, 1970; Simons, 1974; Sachs, 1986b) and reconstituted vesicles (see, for example, Hilden and Hokin, 1975; Karlish *et al.*, 1982), requires inorganic phosphate (P_i) and ATP to perform a one-for-one K^+ exchange. This process has a low affinity for intracellular K^+ (K_{in}) and a high affinity for K_{ext}, suggesting that K_{in} acts as a Na^+ congener to drive this partial reaction. Since ATP is not hydrolyzed, non-phosphorylating

analogues can also support this flux (Simons, 1975). In the absence of nucleotides and P_i, another kind of K/K exchange has been reported in reconstituted vesicles (Karlish and Stein, 1982) and in erythrocyte ghosts (Sachs, 1986b; Kenney and Kaplan, 1985). This process may represent a "slippage" of the pump, such that it acts somewhat like a K^+ carrier or exchanger (see Karlish and Stein, 1982; Läuger, 1991).

When both Na_{ext} and K_{ext} (as well as any other alkali cations) are absent, the pump mediates an efflux of two or three Na^+ for each ATP hydrolyzed, whereby there is no corresponding cation returned to the cytoplasm. This Na/O flux, originally referred to as "uncoupled Na^+ efflux" (Glynn and Karlish, 1976), was observed in erythrocytes (see, for example, Garrahan and Glynn, 1967b; Glynn and Karlish, 1976; Dissing and Hoffman, 1983) as well as in reconstituted vesicles (see, for example, Karlish and Kaplan, 1985; Cornelius, 1989). When the Na,K-ATPase operates by this pathway, E_2P slowly breaks down, spontaneously reverting to E_1 without passing through the K^+-related steps of the reaction cycle. In proteoliposomes containing *Torpedo californica* electroplax or kidney Na,K-ATPase, the process is electrogenic and the Na/O flux decreases as the acidity of the medium increases (Cornelius, 1989; Goldshleger *et al.*, 1990), possibly reflecting a transition from Na/O to Na/H exchange (see below). In erythrocytes, the Na/O flux is partially inhibited by low (5 mM) Na_{ext} (Garrahan and Glynn, 1967b; Glynn and Karlish 1976) and constitutes an electroneutral process coupled to a ouabain-sensitive anion efflux (Dissing and Hoffman, 1983). Furthermore, the fraction of this flux which is not inhibited by low Na_{ext} is coupled to phosphate released directly from the γ-phosphate of substrate ATP molecules (Marin and Hoffman, 1988). Dissing and Hoffman (1990) have recently carried out a detailed study of the stoichiometries and electrogenicities of the Na^+-dependent and Na^+-independent anion fluxes. The significance of anion-coupled transport is that the occlusion sites of the Na,K-ATPase may be capable of accommodating anions as well as cations. Alternatively, if anions are not occluded and transported at the same site as cations, it is possible that this efflux occurs through another membrane component that is

closely associated with the Na,K-ATPase — *e.g.*, the anion transporter band 3, or the putative *γ* subunit, behaving, like phospholemman, as an anion channel (see Section 1.2.3.c.).

Analogous to the uncoupled Na^+ flux, Sachs (1986b) has shown that the Na,K-ATPase can mediate an uncoupled K^+ flux in the absence of intracellular alkali cations. Since the Na^+-dependent pathways of the reaction sequence are not involved, this flux, like K/K exchange, requires only a non-phosphorylating analogue of ATP plus P_i.

Protons appear to be able to substitute for alkali cations as substrates of the Na,K-pump at mildly acidic pH (see Blostein, 1989 for further discussion). H/K exchange in reconstituted vesicles (Hara and Nakao, 1986) and inside-out vesicles (IOV's) derived from human erythrocytes (Blostein, 1985; Polvani and Blostein, 1988), or Na/H exchange in IOV's (Polvani and Blostein, 1988) has been described. At higher pH, along with the pump's use of H^+, Blostein and Polvani (Polvani and Blostein, 1989; Blostein and Polvani, 1992) reported a markedly reduced Na/K(Rb) stoichiometry as well as an altered charge transfer (reversal of the membrane potential) in low-Na^+ medium.

The Na,K-pump can also transport other inorganic cations; at intracellular pump sites, Li^+ serves as a congener for Na_{in}, while at extracellular pump sites, Li^+, Cs^+, and Rb^+ serve as K_{ext} congeners (for discussion, see Hoffman, 1986). Boyer (1988) has suggested that H^+ in the form of hydronium ions (H_3O^+) can mimic the physical structure of these cations and thus also serve as alkali cation congeners (see also Section 1.2.3.e.).

1.2.3.c. Phosphorylation and ATP hydrolysis

As described above, phosphorylation of the Na,K-ATPase by ATP normally occurs after high affinity binding of this nucleotide to the E_1 conformation in the presence of Na^+. Under special conditions (in the presence of 20-40% dimethylsulfoxide (DMSO) at neutral pH), ATP can phosphorylate the enzyme, apparently at the high-affinity (aspartyl residue) site in the absence of Na^+ (Barrabin *et al.*, 1990). This finding, also reported for the Ca-

ATPase (Carvalho-Alves and Scofano, 1983), suggests that the cation binding usually required for phosphorylation by ATP may be mimicked by solvent-induced conformational alterations. Magnesium, which is generally recognized as the cofactor for the phosphorylation of the Na,K-ATPase, appears to bind to the enzyme either as a complex with ATP or after ATP binding (Robinson, 1974; Plesner and Plesner, 1981), although the exact sequence of these events remains to be determined (Robinson and Pratap, 1991). It remains tightly bound to the enzyme through its cycle until it is released during E_2P hydrolysis (Fukushima and Post, 1978). The possibility that free Mg^{2+} has a distinct binding site from that of the Mg^{2+}-ATP complex has been explored by Sachs (1988). Other divalent cations, such as Ca^{2+}, Mn^{2+}, and Cu^{2+}, can substitute for Mg^{2+} to support ATP hydrolysis to varying degrees (see, for example, Fukushima and Post, 1978; Askari and Huang, 1981). The reaction mechanism for E_2 phosphorylation by P_i (and Mg^{2+}) involves a carboxylate (of the active site aspartyl residue) attacking the P atom in the P_i group (Dahms *et al.*, 1973). Dephosphorylation (or hydrolysis) of the EP normally occurs by water attack on the phosphorus atom (Dahms *et al.*, 1973). However, under certain conditions, as mentioned above, the phosphoryl group on the enzyme can be donated to ADP to form ATP in a reversal of the phosphorylation reaction.

As reviewed by Glynn (1985), there are other catalytic activities of which the Na,K-ATPase is capable, mostly involving the exchange of groups among nucleotides. For example, there is the ATP-ADP exchange concomitant with the aforementioned Na/Na exchange without net ATP hydrolysis (Blostein, 1970). Another catalytic activity is the exchange of ^{18}O between P_i and water (see, for example, Dahms and Boyer, 1973). It requires Mg^{2+} and K^+ (or a K^+ congener, but not Na^+), and is much faster than the maximal turnover rate of ATP hydrolysis. Various phosphatase activities have also been reported, involving the release of P_i from non-nucleotide substrates such as *p*-nitrophenyl phosphate (pNPP) or acetyl phosphate (for further review and references, see Glynn, 1985). This activity requires Mg^{2+} and in particular K_{ext} (Drapeau and Blostein, 1980),

suggesting that it is catalyzed by E_2K. Phosphorylation of the enzyme may or may not occur, depending on the substrate used in the reaction.

1.2.3.d. Pump conformations

As shown in the Albers-Post model, the Na,K-ATPase can exist in two major conformational states: E_1, which has a high affinity for Mg^{2+}-ATP and is phosphorylated in the presence of Na^+; and E_2, which has a high affinity for K^+ and is phosphorylated by P_i and Mg^{2+}. The evidence that these functional differences correspond to distinct conformational states of the Na,K-ATPase comes from a variety of studies, including those of tryptic cleavage patterns and those using fluorescent probes (for reviews, see Glynn, 1985; Jørgensen and Andersen, 1988). Jørgensen has carried out extensive studies of Na,K-ATPase proteolytic cleavage patterns and the resulting changes in function under various conditions (Jørgensen, 1975, 1977, 1982; Jørgensen and Collins, 1986). In the presence of Na^+ (E_1 conformation), trypsin cleaves at two sites. The first, called T_2, is associated with a rapid cleavage occurring near the N-terminus at Lys^{30} and causing a 50-60% inhibition of ATPase activity. A slower cleavage, T_3, occurs at Arg^{262} and eliminates ouabain-sensitive Na,K-ATPase and K-phosphatase activity. With K^+ present, trypsin cleaves at a different site, Arg^{438} (T_1), followed later by cleavage at T_2. The T_1 cleavage event abolishes Na,K-ATPase activity, while the T_2 cleavage event is required to eliminate K-phosphatase activity. Chymotrypsin cleaves at Leu^{266} (C_3) in the presence of Na^+, "locking" the enzyme in the E_1 conformation and thereby eliminating Na,K-ATPase and K^+-phosphatase activity. In the presence of K^+, there is no cleavage by chymotrypsin.

Changes in the fluorescence of various reporter molecules during the reaction cycle also provide strong support for conformational changes. These events usually correspond to physical changes in the environment of the fluorescent group, such as movement into or out of the lipid bilayer. For example, the fluorescence intensity of FITC, which binds covalently to the catalytic subunit near the ATP binding region, depends on the presence of

various ligands such as Na^+, K^+, and P_i (see, for examples, Karlish, 1980; Hegyvary and Jørgensen, 1981). The high level of fluorescence quenching induced by K^+ binding makes this probe an especially useful indicator of enzyme conformational changes. Another probe, eosin, binds near or at the ATP binding site (Skou and Esmann, 1981). Its fluorescence and binding affinity change depends on whether the enzyme is in a Na^+ or K^+ solution. The probe 5-iodoacetamidofluorescein (IAF), unlike FITC and eosin, appears to be able to bind to the enzyme and report conformational changes without interfering with its function (Kapakos and Steinberg, 1982). Tryptophan residues, being intrinsically fluorescent, have also been used to detect conformational changes during the Na,K-ATPase reaction cycle (see, for example, Karlish and Yates, 1978; Tyson and Steinberg, 1987).

1.2.3.e. Cation occlusion and binding affinities

One of the key elements of Na,K-pump function is the process of cation occlusion. Glynn (1993) has recently reviewed the history of cation occlusion experiments, while Glynn and Karlish (1990) have presented a detailed review on the nature of cation occlusion sites in P-type pumps.

The first evidence for Rb^+ occlusion came from studies showing that each Na,K-ATPase molecule carried through a cation exchange column was accompanied by two Rb^+ ions (Beaugé and Glynn, 1979). It was subsequently shown that the rate of Rb^+ release correlated with the rate of the conformational change (Glynn *et al.*, 1987). In the presence of P_i, K^+ (Rb^+) occlusion or release occurs at the extracellular surface in an ordered sequence (see, for example, Forbush, 1987a, 1987b; Glynn and Richards, 1989) corresponding to a "fast-exchanging" and a "slow-exchanging" binding site (Forbush, 1987a, 1988). Similar behavior has been reported for the Ca-ATPase (Inesi, 1987). Forbush (1987a) has proposed a "flickering gate"-type model to account for these observations, similar to that proposed for certain ion channels (Hille, 1984). Alternatively,

the ordered behavior may be caused by the single-file passage of ions through a long, narrow channel (see below).

In order to demonstrate Na^+ occlusion, Glynn *et al.* (1984) used enzyme preparations in which the E_1P-E_2P conformational change was blocked by N-ethylmaleimide (NEM) or chymotrypsin, thus minimizing the possibility of Na+ release by newly formed ADP. The authors showed that three Na^+ ions per molecule of enzyme were carried through the ion exchange column. Other studies concerned with the nature of EP forms and their interconversion are consistent with the notion that the release of Na^+ to the extracellular surface of the protein occurs in two steps (Lee and Fortes, 1985; Yoda and Yoda, 1987a). In the latter case there is evidence for the existence of E^*P, which is hydrolyzed after releasing only one Na^+ to the extracellular surface.

The nature of the cation binding sites of the Na,K-ATPase, presumably located within the transmembrane domains (see Section 1.2.2.d.), remains to be resolved (for reviews, see Jorgensen and Andersen, 1988; Glynn and Karlish, 1990; Glynn, 1993). Based on the above information and studies of cation binding sites in ionophores and other proteins (see Miller, 1993), it is reasonable to envision the cation binding site(s) as a pocket or pore-like structure, probably lined with negative charges. Cations, either partly or completely dehydrated, would be held in this pocket by coordination with the negatively charged groups. These groups could be contributed by oxygen-, nitrogen-, or sulfur-containing protein residues, as well as by amide or carbonyl groups in the peptide backbone. In addition, aromatic *pi* bonds and even water could participate in cation stabilization (Miller, 1993).

The interaction of cations with these sites (*i.e.*, reflecting intrinsic cation binding affinities) probably depends on two main factors: the free energy of dehydration and the dehydrated ionic radius (see Glynn and Karlish, 1990; Miller, 1993). Cations such as K^+, which can easily shed their hydrating shell of water molecules, would be energetically favored to move from the bulk aqueous solution into a binding pocket. However, only

those molecules with small dehydrated radii would be expected to fit into a tight binding pocket. In order to accommodate ions of differing radii, the cation binding sites would have to be somewhat flexible. Miller (1993) has discussed how the structure of some known cation binding sites is flexible enough to adjust for "mismatches" (*i.e.*, ions of low affinity for a site) by changing the number of negatively charged groups that interact with the ion. Such structural changes could also be the basis for the differences in cation affinity of the Na,K-ATPase in different conformations. One such model which accommodates the binding of a variety of cations, including protons as H_3O^+, is a crown ether structure (Eisenman and Dani, 1987; Boyer, 1988). This could help to explain how the Na,K-ATPase is able to transport such diverse cations as Rb^+, Cs^+, Tl^+, NH_4^+, Na^+, Li^+, and H_3O^+.

A number of highly conserved, flexible proline residues located within the transmembrane regions may also contribute to the structure of the binding pockets (Brandl and Deber, 1986). Perhaps acting like gates during conformational transitions (Jørgensen and Andersen, 1988), they could account for the changes in the sidedness of cation access. Alternatively, ion translocation could be produced by minor peptide movements such as the twisting of α helices (Tanford, 1982). This model could also account for the alternating exposure of cation binding sites to different sides of the membrane as well as the significant differences in cation binding affinities during different stages of the reaction cycle.

The involvement of two negatively charged groups in the cation binding sites was suggested from studies of charge translocation (see Section 1.2.3.f.). Thus, the outward movement of three Na^+ ions is accompanied by the net efflux of a single positive charge, whereas the inward movement of two K^+ ions is electroneutral. Using the carboxyl group-specific probe DCCD, Shani-Sekler *et al.* (1988) provided evidence that one or possibly two carboxyl-containing glutamate residues in the Na,K-ATPase α subunit are involved in cation occlusion. Since either Rb^+ or Na^+ protected the enzyme from being labeled by this probe, the authors also concluded that these two ions bind to the same site(s). Using

extensive proteolytic digestion to isolate the membrane-embedded regions of the α subunit (the "19-kDa preparation"), Karlish and coworkers (Karlish *et al.*, 1990; Goldshleger *et al.* (1992) further localized the DCCD-binding residues (Glu^{953}, and perhaps Glu^{327} in the pig α subunit) to specific transmembrane domains.

The results of recent site-directed mutagenesis studies of the rat glutamate residues, Glu^{955} and Glu^{956}, that correspond to the DCCD-labeled pig residues tend to argue against a critical role for the carboxyl groups of these residues in cation binding (van Huysse *et al.*, 1993). Changing the amino acids to aspartate or glutamine had little effect on the enzyme's ability to catalyze Na^+,K^+-dependent ATP hydrolysis. One explanation of these results is that Glu^{955} and Glu^{956} are not the critical residues involved in cation binding. Because DCCD interactions with proteins are complex, sometimes leading to intraprotein crosslinking reactions (see Pedemonte and Kaplan, 1990), it is possible that some of the observations reported by Shani-Sekler *et al.* (1988) were due to carboxyl-nonspecific inactivation events. Using a different carboxyl group-specific probe, 4-(diazomethyl)-7-(diethylamino)-coumarin (DEAC), Argüello and Kaplan (1994) have implicated Glu^{779} of the canine enzyme as an essential residue in cation binding. Because of its proximity to the ATP-binding and phosphorylation domains, they argue that it is ideally located to respond to the structural changes that may be associated with these regions during conformational changes (see Lutsenko and Kaplan, 1994). Also, site-directed mutagenesis studies of the structurally related Ca-ATPase have led to the identification of six negatively charged amino acid residues within the fourth, fifth, sixth, and eighth transmembrane domains that are essential for Ca^{2+} translocation and Ca^{2+}-dependent ATP hydrolysis (Clarke *et al.*, 1989, 1990a). Thus, by analogy, Glu^{953} in the ninth predicted transmembrane domain of the Na,K-ATPase may not be very important in cation binding.

Vilsen (1993) recently identified a naturally occurring mutant, Glu^{329} (equivalent to Glu^{327} in the pig enzyme) to Gln^{329}, in the fourth transmembrane domain of the rat α_1 subunit. Measuring the Na,K-ATPase activity of membrane preparations from COS-1 cells

transfected with the cDNA of this mutant, this author found an approximately 2-fold decrease in the enzyme's apparent affinity for Na^+. Vilsen concluded that this change in affinity represented a change in the intrinsic Na^+ binding affinity, thereby placing this residue within the binding site for this cation (see Section 5.3 for further discussion). The small change in the apparent affinity, similar to those reported by van Huysse *et al.* (1993), argues against a prominent role of these glutamate carboxyl groups in the cation binding sites.

Jewell-Motz and Lingrel (1993), using site-directed mutagenesis to generate Glu^{327} mutants of the rat α_2 isoform (analogous to Gln^{329} in the α_1 isoform), found a similar small change in the magnitude of the apparent affinity of the enzyme for Na^+ when Gln^{327} was substituted for Glu^{327}. They also found that certain mutations of other intramembrane glutamate and aspartate residues had much greater affects on apparent affinities for Na^+. In fact, some of the substitutions did not produce functional enzymes (see Jewell and Lingrel, 1991). These studies emphasize the need to further examine the role of negatively charged amino acids in the cation binding sites of the Na,K-ATPase. Perhaps, as suggested by Rose *et al.* (1983) the negative charges involved in cation binding are contributed by peptide backbone carbonyl or amide groups. In this case, the amino acid substitutions chosen in the aforementioned experiments would still provide the required negative charges.

There are probably other factors involved in cation binding domains. This may explain why Jewell and Lingrel (1992), using a series of chimeric α_1/α_3 enzymes of the rat catalytic subunit isoforms, were unable to identify any particular region of the α subunit that is responsible for the differences in cation affinities known to exist between these isoforms (Jewell and Lingrel, 1991). Moreover, a comparison of the amino acid sequences of these rat α subunit isoforms (see Lingrel *et al.*, 1990) shows that all of the negatively charged residues predicted to lie within the transmembrane domains are conserved. This suggests that some of the determinants of apparent cation affinities may be associated with

other, possibly uncharged, residues of the cation binding pockets. It is also possible that the measured apparent cation affinities do not reflect intrinsic cation binding affinities. As discussed by Läuger (1991), intrinsic binding affinities may not be rate-determining due to other kinetic aspects of the pump cycle (see also Section 5.3). Thus, the altered binding affinities in the some of aforementioned studies may not have been significant enough to interfere with the overall enzyme activity.

1.2.3.f. Charge translocation

The occlusion and subsequent translocation of cations implies that individual positive charges are being moved across the membrane. However, experiments designed to measure charge translocation have detected the net movement (outward) of only one positive charge during the Na,K-ATPase reaction cycle (see Glynn and Karlish, 1990). As mentioned above, Goldshleger *et al.* (1987) have hypothesized from their studies of charge translocation in proteoliposomes containing reconstituted Na,K-ATPase that there are two negatively charged sites in the cation binding domain. Each binds to a Na^+ ion, while the third Na^+ ion must somehow be stabilized by a conformational- and possibly charge-linked interaction. Since the previous discussion regarding ion binding sites argues for the existence of more than a single negative charge per cation, other types of charge movement, such as that of α helix dipoles (see Läuger, 1991), must be considered. The net result, however, must still be the movement of a single positive charge through the membrane electrical field.

As an electrogenic enzyme, it is likely that the Na,K-ATPase is affected by transmembrane voltage changes, especially if the rate-limiting step of the reaction cycle is voltage-sensitive (for discussion, see Glynn and Karlish, 1990; Läuger, 1991; Vasilets and Schwarz, 1993). To study this phenomenon, a number of investigators have used voltage clamp techniques on intact cells (Gadsby *et al.*, 1985; Gadsby and Nakao, 1989; Nakao and Gadsby, 1989), dialyzed squid axons (Rakowski *et al.*, 1989), or oocytes (Schwarz

and Gu, 1988; Rakowski and Paxson, 1988). They have shown that the Na^+ translocation pathway of the cycle represents the electrogenic, and hence voltage-sensitive step, whereas the K^+-translocating pathway of the reaction cycle is voltage-insensitive (see, for example, Bahinski *et al.*, 1988). Recently, it has been possible to identify more precisely the details of the charge translocating step. Borlinghaus *et al.* (1987) demonstrated that the phosphorylation and Na^+ occlusion steps are electroneutral events, whereas Gadsby *et al.* (1993) found that the release of Na^+ ions to the extracellular side of the membrane is strongly voltage dependent. They proposed that this represents the actual charge-translocating step, where, under the influence of the membrane electrical field, Na^+ ions must pass through a well or channel-like structure before reaching the extracellular bulk solution. They also suggested that this event corresponds to the true rate-limiting step of the pump's reaction cycle. This model was further refined by Hilgemann (1994), who proposed that the release of only the first of the three Na^+ ions corresponds to the major electrogenic event. In other studies, Nakao and Gadsby (1989) investigated the effects of membrane potential on cation binding affinities using guinea pig cardiac myocytes. By varying the concentrations of Na^+ and K^+, they showed that the binding or release (*i.e.*, apparent affinity) of Na_{ext} is strongly dependent on membrane voltage, whereas the apparent affinity for Na_{in} and K_{ext} are not. Vasilets and Schwarz (1992, 1993) have shown a much stronger dependence of the apparent affinity for K_{ext} on membrane voltage using *Torpedo californica* pumps expressed in *Xenopus laevis* oocytes, perhaps reflecting structural differences among species. These findings are also consistent with the sequential (*i.e.*, single-file) passage of ions through the electrical field of the membrane as part of their binding and release steps.

1.2.3.g. Inhibitors

The Na,K-ATPase is specifically inhibited by members of the cardiac glycoside family of drugs, which includes ouabain and digoxin (for review, see Blaustein, 1993).

Traditionally extracted from certain plants, these compounds, first described by Withering (1785), have been useful as therapeutic agents for several centuries. By far the most important modern clinical use of these drugs is the treatment of congestive heart failure (for review, see Hoffman and Bigger, 1985). The cardiotonic effects of digoxin and digitoxin, the two most frequently administered cardiac glycosides, arise from their inhibitory effects on the Na,K-ATPase, which appears to be the only pharmacological target of these drugs (see Hansen, 1984). The chief mechanism believed to be responsible for the inotropic effects of cardiac glycosides is an increase in the intracellular Ca^{2+} concentration ($[Ca]_{in}$) indirectly caused by the drugs' inhibition of the Na,K-ATPase (for reviews, see Akera *et al.*, 1970; Blaustein, 1993). Presumably, the elevated intracellular Na^+ concentration ($[Na]_{in}$) caused by these drugs increases $[Ca]_{in}$ via the Na/Ca exchanger, leading to an increased contractility of heart muscle and the corresponding positive inotropic effect.

For *in vitro* studies of Na,K-ATPase behavior, cardiac glycosides are unparalleled in their ability to cause rapid and highly specific inhibition of the Na,K-ATPase. Binding to the enzyme, as described in Section 1.2.2.d., is greatly accelerated in the presence of either Na^+ plus Mg^{2+} and ATP, or Mg^{2+} plus P_i (see, for example, Albers *et al.*, 1968; Hoffman, 1969; Akera, 1981). In contrast, K^+ impedes glycoside binding, suggesting that these drugs interact preferentially with the E_2P pump conformation (Erdmann and Schöner, 1973; Matsui and Homareda, 1982). Although complete, inhibition is reversible.

As described in Section 1.2.2.d., the main determinant of cardiac glycoside binding kinetics is drug structure. The overall affinity of the enzyme for these drugs is more dependent on the dissociation rate rather than the association rate (Tobin and Brody, 1972). Aglycone forms of these drugs (*e.g.*, strophanthidin) are useful for kinetics studies since their relatively quick off-rates permit more accurate determinations of dissociation constants. Studies of various tissues and species show that there is a wide range of pump specificities for ouabain. For example, the α_1 isoforms of rodents comprise a class of pumps which is relatively resistant to ouabain (see Tobin and Brody, 1972; Emanuel *et al.*,

1988) — about three orders of magnitude more than the majority of pumps from most other sources, including other rodent isoforms. The structural basis for this difference is largely due to the presence of two charged amino acids in the first extracellular domain of the α subunit (Price and Lingrel, 1988). The extent and physiological significance of varying ouabain sensitivities, if any, remain to be elucidated (see Section 1.2.5.c.).

Another inhibitor of the Na,K-ATPase, vanadate, is capable of inhibiting all members of the P-type ATPase family. A structural analogue of phosphate, vanadate binds to the same aspartyl residue of the pump that is normally phosphorylated by ATP and appears to "lock" it into the E_2 conformation and prevent it from completing its catalytic cycle (see Glynn, 1985). Inhibition requires Mg^{2+} and is accelerated by the presence of K_{in}, which would favor the formation of E_2 (see Cantley *et al.*, 1978).

Oligomycin, which binds to E_1P in the presence of Na^+, is useful both as an inhibitor and as a probe of pump conformation (see Glynn, 1985). Its actions, however, are complex since it exhibits uncompetitive inhibition which is incomplete in the presence of K^+ (see, for example, Robinson, 1971). In addition, its behavior is consistent with interactions with Na,K-ATPase dimers, suggesting half-of-sites kinetics (see Section 1.2.3.a. and Plesner, 1987a). Because it acts on E_1P, the functions effected by K^+-dependent pathways (*e.g.*, K^+-phosphatase activity) are not inhibited as long as Na^+ is not present (see, for example, Israel and Titus, 1967; Sachs, 1980).

A compound which has unique effects on the Na,K-ATPase is palytoxin, derived from coelenterates (Moore and Scheuer, 1971). When applied, it causes pumps to behave as ouabain sensitive cation leaks, almost as if they were channels (Tosteson *et al.*, 1991). Glynn (1993) has speculated that binding of this toxin might change the conformation of the pump such that the gates which modulate cation occlusion are held open.

1.2.4. Isoforms of the Na,K-ATPase α and β subunits

1.2.4.a. Kinetic evidence for tissue-specific enzymes

Some the earliest evidence for the existence of Na,K-ATPase isoforms came from studies which showed tissue-specific differences in the reactivities of Na,K-ATPases to cardiac glycosides (for a detailed review and further references, see Sweadner, 1989). Skou (1962), for example, comparing rabbit brain and kidney enzymes, demonstrated a five-fold difference between their values for half-maximal inhibition by the cardiac glycoside *g*-strophanthin. A much larger difference was noted by Ahmed and Judah (1964) for the strophanthin sensitivities of Na,K-ATPases from rat liver, brain, and kidney. Subsequent studies showed that there is a wide range of cardiac glycoside sensitivities among certain tissues and species. In particular, a nearly 1000-fold difference between kidney pumps from rodents compared to those of other species has been observed (Ahmed and Judah, 1964; Tobin and Brody, 1972). Although some of the diversity reported was most likely due to differences in binding conditions and the antagonistic effects of K^+ (see Sweadner, 1989), reports of complex inhibitory kinetics for rodent brain, but not kidney, provided convincing evidence for the existence of at least two populations of pumps (Marks and Seeds, 1978; Urayama and Nakao, 1979; Sweadner, 1979). In more recent studies, differential inhibition by cardiac glycosides has been used to distinguish the activities of the three α subunit isoforms in rat brain (Berrebi-Bertrand *et al.*, 1990; Blanco *et al.*, 1990; Gerbi *et al.*, 1993).

In early studies investigating the substrate (ATP, P_i, Na^+, K^+) affinities of Na,K-ATPases from various tissues, there were few significant differences reported in either ATP or cation affinities (see, for example, Kimelberg *et al.*, 1978; Urayama and Nakao, 1979; Sweadner, 1985). In general, comparisons between brain and kidney enzymes indicated few or no differences in the apparent affinities for K^+, while the apparent affinities for Na^+ were often lower for brain than for kidney enzymes. However, due to uncertainty regarding the nature of the isoform composition in the various brain

preparations as well as significant differences between the methods of tissue preparation and enzyme activity assays, some of the results are difficult to interpret. Recent studies which circumvent some of these limitations are considered below (see Section 1.2.4.e.).

1.2.4.b. Structural evidence for tissue-specific enzymes

The first clear structural evidence supporting the existence of distinct Na,K-ATPases was provided by high-resolution SDS-PAGE. Using this technique on brine shrimp, Peterson *et al.* (1978) found two closely migrating bands representing the catalytic subunit of the enzyme. Similarly, Sweadner (1979) reported two bands from mammalian brain but only one band from other tissues such as kidney or electroplax. The uniqueness of the bands from brain was confirmed by their differential sensitivities to proteolytic cleavage and to sulfhydryl reagents such as NEM. Subsequent studies demonstrated that the individual bands could be selectively recognized by tissue-specific antisera (Sweadner and Gilkeson, 1985) and that they had distinct N-terminal sequences (Lytton, 1985b). (An important caveat to these analyses, however, is that artifacts arising from proteolysis, heating, or chemical treatment may produce multiple bands during SDS-PAGE when, in fact, there is only one isoform present (Sweadner, 1989; Cortas *et al.*, 1991)). For several years, the more slowly migrating form of the Na,K-ATPase from brain was referred to as the $\alpha(+)$ form, compared to the α form from kidney. The isoforms have since been renamed to reflect the resolution of the $\alpha(+)$ band into two separate entities; α became α_1, and $\alpha(+)$ became α_2 and α_3 (Schneider *et al.*, 1988).

Structural studies have also suggested the existence of multiple β subunit isoforms. In contrast to the α subunit, which displays a relatively constant position with SDS-PAGE analysis, the apparent molecular weight of the β subunit varies widely among tissues and species. As discussed by Sweadner (1989), these differences appear to be due to varying degrees of N-linked glycosylation, since the deglycosylated proteins from rat kidney or axolemma show similar migration patterns and immunoreactivity (Sweadner and Gilkeson,

1985). However, others have reported immunological evidence for the existence of distinct β subunit isoforms in avian (Fambrough and Bayne, 1983; Takeyasu *et al.*, 1989) and mammalian tissues (Hubert *et al.*, 1986).

1.2.4.c. Genetic evidence for tissue-specific enzymes

With the advent of molecular biological techniques, it became possible to confirm the existence of distinct Na,K-ATPase isoforms. As discussed in Section 1.2.2.a., the deduced amino acid sequences for the catalytic α subunits of a variety of species have been published. In addition, Shull *et al.* (1986a) reported the sequences for three distinct isoforms of the α subunit deduced from rat brain cDNA. At least five different genes related to the α subunit have been detected in the human genome (Sverdlov *et al.*, 1987), although only three (those coding for the α_1, α_2, and α_3 isoforms) are known to be expressed at the protein level. Beta subunit cDNA sequences have been obtained for various species (for review, see Sweadner, 1989) and indicate the presence of at least two isoforms in mammals (Martin-Vasallo *et al.*, 1989) and a third in amphibia (Good *et al.*, 1990). For the putative γ subunit, only a single gene has been reported (Mercer *et al.*, 1993).

As reviewed by Fambrough *et al.* (1991), nucleotide sequence homologies are greater between the same isoforms of different species (93-96%) than between different isoforms of the same species (~82%). As shown in Fig. 1, this implies that isoform divergence occurred approximately 200 million years ago, during the period when birds and mammals began to diverge from each other. Additional support for the presumed early divergence of the α subunit isoforms is based on the high homology of the rat α_3 isoform with the single isoform found in *Drosophila* (Lebovitz *et al.*, 1989). At present, a few structural differences among the α isoforms can be deduced from the information contained within the primary amino acid sequences. They include variations in the number of amino acid residues and substantial sequence differences in the N-terminal regions of the various

isoforms (Shull *et al.*, 1986a). Comparisons of β_1 subunits show a greater than 95% amino acid homology among mammalian species and an approximately 60% homology between mammals and amphibia (see Mercer, 1993). In contrast, the homologies between different β subunit isoforms are as low as 56%. Primary sequence analyses show small yet significant differences in the total number of residues and potential N-linked glycosylation sites (Mercer, 1993). As far as the γ subunit is concerned, the deduced amino acid sequences of the sheep and rat are 91% homologous (Mercer *et al.*, 1993).

The genomic organization of the Na,K-ATPase α subunit indicates the existence of many (at least 50) introns and exons (see Lingrel *et al.*, 1990). Some of these exons probably code for the conserved structural domains of the catalytic subunit. Little is known with regard to the upstream promoter regions that most likely regulate transcription. Recent work has shown the existence of cation- and hormone-responsive elements (see, for example, Kawakami *et al.*, 1993).

The chromosomal locations of the subunit isoform genes have been determined, at least for humans and mice. In humans, both the α_1 and α_2 subunits, and most likely the β_1 subunit as well, have been mapped to chromosome 1, while the α_3 subunit is found on chromosome 19 (Kawakami *et al.*, 1986; Chehab *et al.*, 1987; Yang-Feng *et al.*, 1988). In mice, the three α subunit genes are located on separate chromosomes (Kent *et al.*, 1987b).

Analyses of the primary transcripts of the Na,K-ATPase subunits demonstrate that the α_1 and α_3 genes each produce a single mRNA species, while the α_2 gene produces two sizes of mRNA (see Sweadner, 1989; Lingrel *et al.*, 1990). There are at least five different β_1 subunit-specific mRNA's (see Young *et al.*, 1987), probably due to the presence of multiple initiation and polyadenylation sites. The β_2 subunit gene produces one mRNA and the γ subunit gene produces two mRNAs (Mercer *et al.*, 1993).

1.2.4.d. Tissue and cellular distributions of isoforms

The tissue-specific distributions of the isoforms of the Na,K-ATPase have been explored using both Northern blot hybridization to characterize mRNA expression and Western blot analysis to detect protein expression. Several groups (see, for example, Herrera *et al.*, 1987; Young and Lingrel, 1987; Orlowski and Lingrel, 1988; Schneider *et al.*, 1988) have carried out comparative RNA blot analyses of rat tissues. The results are summarized in Table I. The α_1 isoform was detected in virtually all tissues examined, consistent with the hypothesis that it is a constitutively expressed "housekeeping" isoform (see Chapter 4). It appears to be most abundantly expressed in kidney and brain. Muscle and brain express the highest levels of α_2 mRNA, which includes both the 5.3- and 3.4-kilobase (kb) species. Central nervous system tissue, from which three α subunit genes were originally cloned (Shull *et al.*, 1986a), represents the major site of α_3 mRNA expression. The β_1 isoform is expressed in most tissues, whereas the β_2 isoform has only been detected in brain and glial cells (see, for example, Shyjan and Levenson, 1989; Gloor *et al.*, 1990; Watts *et al.*, 1991). Some tissues, such as liver (Orlowski and Lingrel, 1988) and very young rat pineal gland (Shyjan *et al.*, 1990a), express unusually low levels of both β isoforms, suggesting that there may be other uncharacterized isoforms of this subunit.

These mRNA expression patterns correlate fairly well with analyses of α subunit isoform protein expression. In rodent nervous tissue, for example, the presence of the α_1 and $\alpha(+)$ isoforms was shown by Sweadner (1979) through differential cardiac glycoside sensitivities. Urayama *et al.* (1989) later demonstrated the presence of α_2 and α_3 in rat axolemma preparations using differential cleavage of these isoforms by weak acid hydrolysis and monoclonal antibody-specific reactivity of proteolytic digests. Certain anatomical structures in the nervous system show distinct isoform expression patterns. In the rat spinal cord, for example, expression of the α_1 and α_3 isoforms predominates (McGrail *et al.*, 1991). The pineal gland expresses mainly the α_3 subunit, along with the

TABLE I

Tissue distributions and relative expression levels for mammalian subunit isoforms of the Na, K-ATPase.

Relative abundance estimates of mRNA and protein levels shown here are only approximate, and are limited to comparisons within each tissue. Data are taken from Orlowski and Lingrel (1988), Schneider *et al.* (1988), Martin-Vasallo *et al.* (1989), Dhir *et al.* (1990), Lingrel *et al.* (1990), Hundal *et al.* (1992), and Mercer (1993).

Tissue	Isoform				
	α_1	α_2	α_3	β_1	β_2
Kidney	+++	-	-	+++	+/-
Brain	++	+++	+++	++	+++
Heart	++	+	+/-	+	++
Skeletal Muscle	+	+++	-	+	+
Smooth Muscle	+	++	-	+	?
Stomach	+	-	-	+	?
Lung	+	+/-	-	+	?
Liver	+/-	-	-	-	-
Intestine	++	-	-	++	?
Erythrocyte	+	-	-	+/-	-

β_2 subunit and lesser amounts of the α_1 subunit (Shyjan *et al.*, 1990a, 1990b). All three α isoforms have been detected in the retina (McGrail and Sweadner, 1989), with the α_3 isoform predominating in the inner layer. The choroid plexus appears to contain only the α_1 subunit (see Sweadner, 1989; Zlokovic *et al.*, 1993) in combination with either the β_1 or β_2 subunits (Zlokovic *et al.*, 1993), while cerebral microvessels may contain all the α and β isoforms (Zlokovic *et al.*, 1993).

In more recent studies, attempts have been made to assess isoform distributions in the various cells of the nervous system. The α_1 isoform appears to be expressed in all cell types, including those of the nervous system (see, for example, Watts *et al.*, 1991). The α_2 isoform, previously thought to be limited to glia, has also been found in some neurons (McGrail *et al.*, 1991). The α_3 isoform has been found in many neurons (Schneider *et al.*, 1988; Watts *et al.*, 1991) as well as in vascular tissue (Zlokovic *et al.*, 1993), but not in glial cells. That neurons are capable of expressing all three α subunit isoforms argues against previous notions of distinct "neuronal" and "glial" isoforms (for further discussion, see Sweadner, 1989, 1992). Similarly, the presumed restriction of Na,K-ATPase pumps to nodes of Ranvier (see Stahl and Baskin, 1990) has been recently refuted by the detection of internodal ATPase labeling (McGrail *et al.*, 1991; Mata *et al.*, 1991) during different stages of ontogeny.

In addition to tissue-specific isoform distributions, there are also developmentally regulated expression levels. Orlowski and Lingrel (1988) showed that the expression of mRNA for each of the isoforms was high in rat brain during early development. However, the relative increase was much greater for the α_3 isoforms than for the other isoforms. Qualitatively similar results were reported by Urayama *et al.* (1989) who used Western immunoblot analyses with isoform-specific monoclonal antibodies.

In heart, there is evidence for at least two isoforms based on detection of EP on acid gels and protein by SDS-PAGE. The $\alpha(+)$ band was differentiated from the α band based on its sensitivity to pyrithiamine (Matsuda *et al.*, 1984) or NEM (Ng and Akera, 1987a,

1987b). Orlowski and Lingrel (1988) later showed the presence of mRNA specific for all three of the isoforms, depending on the animal's stage of development. Thus, in the hearts of fetal and young rats, the α_1 and α_3 isoforms predominated, whereas in adult animals the α_1 and α_2 isoforms were most apparent. This developmental switch coincides with the appearance of alterations in cardiac electrophysiology (Langer *et al.*, 1975). Also, the ratio of isoform distributions can change in adult hearts following muscle damage due to injury or disease (see Lingrel *et al.*, 1990).

Other tissues which express the α_2 isoform include skeletal muscle and smooth muscle (see Table I). Apart from nervous tissue, the remainder of tissues, including kidney and erythrocytes, contain predominantly or perhaps exclusively α_1 (Inaba and Maeda, 1986; Dhir *et al.*, 1990). However, there is some evidence that the kidney may express low levels of the other isoforms (see, for example, Ahn *et al.*, 1993) or that it may express presently unidentified isoforms (Doucet, 1992).

The tissue-specific expression patterns of the α and β subunits of the Na,K-ATPase suggest that isoform subunit pairing is quite flexible, although relatively few of these have been verified by immunoprecipitation studies. For example (see Table I), the α_1 isoform is expressed with the β_1 isoform in kidney and skeletal muscle, and is expressed with the β_2 isoform in skeletal muscle and pineal gland (Shyjan *et al.*, 1990b; Hundal *et al.*, 1993), suggesting the possible existence of both $\alpha_1\beta_1$ and $\alpha_1\beta_2$ heterodimers in these tissues. Similarly, both $\alpha_2\beta_1$ and $\alpha_2\beta_2$ heterodimers may exist in heart and muscle. The presence of $\alpha_2\beta_2$ complexes in glial cells has been verified by immunoprecipitation studies (Gloor *et al.*, 1990). Large, myelinated neurons contain the α_3 and β_1 subunits (McGrail *et al.*, 1991), whereas pineal glands express the α_3 and β_2 isoforms (Shyjan *et al.*, 1990a, 1990b). Watts *et al.* (1991) recently showed that in rat brain, all three α subunits can be found in the presence of either β subunit, resulting in the possible formation of six different heterodimers. Similar findings were presented for cerebral microvessels (Zlokovic *et al.*,

1993). Evidence that pairing of these various isoforms can produce functional pumps in protein expression systems is presented in the following section.

1.2.4.e. Comparative functional studies of Na,K-ATPase isoforms

Microsomal membrane preparations. ATP hydrolysis, partial reactions such as phosphatase activity, and steady-state EP levels and E_1P/E_2P ratios are typically examined using plasma membrane (microsomal) fractions isolated by differential centrifugation from tissues rich in the Na,K-ATPase (for examples, see Table II). Although these assays have the advantage of being relatively easy and fast to carry out, the lack of sidedness in these preparations implies that Na^+ and K^+ have unrestricted access to both intracellular and extracellular binding sites. The resulting simultaneous activation and inhibition by these cations may complicate kinetic analyses. In addition, the need for detergents to permeabilize the membranes of most of these preparations in order to allow substrates access to their binding sites has raised concerns that changes in lipid-protein or protein-protein associations could cause significant changes in enzyme kinetics (see Chapter 5).

Table II summarizes the essential findings of comparative ATPase hydrolysis studies of Na,K-ATPase isoform activation by cations. As mentioned in Section 1.2.4.d., the presence of two and sometimes three α subunit isoforms, as well as multiple β subunit isoforms, within a given tissue or cell type complicates the interpretation of some of these studies. In particular, this applies to early comparative studies of glial vs. neuronal ATPases in which activation by K^+ was examined (Moonen and Franck, 1977; Grisar *et al.*, 1978; Kimelberg *et al.*, 1978; Atterwill *et al.*, 1984; Reichenbach *et al.*, 1985). In more recent studies, however, most authors have attempted to relate their findings to the relative amounts of isoforms present in the tissues studied. For example, Lytton (1985a) examined the Na^+ activation profiles of Na,K-ATPases from rat kidney, axolemma, and adipocytes. Previous studies showed that the $\alpha(+)$ isoform comprised more than 90% of the Na,K-ATPase activity in axolemma membranes (Sweadner, 1979) and 75% of that in adipocyte

TABLE II

Summary of comparative cation activation studies of Na,K-ATPase isoforms.

The experiments are listed chronologically and grouped according to the type of enzyme activity measurement used. In tissues with more than one isoform present, those isoforms shown in parentheses most likely display low levels of activity, while those that are underlined probably represent the dominant activity.

Study	Tissue	Isoform(s)	Assay conditions; membrane isolation method	Apparent cation affinity (mM)	
A. ATP hydrolysis				**K^+**	**Na^+**
Skou (1962)	Rabbit brain	$\alpha_1/\alpha_2/\alpha_3$	Reciprocal Na^+ for K^+;	1.0	25
	Rabbit kidney	α_1	0.05-0.1% DOC extraction	0.5	60
Kimelberg et al. (1978)	Rat neuron	$\alpha_1/(\alpha_2)/\alpha_3$	10 mM K^+ or 60-110 mM Na^+;	1.7	15.4
	Rat glia	$\alpha_1/\alpha_2/(\alpha_3)$	no detergents	1.2	10.0
	Rat synaptosomes	$\alpha_1/\alpha_2/\alpha_3$		0.7	10.5
Urayama & Nakao (1979)	Rat brain	$\alpha_1/\alpha_2/\alpha_3$	10 mM K^+ or 100 mM Na^+;	1.4	8.7
	Rat kidney	α_1	2 M NaI extraction	1.4	11.3
Sweadner (1985)	Rat brain	$(\alpha_1)/\alpha_2/\underline{\alpha_3}$	20 mM K^+ or 120 mM Na^+;	0.9	7.5
	Rat kidney	α_1	2 M NaI extraction	0.9	9.5

Study	Tissue	Isoform(s)	Assay conditions; membrane isolation method	Apparent cation affinity (mM)	
A. ATP hydrolysis (cont')				**K^+**	**Na^+**
Lytton (1985)	Rat brain	$(\alpha_1)/\alpha_2/\underline{\alpha_3}$	10 mM K^+;	-	4.5
	Rat kidney	α_1	no detergents	-	8.9
	Rat adipocyte	α_1/α_2		-	3.2
Feige (1988)	Rat brain	$\alpha_1/\alpha_2/\alpha_3$	20 mM K^+ , recipr. Na^+ for K^+;	1.8	5.4
	Rat kidney	α_1	2 M NaI plus 1 mg/ml DOC extraction	1.2	12.1
	Rat heart	α_1/α_2		1.4	10.0
Shyjan (1990)	Rat brain	$\alpha_1/\alpha_2/\alpha_3$	20 mM K^+ , 130 mM Na^+;	1.3	15.5
	Rat kidney	α_1	no detergents	1.8	20.6
	Rat pineal gland	$(\alpha_1)/\underline{\alpha_3}$		2.1	11.0
Brodsky and Guidotti (1990)	Rat brain	α_1	10 mM K^+ , 0-100 mM Na^+;	-	9
		α_2/α_3	no detergents	-	12.5
Jewel & Lingrel (1991)	Rat isoforms expressed in HeLa cells	α_1	10 mM K^+ or 30 mM Na^+;	0.45	1.15
		α_2	1 M NaI extraction	0.43	1.05
		α_3		0.27	3.08
Gerbi et al. (1993)	Rat brain	α_1	10 mM K^+ , 0-100 mM Na^+;	-	3.88
		α_{2a}	no detergents	-	4.98
		α_{2b}		-	28
		α_{3a}		-	3.5
		α_{3b}		-	20

Study	Tissue	Isoform(s)	Assay conditions; membrane isolation method	Apparent cation affinity (mM)	
B. Ion transport				**K^+**	**Na^+**
Lytton (1985)	Rat adipocyte	α_1	$[Na^+]in \approx 2$-80 mM	-	17
		α_2		-	52
	(+ Insulin)	α_1		-	14
		α_2		-	33
Schielke et al. (1990)	Rat synaptosomes	$\alpha_1/\alpha_2/\alpha_3$	Rb^+ uptake: $[Na^+]in$ (≈10-15 mM?)	0.5	-
	Rat microvessels	$\alpha_1/\alpha_2/\alpha_3$	Na^+ efflux: >30 mM Na^+	3.8	-
Brodsky and Guidotti (1990)		α_1	Rb^+ uptake: $[Na^+]in \approx 12$-100 mM	-	17
		α_2/α_3		-	33
McGill and Guidotti (1991)	Rat adipocyte	α_1	Rb^+ uptake: $[Na^+]in \approx 5$-130 mM	-	11
		α_2		-	39
	(+ Insulin)	α_1		-	8
		α_2		-	23

membranes (Lytton *et al.*, 1985a). The $\alpha(+)$ isoform of brain has since been shown to consist of a mixture of the α_2 and α_3 isoforms (Schneider *et al.*, 1988). Presumably, the $\alpha(+)$ isoform of the adipocyte is identical to the α_2 isoform, as it appears to be insulin responsive like the α_2 isoform of muscle (Lytton, 1985a; Hundal *et al.*, 1993).

Quantitation of the relative amounts of isoforms has also been achieved to some extent in a comparative study of the Na^+ activation profiles of Na,K-ATPases from rat kidney, brain, and heart. Thus, Feige *et al.* (1988) used (dihydro)ouabain inhibition profiles to determine that their brain and heart membranes contained 75% and less than 10%, respectively, of the $\alpha(+)$ isoform. Brodsky and Guidotti (1990) also used ouabain titration to determine that their rat brain synaptosomal membranes contained ≈80% of the α_2 (and/or α_3) isoform, with the rest consisting of the α_1 isoform.

Taking advantage of their recent discovery that adult rat brain pineal glands express predominantly the α_3 isoform, along with minor amounts of the α_1 but none of the α_2 isoform, Shyjan *et al.* (1990a) compared the cation activation profiles of microsomal membrane Na,K-ATPase activity from this tissue with those of rat kidney and brain. These investigators also used ouabain inhibition profiles to estimate that their brain and pineal gland membranes comprised ≈ 60% and 50%, respectively, of the ouabain-sensitive isoform(s) (α_2 and/or α_3 in brain and α_3 in pineal gland). By carrying out their cation activation assays on pineal gland in the presence of 10 μM and 5 mM ouabain, they distinguished the activity of the α_3 isoform from rat pineal gland membranes and showed that it had a nearly two-fold higher apparent affinity for Na^+ than the α_1 isoform of kidney membranes. These results are both qualitatively and quantitatively similar to most of those from the foregoing studies listed in Table II.

In a recent publication, Gerbi *et al.* (1993) also used the differential ouabain sensitivities of rat brain membranes to distinguish the Na^+ activation profiles of various isoforms. Although complex, their findings indicate the presence of subpopulations of both the α_2 and α_3 isoforms in their preparations, some of which have much lower apparent

affinities for Na^+ than the α_1 pumps. This conflicts with many previous studies, which report that the rat kidney (α_1) Na,K-ATPase has a similar or higher apparent affinity for Na^+ than any of the ouabain-sensitive isoforms (see Table II).

Also in contrast to the majority of cation activation studies are results of Na,K-ATPase assays carried out on membranes of HeLa cells individually transfected with the three rat α subunit isoforms (Jewell and Lingrel, 1991). These authors, using site-directed mutagenesis to change critical ouabain-binding residues in the first transmembrane domain of the α subunit, rendered the α_2 and α_3 rat isoforms resistant to ouabain. They could thus distinguish the exogenous Na,K-ATPase activity from that of the ouabain-sensitive HeLa cell host. Their cation activation profiles demonstrated that the α_3 isoform has a three-fold lower apparent affinity for Na^+ compared to the α_1 or α_2 isoforms. In addition, they found that the α_3 isoform has a 1.5-fold higher apparent affinity for K^+.

Other aspects of Na,K-ATPase kinetic behavior which have been characterized using microsomal preparations of various tissues include measurements of the steady-state levels and distribution of EP forms and pNPP hydrolysis (pNPPase) activity. Differences in the cation sensitivities of brain and kidney EP have been reported. Measurements of the E_1P/E_2P ratios revealed that the kidney enzyme was more likely to be found in the E_2P conformation (under the conditions of these assays), whereas the brain enzyme favored the E_1P conformation (Fukushima and Nakao, 1980; Klodos and Nørby, 1987; Matsuda and Iwata, 1988). There was, however, no apparent difference in the maximal turnover numbers of these pumps (Yamaguchi and Tonomura, 1978). Measurements of K^+-stimulated pNPPase activity supported these findings; the higher ratio of this hydrolytic activity to that of Na^+,K^+-stimulated ATP hydrolysis in kidney compared to brain (Nagume *et al.*, 1986; Matsuda and Iwata, 1988) implied that the kidney pump was more likely to be found in the E_2P conformation. Similarly, this conclusion was supported by the finding that the Na,K-ATPase activity of rat kidney was more sensitive to vanadate than that of rat brain (Feige *et al.*, 1988). Back-door phosphorylation experiments (performed

using Mg^{2+} and P_i) showed that the brain enzyme (mostly $\alpha(+)$ in this case) took longer to reach equilibrium and was less affected by K^+ antagonism than the kidney enzyme (Matsuda and Iwata, 1987). Differential sensitivities of brain and kidney Na,K-ATPases to sulfhydryl reagents have also been reported; these studies have been thoroughly reviewed by Sweadner (1989).

A potential problem with assays using either chaotropic agents or detergents is that the removal of cytosolic and loosely associated membranous elements may change enzyme kinetic behavior. Thus, several authors have reported significant changes in cation activation kinetics caused by purification or detergent treatment of membranes (Lytton, 1985a; Feige *et al.*, 1988; Brodsky and Guidotti, 1990). The nature of such alterations, which could include changes in the lipid association or interactions of pumps with other membrane components, remains unclear.

Subcellular vesicular preparations. One of the unique properties of neurons is that their presynaptic nerve terminals may be pinched off to form intact vesicles (synaptosomes). These structures contain much, if not all, of the original cellular metabolic components, including the enzymes and substrates required for ATP synthesis (Abita *et al.*, 1977). Synaptosomes have recently been used to examine the kinetic behavior of Na,K active transport. Measuring $^{86}Rb^+$ uptake in rat forebrain synaptosomes, Brodsky and Guidotti (1990) found that the α_1 isoform had a nearly three-fold higher apparent affinity for Na_{in} than the α_2 (and/or α_3) isoform. This is in agreement with Gerbi *et al.*'s (1993) recent findings, and may also fit with Jewell and Lingrel's results if the ouabain-sensitive activity of the synaptosomes were attributed mostly to the α_3 isoform. On the other hand, these results could also agree with those of Lytton (1985a) and McGill and Guidotti (1991) if the ouabain-sensitive activity were attributed to the α_2 isoform.

Another aspect of Brodsky and Guidotti's report is the evidence that there are significant differences in pump behavior between intact synaptosomes and membranes derived from them. For example, ouabain titration profiles showed that the α_1 isoform

comprised ≈75% of the total Na,K-pump activity in synaptosomes compared to only 20% of the total Na,K-ATPase activity in synaptosomal membranes. In addition, the difference in the apparent affinities for Na^+ among the isoforms in synaptosomes was not apparent in synaptosomal membranes. They concluded that an unidentified component, present in synaptosomes but lost or inactivated during the preparation of membranes from these vesicles, may be responsible for changes in both the activity and Na^+ responsiveness of α_2 and/or α_3 pumps in neurons.

Cultured cells. Comparative studies of ion transport have been carried out using cultured cells from a wide variety of sources. The challenge of maintaining a relatively fixed $[Na]_{in}$ during the assay has usually been met by using short assay periods (McGill and Guidotti, 1991) or the lipid-soluble Na/H exchanger monensin (see, for example, Haber *et al.*, 1987; Stimers *et al.*, 1990). Again, a serious disadvantage with many cell types is that they often contain more than one isoform of the Na,K-ATPase. Thus, with the exception of rats, whose α_1 isoforms may be distinguished from their α_2 and α_3 isoforms based on their differential ouabain sensitivities, the majority of cultured cells and cell lines expressing more than one isoform are generally unsuitable for isoform-specific pump kinetic studies.

One of the earliest comparative studies of cation activation of Na,K-ATPase isoforms was carried out by Lytton (1985a) using rat adipocytes. Using differential ouabain sensitivity as described above, he concluded that the $\alpha(+)$ isoform of adipocytes was at least three-times less sensitive to Na_{in} than the α isoform. In addition, he demonstrated that this difference was drastically reduced either by insulin or by the process of preparing membranes from these cells. A subsequent re-evaluation of this work (McGill and Guidotti, 1991) essentially confirmed these findings, although differences in the apparent stimulatory effects of insulin were noted. The authors determined that these discrepancies with the previous study were largely artifacts due to significant changes in Na_{in} caused by the inhibitory effects of low doses of ouabain on the $\alpha(+)$ isoform.

Other comparative studies of Na,K-ATPase isoform pumping in cultured cells have focused on neurons and glia. Even though the concept of distinct "neuronal" and "glial" Na,K-ATPase isoforms is probably no longer valid (see Section 1.2.4.b.), differences in the kinetic behavior of these enzymes continue to be reported. Among the most surprising findings is that glial pumps appear to be maximally stimulated at ≈10 mM Na_{in} (see Reichenbach *et al.*, 1992), which is usually below the Michaelis constant (K_m) for pumps in most other cells (see, for exampie, Garay and Garrahan, 1973; Trachtenberg *et al.*, 1981; Pollack *et al.*, 1981). Furthermore, K_{ext} continues to stimulate glial pumps up to ≈10 mM (Reichenbach *et al.*, 1985), whereas neuronal (Marroni *et al.*, 1983) and other pumps (see, for example, Garrahan and Glynn, 1967a) are usually saturated at ≈5 mM K_{ext}. The possibility that glial pumps may transport only K^+ under certain conditions, or may be under the influence of a regulatory factor which is lost during cellular disruption has been discussed by Sweadner (1989, 1994) and Reichenbach *et al.* (1992).

Expression systems. A recent alternative to systems limited by endogenously expressed isoforms is cultured cells transfected with cDNAs coding for various pump subunits. This technology was first applied to mammalian cells, resulting in the expression of foreign α subunits in monkey CV-1 cells (Kent *et al.*, 1987a), mouse Ltk$^-$ cells (Takeyasu *et al.*, 1988), and human HeLa cells (Jewell and Lingrel, 1991). Other systems which have been subsequently developed include yeast (Horowitz *et al.*, 1990a) and insect cells (Blanco *et al.*, 1993). Yeast are especially attractive since the cells are easy to culture and contain no endogenous Na,K-ATPases. The appeal of the insect cell system lies partly in the low levels of endogenous pump activity, as well as in the potential for high levels of exogenous protein expression which is provided by baculovirus promoters. These systems are particularly well suited to studying the effects of interactions between various exogenous α and β subunit isoforms. With the exception of the ATPase study mentioned previously (Jewell and Lingrel, 1991), comparative functional studies of Na,K-ATPase isoforms have only recently been undertaken in these systems.

Another expression system, the amphibian oocyte injected with mRNA encoding Na,K-ATPase subunits, has been widely used for measurements of ouabain-sensitive cation fluxes (see, for example, Noguchi *et al.*, 1987; Schwarz and Gu, 1988) or K^+/Rb^+ currents detected by recording microelectrodes (Rakowski and Paxson, 1988). Most of the isoform-related comparative functional studies using this system have focused on the influence of the β subunit isoform on the apparent affinity for K_{ext} of Na,K-pumps (Jaisser *et al.*, 1992).

1.2.5. Regulation of the Na,K-ATPase

1.2.5.a. Substrates (ATP, Na^+, K^+)

In most cells, the concentration of ATP is high enough to saturate the binding site of the Na,K-ATPase for this substrate. For example, ATP levels in kidney cells are approximately 2 mM, whereas the apparent affinity for ATP ($K_{m(ATP)}$) for the kidney Na,K-ATPase is typically ≈0.4 mM (see, for example, Sweadner, 1985). Most (≈95%) of this ATP is supplied by oxidative phosphorylation (Cohen and Kamm., 1981; Mandel and Balaban, 1981); the small amount of ATP which is generated by glycolysis in kidney cells does not appear to be sufficient to meet the demands of the Na,K-ATPase (Wittner *et al.*, 1984). As expected, there is a direct correlation between endogenous ATP levels, ATP regeneration ability, and pump activity (Soltoff, 1986; Djouadi *et al.*, 1992). In some systems, however, ATP may be a rate-limiting substrate, even when raised above the normal 1-2 mM. Using intact kidney tubules, Soltoff and Mandel (1984) demonstrated a linear correlation between the level of Na^+ pumping and the level of intracellular ATP that did not show saturation kinetics even when intracellular ATP was as high as 3.5 mM. In contrast, permeabilized membranes of the same tubules did show saturation kinetics and had a typical $K_{m(ATP)}$ of 0.4 mM. Similarly, Ikehara *et al.* (1984) found a linear correlation between the level of K^+ transport and the level of intracellular ATP using HeLa cells. A possible explanation for these discrepancies could be that the concentration of ATP

measured from cellular extracts does not accurately reflect the amount of ATP available to the pumps of intact cells (see below).

In erythrocytes, which contain no organelles, the exclusive source of ATP is glycolysis. Several investigators have reported an association between two membrane-associated glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase, and the Na,K-ATPase (Schrier, 1966; Parker and Hoffman, 1967). Hoffman's group subsequently showed that a membrane-associated pool of ATP exists in red cells and that it is the preferential energy source of Na,K-ATPases. However, "bulk" cytoplasmic ATP could also be used in place of this preferred pool (Hoffman and Proverbio, 1974; Proverbio and Hoffman, 1977). Mercer and Dunham (1981) unequivocally demonstrated ATP synthesis by these membrane-bound glycolytic enzymes and further defined the nature of the ATP pool in the membrane. A similar preferential fueling of pump activity by glycolytic ATP was recently demonstrated using cardiac myocytes (Glitsch and Tappe, 1993). As discussed by Welch (1977), coupled multienzyme systems such as these confer several advantages with respect to efficiency and regulation; these include substrate-reactant channeling, compartmentalization, and increases in the effective local concentrations of reactants.

There is also evidence that other membrane proteins are linked with glycolytic enzymes in multienzyme systems. For example, ATP-sensitive K^+ channels in cardiac myocytes can be preferentially inhibited by ATP generated via glycolysis (Weiss and Lamp, 1987). Furthermore, using intact kidney tubules, Hurst *et al.* (1993) reported a reciprocal relationship between ATP-sensitive K^+ channel activity and Na,K-ATPase activity. These results demonstrate that membrane-associated ATP is an important determinant of the activity of other membrane proteins aside from the Na,K-ATPase, and that these proteins probably interact with ATP from a common pool. Thus, the possibilities for regulation of the Na,K-ATPase by substrate ATP are complex, suggesting interactions between various transport systems and the energy metabolism machinery of the cell.

Changes in substrate cation concentrations, may, under some circumstances, be rate-limiting for the Na,K-pump. Extracellular K^+, which is 4-5 mM in most tissues, is normally present at saturating levels, and thus would not be expected to modulate pump activity *in vivo*. An exception, however, is the tissues of the central nervous system, where resting K_{ext} concentrations ($[K]_{ext}$) are 3 mM or less (see Syková, 1983). A similar situation may occur locally for skeletal muscle, another excitable tissue. During repolarization of these tissues, $[K]_{ext}$ may drop below resting levels (Krnjevic and Morris, 1975), perhaps becoming rate-limiting for pump activity (see Chapter 4 for further discussion). It is also plausible that, on a long-term basis, hypokalemia may cause a decrease in Na,K-pumping capacity. However, some tissues, such as skeletal muscle, can compensate for lower pump activity through an increase in the total number of pumps expressed (see McDonough *et al.*, 1992).

In contrast, the $[Na]_{in}$ is typically close to the K_m for pump activation by this cation. Stimulation of Na,K-pump activity by elevated $[Na]_{in}$ has been demonstrated in nearly all systems examined to date, with the possible exception of glial cells (see Reichenbach *et al.*, 1987; Sweadner, 1994). A large variety of physiological stimuli can cause significant and sometimes very rapid changes in $[Na]_{in}$, usually leading to Na,K-pump stimulation. Examples of these events include nerve or muscle firing, excitotoxicity, and the effects of certain hormones as discussed in Section 1.2.5.e.

1.2.5.b. Calcium ions

An inhibitory effect of Ca^{2+} on the Na,K-ATPase was first described by Skou (1957). Subsequently reported effects include the inhibition of ATP-ADP exchange (Tobin *et al.*, 1973), the inhibition of K^+-stimulated dephosphorylation (Knauf et al., 1974), competition with Na^+ (Blostein and Burt, 1971; Tobin *et al.*, 1973; Fukushima and Post, 1978), and substitution of Ca^{2+} for Mg^{2+} (Tobin *et al.*, 1973; Fukushima and Post, 1978).

More recent work, instead of using permeabilized membrane preparations, has focused on the influence of micromolar Ca^{2+} concentrations (such as those present in intact cells) on the Na,K-ATPase (for review, see Yingst, 1988). In human red cells, a specific protein named calnaktin, which reversibly modulates the inhibitory effects of Ca^{2+} on the Na,K-ATPase, has been described (Yingst and Marcovitz, 1983; Yingst *et al.*, 1985). This membrane-associated protein, with an approximate molecular weight of 35 kDa, is heat-sensitive and is lost during extensive washing or purification of membranes. Calnaktin, or analogues thereof, may exist in other tissues including brain, heart, and kidney (see Yingst, 1988) as well as in myometrium (Turi *et al.*, 1991). Its role as an *in vivo* pump regulator may be that of a constitutive inhibitor such that at normal $[Ca]_{in}$ it functions as a repressor of pump activity. Evidence that another Ca^{2+}-regulatory protein, calmodulin, may regulate Na,K-ATPase activity is complicated by the possibility that preparations may contain residual calnaktin (Yingst, 1988). Calmodulin alone has no effect on purified dog kidney Na,K-ATPase (Huang and Askari, 1982).

1.2.5.c. Endogenous cardiac glycosides (endo-ouabains)

Evidence accumulated over the past 25-30 years strongly suggests that endogenous forms of the cardiac glycoside drugs, known as endo-ouabains, play a major role in the regulation of pump activity *in vivo*. First proposed to exist by Szent-Györgyi (1953), these compounds have recently been purified from several sources including mammalian plasma (Harris *et al.*, 1990; Hamlyn *et al.*, 1991), urine (Goto *et al.* 1989), and toad skin (Lichtstein *et al.*, 1986). The mammalian-derived compounds are very similar, if not identical, to ouabain and digoxin. The most highly concentrated source of endogenous ouabain appears to be the adrenal gland (Hamlyn, *et al.*, 1991). Due to their possible involvement in the etiology of hypertension (Kramer *et al.*, 1991; Blaustein, 1993), these compounds are currently being investigated in both human and animal models.

1.2.5.d. Membrane environment

Lipids. The lipid dependence of Na,K-ATPase activity was first recognized during early attempts to purify the enzyme from biological membranes using detergents, organic solvents, and phospholipases (for references, see Kimelberg and Papahadjopoulos, 1972). The earliest comprehensive study to determine the nature of the lipid requirement was performed by Kimelberg and Papahadjopoulos (1972) who worked with a well defined, 90% delipidated Na,K-ATPase preparation from rabbit kidney. They observed that phosphatidylserine, followed by phosphatidylglycerol, both of which carry a net negative charge, supported by far the best activity of the enzyme. This finding was later confirmed by DePont *et al.* (1978).

The effects of bilayer thickness were explored by Johannsson et al. (1981) using purified pig kidney enzyme reconstituted with defined phospholipids. Acyl chains containing 16-22 carbon atoms provided the best activity, suggesting the importance of membrane bilayer thickness for ATPase activity. These findings were more recently extended by Marcus *et al.* (1986), who showed that di-(20:1) and di-(22:1) acyl chains supported optimal pumping in reconstituted vesicles and that the presence of more than one double bond reduced activity. Thus, the importance of membrane thickness and fluidity has been confirmed.

In cellular systems, Kimelberg and Mayhew (1975) showed that a transformed cell line with a more fluid lipid environment had higher enzyme activity than one with a less fluid environment. General effects of membrane fluidity on transport proteins and other membrane systems have recently been reviewed by Le Grimellec *et al.* (1992). They pointed out that the fluidity of the membrane environment could also influence pump function in polarized epithelial cells where the apical membrane has a reduced fluidity relative to the basolateral domain. This could explain why pumps sometimes expressed in apical membranes are less active than those in basolateral membranes (see Hammerton *et*

al., 1991) as well as account for the significant increase in pump activity which is observed when apical membranes are rendered more fluid (Sutherland *et al.*, 1988).

Lipid-specific effects on the reaction mechanism of the Na,K-ATPase have also been reported. Hegyvary *et al.* (1980), for example, investigated various aspects of pump behavior using native and delipidated dog heart membranes. Even though the enzyme appeared to be locked in the E_1 conformation, they reported that up to 80% of the maximum velocity of partial reactions (phosphorylation, ouabain binding, K^+-activated pNPPase) could be achieved. They ascribed the changes in ouabain binding kinetics which they observed to the reduced affinity of E_1 for ouabain. Others have also reported that lipids can modulate ouabain binding affinities (Abeywardeena and Charnock, 1983), although these authors did not distinguish whether the differences in binding were due to shifts in enzyme conformational ratios or to direct effects of lipids on cardiac glycoside binding.

The effects of another lipidic membrane component, cholesterol, have also been extensively investigated. Giraud *et al.* (1981), showed that cholesterol depletion or addition of amphiphiles (*e.g.*, chlorpromazine) caused increased disordering (fluidization) of erythrocyte lipid bilayers. Yeagle (1983), also investigating cholesterol effects on red cell membranes, concluded that endogenous levels of cholesterol afforded optimal enzyme activity. In subsequent work on the kidney enzyme, Yeagle *et al.* (1988) demonstrated specific protein-cholesterol interactions that enhanced ATPase activity and were independent of membrane fluidity. Also, Yoda and Yoda (1987b) demonstrated an effect of cholesterol on the equilibrium ratios of EP intermediates.

Free fatty acids, another lipidic membrane component, can modulate Na,K-ATPase activity. In very early experiments, Skou (1964) demonstrated Na,K-ATPase inhibition by oleic acid. More recently, Tal *et al.* (1989), in a search for potential endogenous pump modulators, isolated several fatty acids (including oleic acid) which caused nonspecific ATPase inhibition.

Interest in lipids as second messengers of cellular responses has prompted further investigation into the effects of fatty acids on pump activity. Oishi *et al.*, (1990) have shown that micromolar concentrations of oleic acid, sphingosine or lyso-phosphatidylcholine can inhibit Na^+,K^+-dependent ATP hydrolysis, Na-ATPase, K^+-activated pNPPase in rat brain microsomes, $^{22}Na^+$ uptake in human erythrocyte IOV's, and $^{86}Rb^+$ uptake in human leukemic (HL 60) cells. Although fatty acids added directly to intact human erythrocytes in suspension had no effect on Rb^+ uptake (Dwight *et al.*, 1992), it should be noted that in this study fatty acids were added to intact cells, which could have different fatty acid partitioning or buffering abilities compared to the inside-out membranes of the previous study. Thus, it is possible that endogenously produced fatty acids, such as those resulting from phospholipase C (PKC) activation, act locally on neighboring Na,K-ATPases before they are inactivated by the buffering elements in cell membranes.

Cytoskeleton. Interactions between the Na,K-ATPase and components of the cytoskeleton have been clearly documented. For example, there is evidence that the Na,K-ATPase is associated with the cytoskeleton via ankyrin (Nelson and Veshnock, 1987; Nelson and Hammerton, 1989; Morrow *et al.*, 1989). Associations between the Na,K-ATPase and spectrin (Koob *et al.*, 1990), actin (Molitoris *et al.*, 1991), and fodrin (Morrow *et al.*, 1989) have also been suggested. The cell adhesion molecules E-cadherin (Nelson *et al.*, 1990; Wollner *et al.*, 1992) and AMOG (Gloor *et al.*, 1990) appear to interact with the Na,K-ATPase. In addition, a new group of peripheral membrane proteins, designated "pasins", colocalize with the Na,K-ATPase in epithelial basement membranes (Kraemer *et al.*, 1990).

Although some cytoskeletal proteins may directly modulate Na,K-ATPase activity (see, for example, Bertorello and Cantiello, 1992), current evidence demonstrates that they are mainly involved in maintaining the cellular distribution of Na,K-ATPases. A non-specific means by which polarized epithelial cells restrict the diffusion of membrane proteins (and sometimes lipids) is via the *zonula occludens* (tight junction) proteins

(Madara *et al.*, 1986; van Meer and Simons, 1986). These entities help to define the apical and basolateral domains. As mentioned above, restricting pumps to the basolateral domain keeps them in a more fluid lipid environment which is conducive to optimal activity. However, specific interactions between the Na,K-ATPase and cytoskeletal proteins such as ankyrin and fodrin suggest other mechanisms for the generation and maintenance of epithelial cell polarity.

The general issues of membrane protein sorting have not been completely resolved. With regard to the Na,K-ATPase of polarized epithelial cells, it is possible that pumps are delivered from Golgi vesicles directly to basolateral membranes. Evidence for such a mechanism has been demonstrated using polarized cell lines derived from canine kidney epithelium (Caplan *et al.*, 1986) or from rat thyroid epithelium (Zurzolo and Rodriguez-Boulan, 1993). These authors detected few or no pumps at apical membranes. In contrast to these findings, Hammerton *et al.* (1991) showed that up to half of newly synthesized pumps are delivered to the apical membrane of MDCK cells. They hypothesized that the overall polarity of the cells was maintained by selective stabilization (decreased loss from the membrane) of basolateral pumps, perhaps through cytoskeletal interactions. In a recent review of this work, Hammerton and coworkers ruled out several methodological artifacts as possible explanations of their findings (Siemers *et al.*, 1993). Instead, they suggested that there might be a general membrane sorting defect in their particular clone of MDCK cells. Thus, in some cells, Na,K-ATPases may be sorted according to bulk membrane flow. An interesting exception to basolateral Na,K-ATPase targeting occurs in retinal pigment epithelial cells. Gundersen *et al.* (1991) have shown that apical targeting of this enzyme is associated with a general reversal of the membrane cytoskeleton polarity in these cells.

The membrane cytoskeleton and its interactions with the Na,K-ATPase have also been associated with several disease processes (for review, see Leiser and Molitoris, 1993). During experimentally induced ischemia or anoxia in kidney cells, which causes a

drop in cellular ATP levels, the Na,K-ATPase dissociated from cytoskeletal components and redistributed within the cell, resulting in a loss of membrane polarization (Molitoris *et al.*, 1991). Another group (Doctor *et al.*, 1993) showed that ischemic effects were tissue-specific, causing ankyrin breakdown in whole brain and kidney, but not in heart or three renal cell lines. Although the Na,K-ATPase dissociated from the cytoskeleton, it did not appear to be degraded. A cytoskeletal reversal similar to that reported above for retinal pigment epithelial cells has been observed in the pathological condition known as polycystic kidney disease. Avner *et al.* (1992) and Ogborn *et al.* (1993) have recently demonstrated that, as a result of this disease, kidney epithelial Na,K-ATPases are targeted to the apical rather than the basolateral membrane, leading to the formation of fluid-filled cysts and eventually to renal failure.

L-antigen of sheep erythrocytes. The existence of a distinct but as yet undefined membrane component capable of modulating Na,K-ATPase activity has been documented in erythrocytes of sheep, goats, and cattle (for review, see Lauf, 1978). While the erythrocytes of most mammalian species contain K^+ as the predominant cation, those of the aforementioned ungulates are heterogeneous with respect to their $[Na]_{in}$ and $[K]_{in}$ (see Hoffman, 1986). Thus, the mature cells of sheep have either high-K^+, low-Na^+ (HK phenotype) erythrocytes or high-Na^+, low-K^+ (LK phenotype) erythrocytes. Associated with these phenotypes is a genetic polymorphism of blood group cell surface antigens, the M/L antigens (Ellory, 1977). These antigens were discovered through immunization of HK sheep with LK cells and vice-versa. Cells displaying only the M-antigen are HK, whereas those displaying the L-antigen or both antigens are LK. Although the L allele is considered to be dominant (Evans *et al.*, 1956), the $[K]_{in}$ of LM heterozygotes is slightly higher than those of LL homozygotes, perhaps indicating incomplete dominance or the involvement of multiple genes in the determination of these phenotypes.

There are significant kinetic differences between the ion transport activities of HK and LK cells (see Xu, 1994). Active K^+ transport (Na,K-pump activity) is several fold

higher in HK than in LK cells (Tosteson and Hoffman, 1960; Dunham and Hoffman, 1971). In contrast, the passive K^+ fluxes of HK cells are lower (Tosteson and Hoffman, 1960). These authors explained their findings in a model whereby cation and volume homeostasis are maintained by a balance of pump and leak fluxes. While pumps from both cell types appear to have similar apparent affinities for K_{ext}, K_{in} is strongly inhibitory to LK pumps (Hoffman and Tosteson, 1971; Dunham and Blostein, 1976). Dunham and Anderson (1987) have established that K_{in} acts as a noncompetitive inhibitor, reducing the apparent maximum velocity (V_{max}) of the Na,K-pump. This lower turnover number for LK pumps may explain why the rate of ouabain binding to LK cells is slower (see Lauf, 1978); as discussed in Section 1.2.3.g., ouabain binds to the E_2 conformation, which is less abundant when $[K]_{in}$ is high. With regard to protein structure, significant differences between HK and LK pumps have not been reported. In fact, precursors of both types of cells contain the same α_1 subunit isoform mRNA (Dhir *et al.*, 1990).

The evidence for a modulatory effect of the L-antigen on the Na,K pump comes in part from the following observations: Antisera raised in HK sheep against LK cells can have dramatic stimulatory effects on LK pumps, causing their activity to rise several fold (Ellory and Tucker, 1969). The mechanism may be described as a "relaxation" of the K_{in} inhibitory effect, leading to an increase in the V_{max} of the pumps (Dunham and Anderson, 1987) and an HK-like rate of ouabain binding (Joiner and Lauf, 1975). These antibodies presumably interact with their target antigen, designated L_p, which then causes the observed functional changes in Na,K-pump behavior. Although treatment of the cells with relatively high concentrations of trypsin eliminates the stimulatory effects of anti-L_p antisera, treatment with trypsin after anti-L_p antiserum exposure does not abolish the stimulation, demonstrating that antibody binding protects the proteolytic site of this antigen (Lauf *et al.*, 1971).

The exact nature and identity of the putative L_p-antigen remains unknown. As described by Dunham (1976), anti-L antisera can affect active Na,K-pumping or passive

K^+ transport, suggesting at least two distinct sites of action in the membrane. The L_p antigen is most likely an integral membrane protein which, if like the M-antigen, requires phospholipids and perhaps cholesterol to function (Shrager *et al.*, 1972). Glycosylation, if present (see Lauf, 1978; Xu, 1994), does not appear to be important. Attempts to identify the antigen using cell surface labeling or immunoprecipitation techniques have met with limited success. A recent study by Pittman *et al.* (1990) suggested that a 25-kDa polypeptide, which immunoprecipitates with anti L_p-treated LK but not HK erythrocytes, may be involved in the L-antigen binding site.

1.2.5.e. Short-term regulation (posttranslational modifications)

There is now evidence that, in addition to stimulation by increasing cation or ATP concentrations, Na,K-ATPases can be modulated by hormones and their associated cellular signaling systems. These events, occurring over a relatively short time period (minutes), involve pre-existing pumps rather than the synthesis of new pumps (for reviews, see Rossier *et al.*, 1987; Gick *et al.*, 1988a; Bertorello and Katz, 1993). Among the various modulatory mechanisms are direct pump modifications, such as phosphorylation by protein kinases, and rapid changes, possibly involving the cytoskeleton, in the number of pumps expressed at the surface of certain cell types. The relationship between hormonal receptor activation and Na,K-ATPase modulation is currently being explored in many laboratories.

The complex effects of catecholamines on Na,K-ATPase activity have recently been reviewed by Hernández-R. (1992). Many early studies using neural tissue showed that norepinephrine (NE) and dopamine (DA) stimulated Na,K-ATPase activity (see, for example, Herd *et al.*, 1970; Schaefer *et al.*, 1972; Sulakhe *et al.*, 1977). These effects were subsequently attributed to a combination of specific receptor-mediated activation (Philiis *et al.*, 1982; Phillis, 1992) and non-specific chelation of inhibitory divalent cations (Schaefer *et al.*, 1974; Hexum, 1977) by these compounds. An early study using kidney membrane preparations showed that catecholamines had no effect on Na,K-ATPase activity (Desaiah

and Ho, 1976). However, more recent studies using intact kidney tubule segments and cells have clearly demonstrated that DA is inhibitory whereas NE is stimulatory (Aperia *et al.*, 1987; Bertorello *et al.*, 1988). Inhibition by DA has also been reported in MDCK cells, which is a cell line derived from cortical collecting duct (CCD) epithelium (Shahedi *et al.*, 1992). A more detailed analysis of kidney revealed that DA inhibits the Na,K-ATPase of rat kidney proximal convoluted tubules (PCT) through either DA_1 receptors (Chen and Lokhandwala, 1993) or DA_1 and DA_2 receptors (Bertorello and Aperia, 1990; Satoh *et al.*, 1993a), whereas in medullary thick ascending limb (MTAL) and CCD the inhibition occurs through DA_1 receptors alone (Satoh *et al.*, 1993a). Similarly, DA inhibits the Na,K-ATPase of neostriatal neurons, also via DA_1 and DA_2 receptors (Bertorello et al., 1990).

Other hormones and cytokines which have been implicated in the direct short-term regulation of Na,K-ATPase activity include serotonin (reviewed by Hernández-R., 1992), endothelin (Zeidel *et al.*, 1989; Garvin and Sanders, 1991), parathyroid hormone (Ribeiro and Mandel, 1992), aldosterone (Welling *et al.*, 1993), insulin (Brodsky and Guidotti, 1990; Hundal *et al.*, 1993), interleukin-1 (Zeidel *et al.*, 1991), adenosine (Marver and Bernabe, 1991), and angiotensin II and atrial natriuretic peptide (see Aperia *et al.*, 1994).

Some of the cellular mechanisms responsible for Na,K-ATPase regulation have recently been elucidated (for review and further references, see Bertorello and Katz, 1993). The majority of these appear to involve "second" and "third messenger" systems which are usually coupled to membrane hormonal receptors. For example, Bertorello and Aperia (1989) demonstrated that guanine regulatory proteins are involved in the modulation of rat kidney PCT Na,K-ATPase. Also, cAMP is inhibitory in many tissues including rat kidney MTAL and CCD (Satoh *et al.*, 1992, 1993a), liver (Tria *et al.*, 1974), brain (Lingham and Sen, 1982), rabbit ciliary epithelium (Delamere and King, 1992), and *Xenopus* oocytes (Vasilets and Schwarz, 1992). In contrast, it has no effect in rat kidney PCT (Bertorello and Aperia, 1990; Satoh *et al.*, 1993a) and appears to be stimulatory in *Squalus acanthias* rectal glands (Lear *et al.*, 1992). The phospholipids diacylglycerol (DAG), along with other

activators of PKC, and phosphatidylinositol (IP_3) have been linked to either stimulation or inhibition of the Na,K-ATPase, depending on the tissue studied (see, for example, Simmons *et al.*, 1986; Bertorello, 1992; Satoh *et al.*, 1993a). In addition, lysophospholipids (Karli *et al.*, 1979; Satoh *et al.*, 1992) and arachidonic acid and several of its metabolites such as hydroxyeicosatetraenoic acid and prostaglandin E_2 (PGE_2) (see, for example, Schwartzman *et al.*, 1985; Escalante *et al.*, 1988; Satoh *et al.*, 1993b; Warden and Stokes, 1993) modulate Na,K-ATPases from various tissues.

Since many of these "second messengers" have no effect on Na,K-ATPase activity when added to purified enzyme (see Bertorello and Katz, 1993), it is likely that they act through other cellular effectors (*i.e.*, "third messengers") such as protein kinases or protein phosphatases. This notion is compatible with previous studies demonstrating that certain soluble cellular fractions, lost after strong homogenization or membrane purification, restored catecholamine effects in membrane preparations (Sulakhe *et al.*, 1977; Rodriguez de Lores Arnaiz and Antonelli de Gómez de Lima, 1981). While previous studies have suggested that the Na,K-ATPase may be phosphorylated by membrane-associated protein kinases (Mårdh, 1979; Ling and Cantley, 1984; Turi and Somogyi, 1988), more definitive evidence of protein kinase-mediated phosphorylation of the Na,K-ATPase has recently been published. Lowndes *et al.* (1990), examining the kinetics of dog kidney and duck salt gland Na,K-ATPase phosphorylation by cAMP-dependent PKA, found that this modification of the Na,K-ATPase had an effect on Na^+-dependent phosphorylation but not on K^+-dependent dephosphorylation of the EP. Using purified shark rectal gland Na,K-ATPase, Bertorello *et al.* (1991) demonstrated direct phosphorylation of the α subunit by cAMP-dependent PKA and PKC. These phosphorylation events inhibited enzyme activity by approximately 50%. Protein kinase A phosphorylated at a single serine residue, while PKC phosphorylated two residues, either serine or threonine, or possibly both. The authors claimed to have achieved similar results using purified and microsomal rat kidney Na,K-ATPase preparations. Fisone *et al.* (1994) recently identified Ser^{943} of the rat α_1

isoform as the site of phosphorylation by PKA. Using site-directed mutagenesis followed by transfection into COS cells, they demonstrated that changing this residue to Ala^{943} decreased the mutant enzyme's response to modulation by PKA. In other studies, Chibalin *et al.* (1992, 1993) reported the phosphorylation of purified avian and amphibian pumps by PKA and PKC. The residue phosphorylated by PKC was localized to the large, cytoplasmic loop between the T_1 and C_3 cleavage sites, which is in the N-terminal half of the protein and thus distinct from the aforementioned protein kinase A site. The importance of these studies is the demonstration that two common protein kinases can directly phosphorylate the Na,K-ATPase and alter its activity, strongly implying that phosphorylation by these kinases *in vivo* is a genuine mechanism of posttranslational pump modification. However, regulatory studies of the Na,K-pump using intact cells require that $[Na]_{in}$ be rigorously controlled. Two recent publications emphasize that apparent changes in pump behavior associated with protein kinase modulators were, in fact, due to changes in the $[Na]_{in}$ (Mito and Delamere, 1993; Miller *et al.*, 1993).

Heterogeneity of protein kinase responses has been described for various regions of kidney tubules. For example, as mentioned above, either DA_1 or DA_2 receptor activation may stimulate or inhibit the Na,K-ATPase, depending on its location along the tubules. Satoh *et al.* (1993a) concluded that the PK A response predominates in CCT and MTAL, whereas the PKC response dominates in PCT. Furthermore, Middleton *et al.* (1993) examined PKC-mediated phosphorylation of pumps in kidney cell lines and concluded that pump responses to this kinase are heterogeneous for different cell lines.

Evidence for the expected counterpart to regulatory phosphorylation by protein kinases, *i.e.*, dephosphorylation by protein phosphatases, is beginning to emerge. Bertorello *et al.* (1991) demonstrated that protein phosphatases can mediate dephosphorylation of the purified Na,K-ATPase. In addition, MTAL cells contain an endogenous protein known as DARPP-32 (Meister *et al.*, 1989) which, when phosphorylated by protein kinases, is a potent inhibitor of protein phosphatase-1

(Hemmings *et al.*, 1984). Phosphorylation of DARPP-32 has also been associated with inhibition of the Na,K-ATPase (Aperia *et al.*, 1991). Aperia *et al.* (1994) have recently proposed a model through which the renal proximal tubule Na,K-ATPase may be concomitantly regulated by the interactions of protein kinases, protein phosphatases, and DARPP-32.

Another way in which some cells rapidly modulate their Na,K-ATPase activity is by altering the number of active enzymes expressed at their surface. Recruitment of pumps from intracellular locations in response to insulin has recently been demonstrated in rat skeletal muscle (Hundal *et al.*, 1993). Specifically, the authors reported that the α_2 and β_1 isoforms were recruited to plasma membranes, each being derived from a distinct intracellular pool. In other reports of pump recruitment, Blot-Chaubaud *et al.* (1990) and Barlet-Bas *et al.* (1990) have described the role of mineralocorticoid hormones in the activation of latent kidney Na,K-ATPases. Welling *et al.* (1993) subsequently showed that only the α_1 isoform was effected, as the α_2 and α_3 isoforms could not be detected in this tissue. In studies of amphibian oocytes, Vasilets and Schwarz (1992), along with Schmalzing *et al.* (1991) found that pump surface expression appears to be regulatable by protein kinases. The cellular mechanism(s) responsible for these events has not yet been defined. Bertorello and Katz (1993) have raised the possibility that modulation of pump surface expression may be mediated by the membrane cytoskeleton. As discussed in Section 1.2.5.d., the Na,K-ATPase associates with several cytoskeletal components including ankyrin, fodrin, and actin. In addition, Ohta *et al.* (1987) have demonstrated that actin polymerization is affected by protein kinases. These findings, combined with those of Vasilets and Schwarz (1992), suggest a mechanism whereby pump surface expression may be governed by protein kinase activity.

1.2.5.f. Long-term regulation

Certain hormones, as well as changes in cellular cation concentrations, are associated with long-term modulation of the levels of Na,K-ATPase expression. Requiring hours or even days to exert their effects, the cellular mechanisms involved in these slow responses include changes in the rate or level of mRNA transcription, translation, and stability, as well as changes in the rate of subunit synthesis, assembly, and degradation (for reviews, see Rossier *et al.*, 1987; Lingrel *et al.*, 1990).

Among the hormones that have been implicated in these processes are steroid hormones and thyroid hormones, which generally regulate gene transcription through their actions at nuclear receptors. Thyroid hormone-mediated regulation of Na,K-ATPase activity and expression in tissues such as kidney, liver, and heart has been reported (Lo *et al.*, 1976; Lin and Akera, 1978; Gick *et al.*, 1988b; Horowitz *et al.*, 1990b). These processes appear to be the result of both α_1 and β subunit mRNA induction (McDonough *et al.*, 1988). In mammalian skeletal muscle, the increase in Na,K-ATPase activity by thyroid hormone (see, for example, Kjeldsen *et al.*, 1986) is due specifically to the induction of α_2 and β_2 subunits (Azuma *et al.*, 1993), demonstrating tissue-specific responses to this hormone.

In addition to the rapid effects mediated by aldosterone (see above), this hormone also stimulates *de novo* pump synthesis in amphibian and mammalian kidney CCD cells (Knox and Sen, 1974; Verrey *et al.*, 1987; Barlet-Bas *et al.*, 1988). Although usually associated with a rise in apical Na^+ conductance through aldosterone's action on Na^+ channels (see, for example, Crabbé, 1963; Jørgensen, 1972; Clauss *et al.*, 1987), this event is not absolutely required for aldosterone-mediated Na,K-ATPase biosynthesis (Geering *et al.*, 1982; Johnson *et al.*, 1986). It is, however, necessary for the functional expression of the newly synthesized pumps (Petty *et al.*, 1981; Sansom *et al.*, 1987). The aforementioned findings of Blot-Chaubaud *et al.* (1990) and Barlet-Bas *et al.* (1990), as well as those of Rayson and Gupta (1985), suggest that both the rise in Na^+ permeability

and the increase in pump synthesis associated with aldosterone are required for the upregulation of Na,K-ATPase activity.

Manipulations of cells *in vitro*, which raise $[Na]_{in}$, can induce Na,K-ATPase expression (for review, see Pressley, 1988). For example, chronic exposure of cells to sublethal concentrations of ouabain or low $[K]_{ext}$ (see, for example, Boardman *et al.*, 1974; Pollack *et al.*, 1981; Pressley *et al.*, 1986, 1988; Bowen and McDonough, 1987; Lescale-Matys *et al.*, 1990) inhibits the Na,K-ATPase, resulting in increased $[Na]_{in}$. Alternatively, exposure to veratridine or monensin (Wolitzky and Fambrough, 1986; Unkles *et al.*, 1988) causes substantial Na^+ leaks, again resulting in increased $[Na]_{in}$. Both α and β subunits appear to be coordinately induced in most tissues, as exemplified by the rat liver ARL-15 cell line (Pressley *et al.*, 1988) as well as the dog kidney MDCK cell line (Bowen and McDonough, 1987). However, the cellular mechanisms responsible for the induction vary depending on the individual cell line studied. For example, both α and β subunit mRNAs increased coordinately in MDCK cells (Bowen and McDonough, 1987), whereas in porcine LLC-PK_1 cells only β subunit mRNA increased (Lescale-Matys *et al.*, 1990, 1993). Moreover, in primary cultures of proximal tubule cells, α and β subunit mRNA upregulation was discoordinate, with the β subunit message in significant excess (Tang and McDonough, 1992). Based on these findings, McDonough *et al.* (1992) hypothesized that the plasma membrane expression of newly synthesized pumps was governed by the amount of β subunit available for heterodimer formation. In the absence of an increase of α subunit synthesis, pumps could still be upregulated via a decrease in their rate of degradation (Lescale-Matys *et al.*, 1993; see also Rayson, 1989).

The physiological relevance of these events is currently being explored. For example, part of the mammalian response to hypokalemia (McDonough *et al.*, 1992) appears to be a specific downregulation of the α_2 isoform of the Na,K-ATPase in skeletal muscle, which contrasts with the upregulation seen in kidney outer medullary collecting ducts and described above for cultured cells. The authors have suggested that these

responses are designed to maximize plasma K^+ levels by allowing muscles to release some of their reserve K^+ and the kidneys to optimize K^+ reuptake. It is also possible that the decreased $[K]_{ext}$ may contribute to pump distributions in normal and diseased retinas (Suson and Burke, 1993). Alternatively, increases in $[Na]_{in}$ may be the means by which various catecholaminergic hormones induce expression of Na,K-ATPases following long-term infusions *in vivo* (see, for example, Swann and Steketee, 1989) and the hormone insulin influences the tissue-specific expression of different Na,K-ATPase isoforms in diabetic rats (Ng *et al.*, 1993).

1.3. THESIS RATIONALE AND GOALS

Although functional differences between Na,K-ATPases from various tissues have been extensively studied and documented (see Section 1.2.5.), there has been relatively little advancement in understanding the molecular basis of this diversity. In many cases, such as the Na,K-ATPases from brain and kidney, it appears likely that behavioral differences are related to the expression of distinct isozymes of the Na,K-ATPase. This holds true for the discrete responses of the rodent isoforms to cardiac glycosides. In this species, identification of the structural elements involved has been achieved (see Sections 1.1.2.d. and 1.2.3.g.). The molecular basis of other functional differences between pumps from various tissues, such as their dissimilar responses to activation by alkali cations (see Section 1.2.4.), may be due, in part, to isoform-specific structural features. However, detailed experiments addressing this issue have only recently become feasible with the development of expression systems and the application of techniques such as chimeric protein construction and site-directed mutagenesis (see Section 1.2.4.e.). It remains to be determined whether and to what extent functional differences between brain and kidney enzymes, such as the differences in EP equilibria, are related to distinct membrane lipid environments or to the primary structure of the distinct Na,K-ATPase isoforms present in these tissues.

In contrast to comparative studies in tissues which express different Na,K-ATPase isoforms, there are reports of functional differences among the same isoform expressed in different tissues. For example, behavioral differences between the Na,K-ATPases from erythrocyte and kidney, both of which express the same catalytic isoform (α_1), have been reported (for review, see Blostein, 1989). It is possible that these pumps undergo differential tissue-specific posttranslational processing, including changes that may be associated with the long-term maturation of the erythrocyte enzyme. Alternatively, it may be that distinct components of the membrane environment (such as lipids or proteins) are responsible for these behavioral differences. An example where this holds true is the significantly different pump kinetics of HK and LK sheep erythrocytes (see Section 1.2.5.d.). Thus, the unusually low activity and inhibition by K_{in} observed in LK cells is associated with a particular plasma membrane blood group antigen (the L-antigen) which is, presumably, either a pump component or a modulator of the pump.

There are several aspects regarding the aforementioned functional studies of the Na,K-ATPase which make their findings difficult to interpret. One is the use of detergents or chaotropic agents during the isolation of enzyme-enriched preparations, in particular for the purification and reconstitution of the Na,K-ATPase into proteoliposomes. While some investigators have shown that these procedures have no significant effect on the enzymes in their preparations (see, for example, Karlish and Stein, 1982), others claim that they are responsible for changes in kinetic constants (Feige *et al.*, 1988; Brodsky and Guidotti, 1990) and for the loss of cellular regulatory components of the Na,K-ATPase (Lytton, 1985a). It could be argued that these treatments alter the enzyme's function by disrupting its native membrane environment.

Also with regard to the approaches to comparative functional studies, results obtained from unsided preparations are subject to certain caveats. Thus, the well known competition between Na^+ and K^+ for the enzyme's cation binding sites may complicate the evaluation of the apparent affinities for these ions. In studies with sided preparations,

particularly those involving flux measurements in reconstituted proteoliposomes, the small surface-to-volume ratios may preclude accurate determinations of initial flux rates. Moreover, methods of purifying and reconstituting the distinct α subunit isoforms into the same lipid environment have not yet been developed.

A system which has proven extremely useful for Na,K-ATPase functional studies is the mature mammalian erythrocyte (for review, see Hoffman, 1986). Because of their structural simplicity, metabolic stability, and ease of manipulation, these cells have been used extensively over the years for pump functional studies (see, for examples, Polvani and Blostein, 1988; Sachs, 1988; Dissing and Hoffman, 1990). It is also relatively simple to manipulate erythrocytes *in vitro*, particularly with respect to altering their cation and substrate contents (see Schatzmann, 1988). Compared to reconstituted proteoliposomes or other vesicular preparations with large surface-to-volume ratios (*e.g.*, synaptosomes), erythrocytes comprise a single, large compartment with a small surface-to-volume ratio, and are thus especially well suited to flux studies.

Based on the usefulness of the mammalian erythrocyte for pump transport studies, the first goal of this research was to develop a method of delivering ion pumps, namely the Ca-ATPase of the SR and the Na,K-ATPase from various tissues into mammalian erythrocyte membranes using polyethylene glycol- (PEG-) mediated membrane fusion. An important advantage of this procedure is that enzymes are delivered into erythrocytes without the use of detergents. Another advantage is that, compared to liposomes, erythrocytes provide newly delivered enzymes with a natural or "biological" membrane environment. Moreover, these fused cells have all the advantages of red cells as discussed above.

In the case of the Na,K-pump, interference by endogenous pumps can be an issue. However this problem was circumvented by using erythrocytes from dogs, which are nearly devoid of endogenous Na,K-pumps. Alternatively, rat kidney pumps (α_1 isoform) were delivered into human or sheep erythrocytes and, using the markedly different

sensitivities of the endogenous and exogenous pumps to inhibition by ouabain, their activity was distinguished from that of endogenous pumps.

The next phase of this work was to use the microsome-fused red cell system to carry out comparative studies of pump catalytic isoforms. In one series of experiments, kidney and erythrocyte pumps, both of which contain the α_1 catalytic isoform, were used for determinations of apparent cation affinities. In another set of experiments, apparent cation affinities of rat axolemma and kidney pumps, which contain different α subunit isoforms, were compared using pumps fused into dog erythrocytes. To complement these studies, transport assays using intact HeLa cells transfected with the individual rat α subunit isoforms were also carried out to determine the apparent cation affinities of these pumps (these cells were kindly provided by Drs. J. Lingrel and E. Jewell-Motz of the University of Cincinnati).

Finally, a collaboration with Z.-C. Xu of this laboratory was carried out to investigate the possible effects of the L_p-antigen on exogenous rat kidney pumps delivered into sheep erythrocytes.

CHAPTER 2

DELIVERY OF ION PUMPS FROM EXOGENOUS MEMBRANE-RICH SOURCES INTO MAMMALIAN RED BLOOD CELLS

ABSTRACT

Using polyethylene glycol-mediated fusion of ATPase-enriched (native) microsomes with red blood cells, we have delivered sarcoplasmic reticulum (SR) Ca-ATPase and kidney Na,K-ATPase into the mammalian erythrocyte membrane. Experiments involving delivery of the SR Ca-ATPase into human red cells were first carried out to assess the feasibility of the fusion protocol. Whereas there was little detectable $^{45}Ca^{2+}$ uptake into control cells in either the absence or presence of extracellular ATP, a marked time-dependent uptake of $^{45}Ca^{2+}$ was observed in the presence of ATP in cells fused with SR Ca-ATPase. Comparison of the kinetics of uptake into microsome-fused cells *versus* native SR vesicles supports the conclusion of true delivery of pumps into the red cell membrane. Thus, the time to reach steady state was more than two orders of magnitude longer in the (large) cells *versus* the native SR vesicles. Na,K-ATPase from dog and rat kidney microsomes was fused with red cells of humans, sheep, and dogs. Using kidney microsomes fused with dog red cells which are practically devoid of Na,K-ATPase, functional incorporation of sodium pumps was evidenced in ouabain-sensitive Rb^+ uptake and Na^+ efflux energized by intracellular ATP, as well as in ATP-stimulated Na^+ influx and Rb^+ efflux from inside-out membrane vesicles prepared from the fusion-treated cells. From analysis of the biphasic kinetics of ouabain-sensitive Na^+ efflux under conditions of limited intracellular Na^+ concentration, it is concluded that the kidney pumps are incorporated into a relatively small fraction (≈15%) of the red cells. This system provides a uniquely useful system for studying the behavior of native sodium pumps in a compartment (red cell) of small surface/volume ratio. The newly incorporated native kidney pumps, while of the same isoform as the endogenous red cell pump, behave differently from the endogenous red cell sodium pump with respect to their very low "uncoupled" Na+/O flux activity.

INTRODUCTION

Although a large body of information about the behavior of membrane transport proteins has been obtained from studies of purified protein reconstituted into proteoliposomes (see, for examples, Refs. 1 and 2), it is not clear to what extent delipidation and detergent extraction alter the structure and function of such proteins. In addition, there are some serious limitations to the information obtainable using typical reconstituted membrane protein preparations. In particular, the high transporter density yet large surface-to-volume ratios characteristic of reconstituted vesicle preparations often preclude accurate assessments of initial transport rates due to very rapid changes in the intravesicular concentrations of the transported solutes under assay conditions.

To circumvent these disadvantages and to complement the information already available from studies of purified transporters reconstituted into liposomes, we have evaluated the use of polyethylene glycol (PEG) to deliver ion pumps from an enriched membrane source (microsomes) into mammalian red cells. Previous efforts to incorporate ion transporters into mammalian cells have employed reconstituted proteoliposomes as the source of the transporter. Thus, Eytan and Eytan (3) used Ca^{2+} as the fusogen to incorporate the detergent-solubilized sarcoplasmic reticulum (SR) Ca-ATPase reconstituted into phosphatidylethanolamine/cardiolipin proteoliposomes into human red blood cells. In another report of fusion of a membrane transport system into mammalian cells, Volsky and co-workers (4) fused the band 3 anion transporter, which had been co-reconstituted with Sendai virus envelopes, into plasma membranes of Friend erythroleukemic cells.

In this study, the feasibility of fusion was first demonstrated by functional transfer of the SR Ca-ATPase from SR microsomes into human erythrocytes. Delivery of Ca-ATPase into the red cell under these conditions was indicated by Ca^{2+} influx driven by ATP added to the extravesicular milieu. We then transferred the Na,K-ATPase from kidney microsomes into the plasma membranes of red cells of various species including human, low K^+ sheep, and dog, the latter two having the advantage of very low

endogenous Na,K-ATPase activity. Functional incorporation of Na,K-ATPase molecules into the red cell membrane was demonstrated by ouabain-sensitive alkali cation fluxes in cells energized by intracellular ATP, as well as by ATP-stimulated Na^+ influx and Rb^+ efflux in inside-out membrane vesicles prepared from the fused cells.

EXPERIMENTAL PROCEDURES

Ca-ATPase-enriched sarcoplasmic reticulum vesicles were prepared from rabbit fast skeletal muscle according to the method of MacLennan (5), substituting MOPS for imidazole. The vesicles were rapidly frozen in a dry ice-acetone bath and stored at -70 °C in 30% sucrose (w/w), 10 mM Tris-MOPS, (pH 6.9) until use. Na,K-ATPase-enriched kidney microsomes were prepared as described by Jørgensen (6). The specific activities of the dog and rat microsomal preparations were 5-6 and 3-4 units/mg, respectively, of which approximately 70-90% was latent, representing sealed right-side-out microsomes. These vesicles could be stored up to 4 weeks in 250 mM sucrose, 25 mM imidazole, 1 mM Tris EDTA (pH 7.2) at 0 or at -70 °C for several months without loss of activity or latency.

Blood was obtained from dogs and sheep by venipuncture using heparin as anticoagulant. Blood obtained by venipuncture from healthy human volunteers was anticoagulated with 0.1 volume of 0.1 M Tris EDTA (pH 7.4). The blood was either used immediately or stored for one or two days at 0 °C, either as whole blood or washed in isotonic saline containing 10 mM Tris-HCl (pH 7.4) and 5 mM glucose, without any apparent deleterious effect. In the latter case, the cells were resuspended and stored in 5-10 volumes of repletion buffer comprising, for human cells, 10 mM NaCl, 116 mM KCl, 27 mM potassium phosphate buffer, pH 7.4, 2 mM $MgCl_2$, 0.5 mM adenine, 1 mM inosine, and 10 mM glucose. For low-K^+ dog or sheep red cells, the repletion buffer comprised 132 mM NaCl, 8 mM KCl, 10 mM sodium phosphate buffer, pH 7.4, 2 mM $MgCl_2$, 1 mM adenosine, and 5 mM glucose.

Fusion of human red cells and SR was carried out as follows: cells were washed four times with 5 volumes of 150 mM NaCl,10 mM Tris-HCl, pH 7.4, and 5 mM glucose. The packed cells (≈70% hematocrit) thus obtained were incubated with SR vesicles for 15 min at room temperature at a ratio of 0.3-0.4 mg microsomal protein per ml of packed cells. PEG 4000 (70% w/v, dissolved in 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), and 5 mM glucose) was added gradually to the mixture to give a final concentration (w/v) of 35%. After 90 s of gentle mixing at 37 °C, the mixture was diluted with 25 volumes of either the repletion buffer prewarmed to 37 °C, or the cell wash buffer, and incubated for one h at 37 °C, or a depletion buffer in which glucose and adenosine were omitted and 5 mM 2-deoxyglucose and 5 mM iodoacetamide were added.

Fusion of red cells and kidney microsomes was carried out as described above for the SR Ca-ATPase, but at a ratio of 0.5-1.5 mg of microsomal protein per ml of packed human red cells, and for dog or sheep cells, 45% PEG was used. Dog red cells were depleted of ATP by substituting repletion buffer by an equivalent amount of depletion buffer comprising 150 mM NaCl,10 mM Tris-HCl (pH 7.4), 5 mM 2-deoxyglucose, 10 mM Na_2HAsO_4, and 68 mM sucrose, and incubation was carried out for 1.5 h at 37 °C. Following incubation, the cells were centrifuged at 1100 x *g* for 5 min at room temperature and then washed twice with 25 volumes of 150 mM NaCl,10 mM Tris (pH 7.4), 5 mM glucose, and 68 mM sucrose. Cells were finally suspended at 20-40% hematocrit as described in the figure legends.

IOV were prepared from control (mock-fused) or fused cells according to the method of Steck (7) with minor modifications (8). The percentage of IOV was estimated from the measurement of latent acetylcholinesterase activity as described elsewhere (7).

Latent and total Na,K-ATPase activity of kidney microsomes were assessed following pretreatment without and with SDS, respectively, as described by Forbush (9). ATP hydrolysis was determined by measuring $^{32}P_i$ release from [γ-^{32}P]ATP as described previously (8). The assay medium comprised 120 mM NaCl, 30 mM KCl, 30 mM

imidazole, pH 7.4, 3 mM $MgCl_2$, and 2 mM ATP. Ca-ATPase activity of the SR microsomes was carried out essentially as described by Brotherus and coworkers (10) using [γ-^{32}P]ATP and measuring $^{32}P_i$ release as described above for Na,K-ATPase. The assay medium comprised 100 mM KCl, 5 mM $MgCl_2$, 50 mM MOPS·KOH, pH 7.2, 0.1 mM $CaCl_2$, and 10 mM ATP.

Cation transport assays with cells were carried out as follows: net uptake of $^{86}Rb^+$ used as a K^+ congener was measured at 37 °C in the presence and absence of ouabain as indicated in the figure legends. Net $^{22}Na^+$ efflux from red cells was measured essentially as described by Dissing and Hoffman (11) and indicated in the figure legends. When indicated, intracellular Na^+ and K^+ content were largely replaced by choline chloride using the reversible PCMBS permeabilization method (12). Following incubation, separation of cells from medium was carried out by diluting 0.1 or 0.2 ml cells into 0.9 or 0.8 ml, respectively, ice-cold stop solution of the same composition as the medium used for assay and briefly centrifuging the cells through 0.2 ml of dibutylphthalate. Following removal of the aqueous supernatant, the walls of the tube and the oil surface were washed twice with the same buffer as that used for dilution, after which the wash and the oil were aspirated and the underlying cell pellet lysed with 1.0 ml of distilled water. One portion was taken for measurement of radioactivity by liquid scintillation counting and another, for measurement of hemoglobin concentration. The amount of cell-associated radioactivity per volume of cells was calculated either directly from the hematocrit, or on the basis of the hemoglobin concentration of each sample prior to the onset of the transport assay.

Sodium pump-mediated (ATP-dependent) $^{22}Na^+$ influx and $^{86}Rb^+$ efflux from IOV were assayed as described by Blostein (8) following equilibration of the vesicles with KCl or ^{86}RbCl overnight on ice, respectively. For $^{22}Na^+$ influx measurements, to ensure constant intravesicular K^+ concentration, the vesicles were also preincubated for 5 min with 2 μM valinomycin; the assay medium contained the same concentration of KCl as used for equilibration. For $^{86}Rb^+$ efflux assays, vesicles were diluted into a $Rb^+(K^+)$-free medium.

ATP-dependent $^{45}Ca^{2+}$ influx into SR vesicles was carried out as described by Beeler and Keffer (13). Briefly, the SR were separated from the medium by rapid filtration on Millipore type HA 0.45-μM filters and after washing with ice-cold stop solution comprising 150 mM KCl, 2 mM MOPS (pH 7.4), and 1 mM EGTA, the filter-associated radioactivity was measured by liquid scintillation counting.

Specific [^{3}H]-ouabain binding was carried out as described earlier (14). Intracellular sodium and potassium were measured by flame photometry (Instrumentation Laboratory Model 443 photometer) using lithium as internal standard. Intracellular ATP concentration was measured in neutralized trichloroacetic acid extracts of the cell suspensions by the luciferin-luciferase method (15). Protein was determined using a modification of the method of Lowry (16) and hemoglobin, by absorption at 527 nm.

Reagents were of the highest purity available. PEG 4000 (No. P-3640), was obtained from Sigma, $^{45}Ca^{2+}$ was from Du Pont-New England Nuclear and ^{22}NaCl (carrier-free), ^{86}RbCl, and $^{32}P_i$ (carrier-free) were purchased from Amersham. [γ-^{32}P]ATP was synthesized from $^{32}P_i$ according to the method of Post and Sen (17).

RESULTS

Delivery of SR Ca-ATPase into Human Erythrocytes

Transfer of Ca-ATPase from rabbit muscle SR vesicles into human erythrocytes was initially examined to assess the feasibility of incorporating functional transport proteins into red blood cells by PEG-mediated fusion. Since the calcium pumps of SR effect Ca^{2+} accumulation energized by cytoplasmic ATP, fusion should result in extracellular ATP-dependent Ca^{2+} uptake, a calcium flux opposite to that mediated by the endogenous plasma membrane Ca-ATPase.

Preliminary experiments were first carried out to determine suitable fusion conditions. The concentrations of PEG used for the various cell-microsome systems were chosen to provide optimal pump incorporation along with reasonable cell recoveries. As

shown in Fig. 1, addition of ATP to the microsome-fused cells effects a time-dependent uptake of Ca^{2+}. In contrast, in the absence of extracellular ATP, as well as with mock-fused cells (PEG-treated in the absence of microsomes), Ca^{2+} uptake was not detected. In order to ensure that the observed Ca^{2+} accumulation was truly intracellular rather than into the luminal spaces of adherent or hemifused microsomes, the kinetics of Ca^{2+} uptake into fused cells were compared with uptake into SR microsomes. As described previously (13, 18, 19) and shown in Fig. 2*a*, Ca^{2+} accumulation into SR microsomes is very rapid and reaches a plateau in less than 1 min due, presumably, to the relatively large pump density and small intravesicular volume. In contrast, Ca^{2+} uptake into the microsome-fused cells is slow (Fig. 2*b*) and close to linear with time for at least 30 min reaching a plateau only after 1-2 h (not shown), suggesting uptake into a much larger volume. With three separate preparations, it was observed that the initial rate of Ca^{2+} uptake into microsome-fused red cells ranged from 35-160 μmol/(L cells x h). Other features of Ca^{2+} uptake into cells which distinguished the process from that expected for uptake into (adherent) SR vesicles included the effects of oxalate and caffeine. Thus, the addition of oxalate which combines irreversibly with Ca^{2+} and causes a 10-fold increase in Ca^{2+} accumulation in SR vesicles, had no significant effect on Ca^{2+} uptake into the microsome-fused cells (Fig. 2*b*) even though it permeates the red cell with a half-time of 1 min (20). Similarly, as shown in Fig. 2*a*, Ca^{2+} accumulated in SR microsomes can be released upon addition of caffeine which causes a rapid Ca^{2+} efflux through ryanodine-sensitive channels. In the microsome-fused cells, however, caffeine had no such effect (Fig. 2*b*, *inset*). The lack of effect of oxalate and caffeine would not be the case if Ca^{2+} were merely accumulated in SR vesicles adhering or hemifused to the cells. The lack of caffeine effect also indicates that either the density of (newly-incorporated?) Ca^{2+} release channels is too low to effect noticeable Ca^{2+} loss or that the Ca^{2+} which enters the cells is complexed.

Fig. 3*a* shows that the initial rate of Ca^{2+} uptake into SR vesicle-fused red cells was dependent on the concentration of extracellular Ca^{2+}. Moreover, there seems to be a distinct

plateau of accumulation for each concentration of extracellular Ca^{2+} tested. This probably reflects a balance between the steady-state rates of Ca2+ influx and efflux mediated by the exogenous and endogenous pump molecules, respectively, the latter mediated by intracellular ATP (Fig. 3*b*). To further investigate this hypothesis, we tested the effect of repletion *versus* depletion of intracellular ATP. As shown in Fig. 3*b*, cells incubated in repletion buffer contained 2.5 mM ATP and accumulated much less Ca^{2+} compared to cells depleted of ATP by incubation in depletion buffer containing 2-deoxyglucose and iodoacetamide, in which case intracellular ATP was reduced to 0.01 mM.

Based on the initial amount of SR vesicles mixed with red cells (0.4 mg/ml packed cells) and the specific activity of our preparation (3 μmol of ATP hydrolyzed per mg per min at 37 °C), the efficiency of delivery of transport-competent pumps can be calculated. Thus, with the initial Ca^{2+} uptake rate ranging from 35-160 μmol/(L cells x h), if we assume that two Ca^{2+} are pumped per molecule ATP hydrolyzed, we calculate that 0.02-0.08% of the total Ca-ATPase added becomes functionally incorporated into the red cell membrane.

Some investigators (21, 22) have suggested that antioxidant impurities found in commercially available PEG, such as tocopherol, are responsible for cell lysis as well as membrane fusion. In contrast, others have failed to observe an effect of purification on the majority of commercial sources of PEG (23). As we have not detected differences in either pump incorporation or postfusion cell recovery using PEG purified according to Honda's protocol (22), we routinely used PEG as provided by the supplier.

Delivery of Kidney Na,K-ATPase into Erythrocytes

Na/K-ATPase from dog kidney microsomes was delivered into a variety of mammalian erythrocytes by PEG-mediated fusion, and the cells subsequently repleted with ATP as described under "Experimental Procedures." Given the right-side-out orientation of sodium pumps of kidney microsomes, it is expected that following delivery, like the

FIG. 1. **Time course of calcium uptake into SR microsome-fused human erythrocytes.** Fused (*triangles*) and mock-fused control (*circles*) cells equilibrated at 0 °C were added to ice-cold 154 mM NaCl containing 1 mM $MgCl_2$, 2 mM Tris MOPS (pH 7.4), and 30 μM $^{45}CaCl_2$ (1 μCi/ml) so that the final hematocrit was less than 5%. The mixture was warmed for 2 min at 37 °C, and the reaction initiated by addition of ATP (▲, ●), final concentration 1 mM, or no addition (Δ, O). Aliquots (0.1 ml) were removed at the indicated times, and cell-associated radioactivity and hemoglobin were measured as described under "Experimental Procedures."

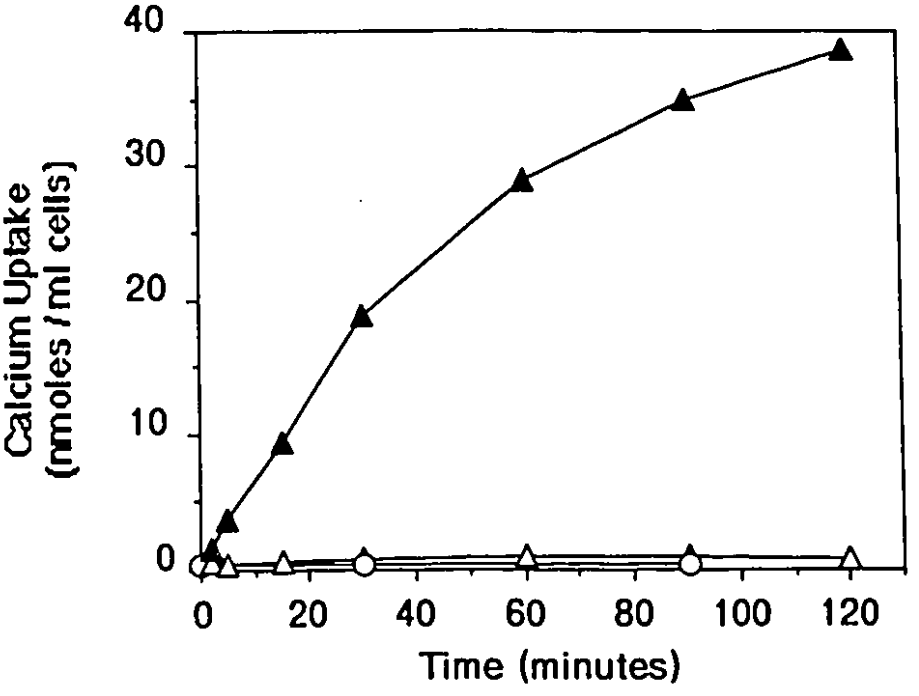
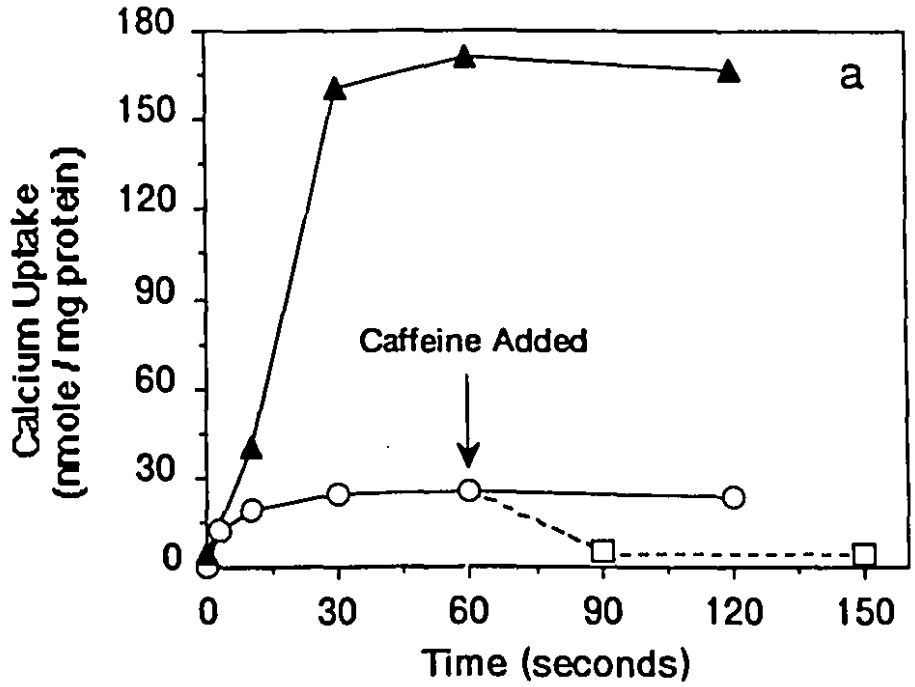

Calcium Uptake
(nmoles / ml cells)
40
30
20
10
0
0
20
40
60
80
100
120
Time (minutes)

FIG. 2. ***a.*** **Time course of calcium uptake and release in SR vesicles: effects of oxalate and caffeine.** SR vesicles (30 μg in 37.5 μl) were added to 222.5 μl of the following medium on ice: KCl (100 mM), $MgCl_2$ (5 mM), MOPS·KOH, pH 7.4 (50 mM), $^{45}CaCl_2$ (10 μM; 1 μCi/ml), in the absence (O) or presence of 5 mM oxalate (▲). The mixture was preincubated for 30 s at 30 °C, after which the reaction was initiated by the addition of 1 mM ATP. The reaction was terminated by dilution with 10 volumes of ice-cold buffer containing 150 mM KCl, 2 mM MOPS·KOH (pH 7.4) and filtered as described under "Experimental Procedures." Ten mM caffeine was added at 60 min and at the indicated times, samples removed (□).

FIG. 2. ***b.*** **Effects of oxalate and caffeine on calcium uptake into SR microsome-fused human erythrocytes.** $^{45}Ca^{2+}$ influx was measured as described in Fig. 1, in the absence (O) and presence (●) of 5 mM oxalate. *Inset*, following 90 min of incubation, aliquots were removed and diluted in 10 volumes of warmed (37 °C) medium (150 mM KCl, 2 mM MOPS, pH 7.4) containing 10 mM caffeine. Incubation was continued for 1 min.


180
150
120
90
60
30
0
Calcium Uptake
(nmole / mg protein)
a
Caffeine Added
0
30
60
90
120
150
Time (seconds)

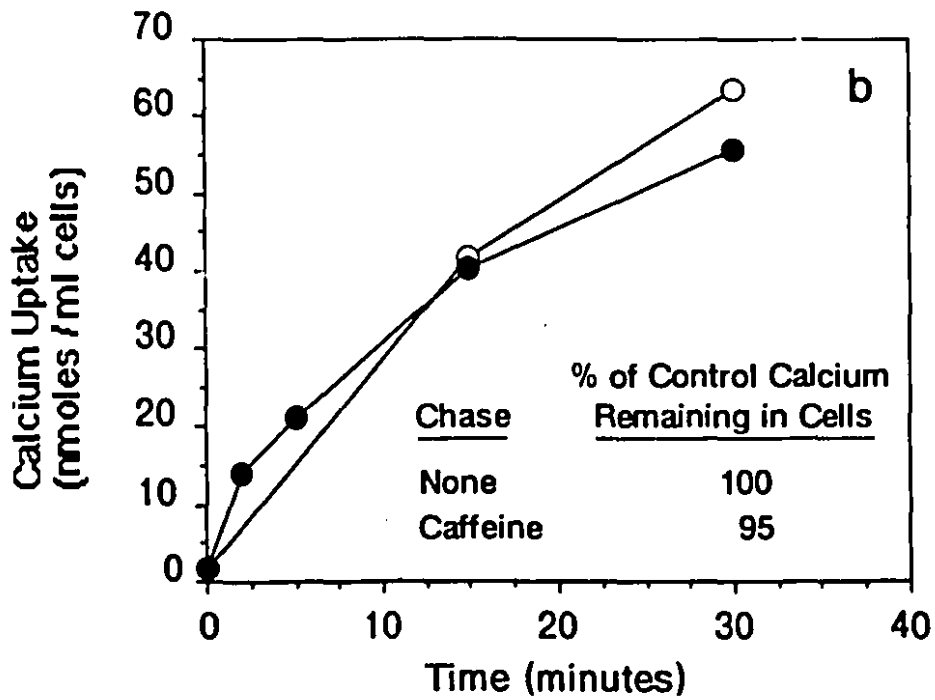
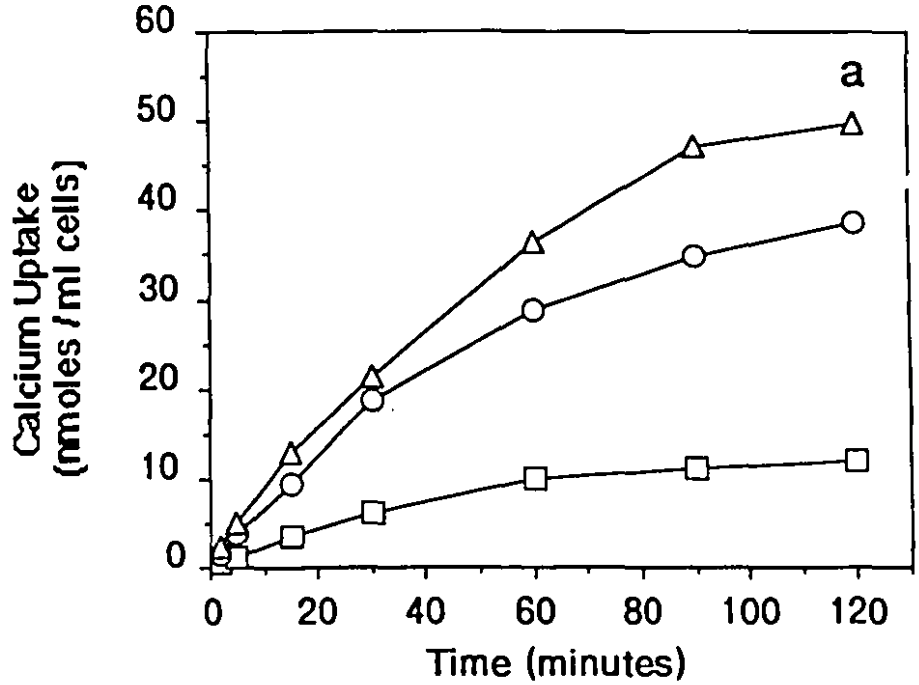
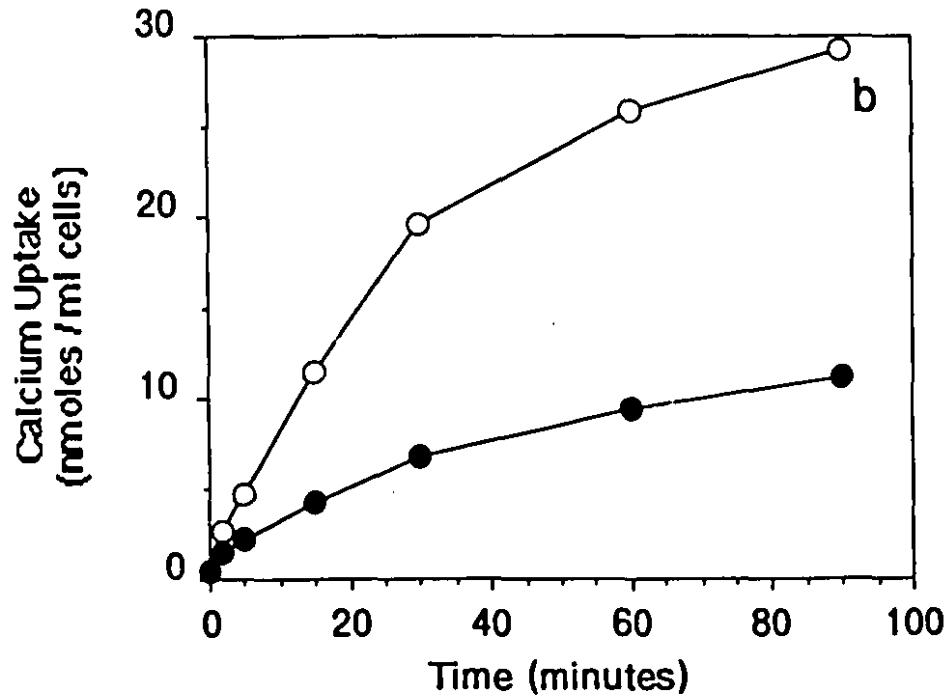

70
60
50
40
30
20
10
0
Calcium Uptake
(nmoles / ml cells)
b
% of Control Calcium
Chase
Remaining in Cells
None
100
Caffeine
95
0
10
20
30
40
Time (minutes)

FIG. 3. ***a.*** **Effect of extracellular calcium concentration on calcium accumulation by SR microsome-fused human erythrocytes.** Assays were carried out as described in Fig. 1 at the indicated concentration of $^{45}CaCl_2$: •, 5 μM; O, 30 μM; Δ, 100 μM.

FIG. 3. ***b.*** **Effect of intracellular ATP levels on calcium accumulation by SR microsome-fused human erythrocytes.** Assays were carried out as described in Fig. 1 using SR-fused cells which were depleted or repleted of ATP as described under "Experimental Procedures," resulting in intracellular ATP concentrations of 0.01 (O) and 2.52 (●) mmol/L cell water, respectively.


a
Calcium Uptake
(nmoles / ml cells)
60
50
40
30
20
10
0
0
20
40
60
80
100
120
Time (minutes)


b
Calcium Uptake
(nmoles / ml cells)
30
20
10
0
0
20
40
60
80
100
Time (minutes)

endogenous red cell pumps, they will be energized by intracellular ATP and inhibited by addition of extracellular ouabain.

Dog Red Cells. In initial experiments carried out with human erythrocytes, the increase in pump activity due to "new" (kidney) pumps was relatively small (≤50% of the endogenous pump activity). To circumvent this problem of high background activity, many of the subsequent fusion experiments were carried out by fusing dog kidney microsomes into dog erythrocytes which, when mature, are practically devoid of Na,K-ATPase activity, or by fusing rat kidney microsomes into human or sheep red cells enabling distinction of exogenous and endogenous Na,K-ATPases on the basis of their markedly different sensitivities to ouabain (see Fig. 8 below). In each case, the concentration of PEG used and time of the fusion reaction were chosen to optimize the balance between transfer efficiency and cell recovery, the latter often being the limiting factor. Thus with human red cells, PEG concentrations greater than 35% resulted in considerable (≥80%) lysis. Dog and sheep red cells were less fragile and could withstand PEG concentrations up to 50%, although the improvement in delivery with concentrations ranging from 35 up to 50% was low. Increasing the time of fusion incubation from 0.5 to 5 min did not enhance pump delivery and tended to decrease the final cell recovery (not shown). Delivery efficiency increased with the time of vesicle-cell incubation after dilution following PEG treatment; the 1-h postfusion incubation was long enough to satisfy this requirement (24) and to allow repletion or depletion of intracellular ATP.

Fig. 4 shows the ouabain-sensitive uptake of the potassium congener, $^{86}Rb^{+}$, into control (mock-fused) *versus* dog kidney microsome-fused dog erythrocytes. As expected, a significant pump activity could not be detected in control cells, whereas a substantial ouabain-sensitive $^{86}Rb^{+}$ uptake was observed in the fused cells. As shown, this uptake depends on the presence of intracellular ATP, indicating that the kidney pumps have been incorporated into the erythrocyte membrane in a normal, transport-competent fashion. If only adhering or hemi-fused vesicles were present, the catalytic sites of the ATPase

molecules would not have access to intracellular ATP and hence no $^{86}Rb^+$ uptake would be observed.

In experiments aimed to test the effect of varying the ratio of microsomes to red cells (Fig. 5), it was observed that a maximal incorporation of functional pump molecules was obtained using about 2.5 mg of microsomal membranes per ml red cells (≈70% hematocrit). Varying the specific activity of the vesicle preparation at this level of microsomes to red cells altered almost proportionately the level of incorporated pump molecules. Thus, incubating red cells (at 2.5 mg of microsomal protein per ml packed cells) with microsomes obtained from the kidney cortex (0.8 units/mg) and from the red outer medulla (2.5 units/mg), the activities of the fused cells were 130 and 300 μmol of Rb+/(L cells x h), respectively (experiment not shown). It thus appears that the main limit to pump incorporation in the experiment shown in Fig. 5 reflects an upper limit to the amount of microsomal protein that can be incorporated. However, if one compares the number of pumps delivered based on measurements of Rb^+ transport calculated as described below, to the number based on ouabain binding, it is clear from the results shown in Fig. 5 that the number of pumps associated with the membrane exceeds the number delivered by 3- to 7-fold as the microsomal protein added was increased from 0.16 to 0.80 mg/ml cells and that the incorporation reached a plateau, whereas adsorption rose steadily with increasing input of microsomal protein. In other experiments (not shown) the addition of the lectin concanavalin A prior to PEG treatment increased the number of cell-associated pumps (ouabain binding) further, without increasing delivery, such that a 15-fold ratio of associated pumps:delivered pumps was obtained.

Based on the premise that the kidney pumps have become integrated normally into the erythrocyte membrane following PEG treatment, it was reasoned that the activity of the new pumps should also be observed in inside-out vesicles derived from the microsome-fused cells. In this system, the direction of pumping is reversed; Rb^+ (K^+) is extruded from the vesicular lumen and Na^+ is accumulated in the presence of extravesicular ATP.

FIG. 4. **Ouabain-sensitive Rb^+ uptake into dog kidney microsome-fused dog erythrocytes.** Washed fused and control (mock-fused) cells were added at 0 °C to 150 mM NaCl, 10 mM Tris (pH 7.4), 5 mM glucose, 2 mM $MgCl_2$, 68 mM sucrose ± 0.1 mM ouabain. After a 2-min preincubation at 37 °C to facilitate ouabain binding, the reaction was initiated by adding 0.05 volume of 2 mM $^{86}RbCl$ (specific activity, 1 mCi/mmol). Aliquots were removed at the indicated times and assayed for cell-associated radioactivity and hemoglobin as described under "Experimental Procedures." Δ, fused, ATP-depleted cells; O, mock-fused, ATP-depleted cells; ▲, fused, ATP-replete cells; ●, mock-fused, ATP-replete cells. Intracellular ATP levels in mmol/L cell water, assuming 70% cell water, were 0.03, 0.08, 1.08, and 1.38 in each of these samples, respectively.

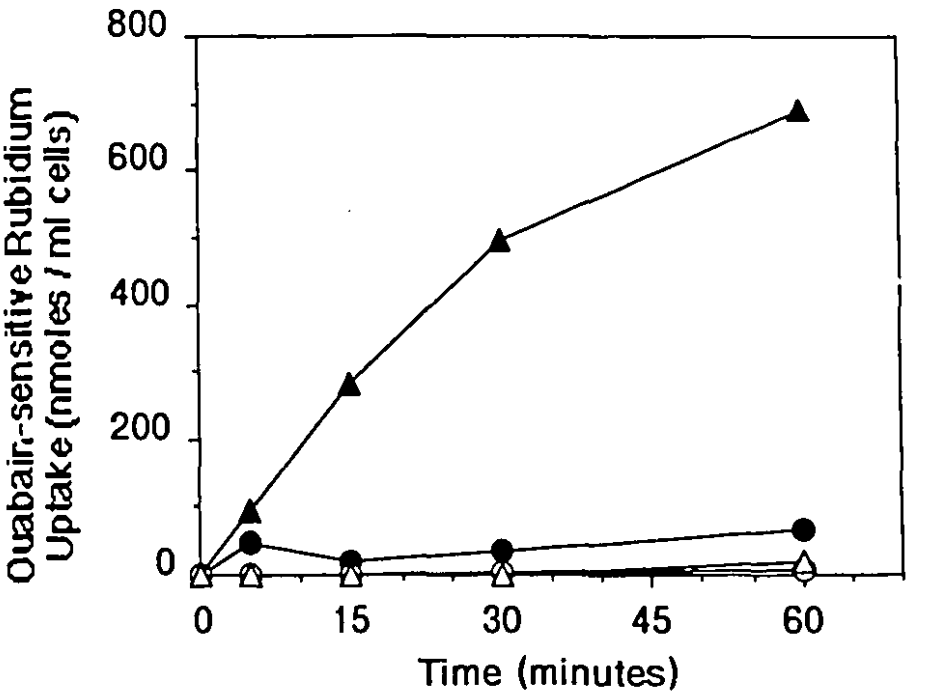
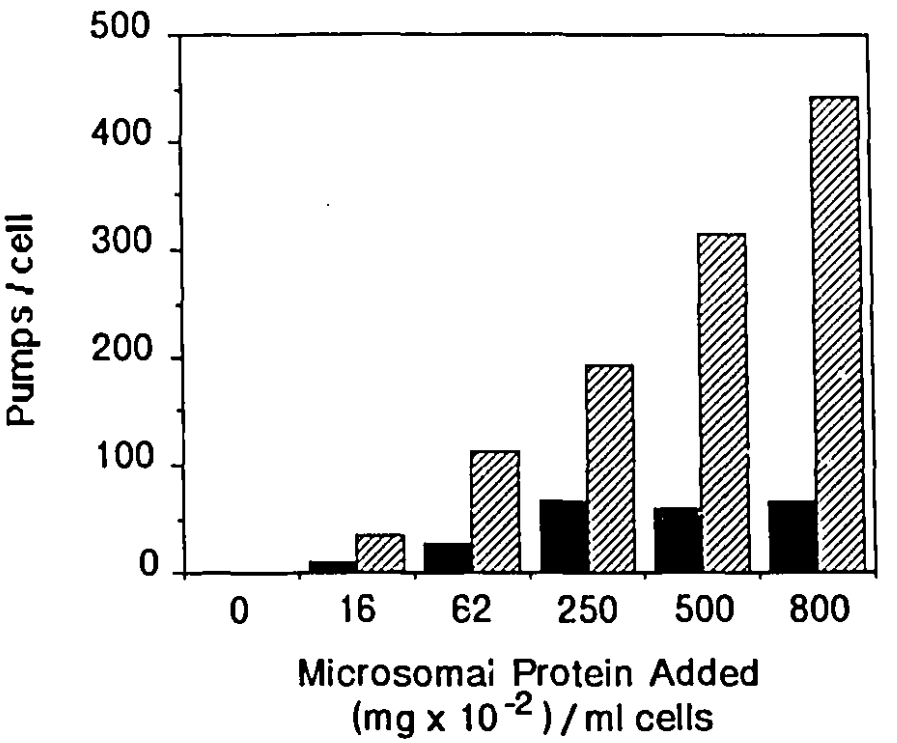

Ouabain-sensitive Rubidium Uptake (nmoles / ml cells)
800
600
400
200
0
0
15
30
45
60
Time (minutes)

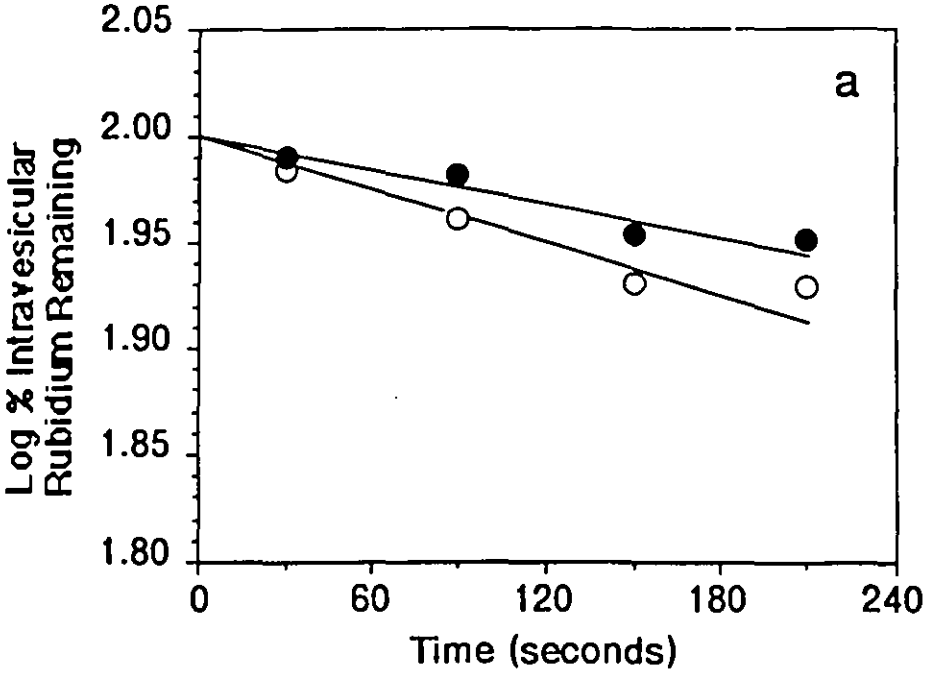
FIG. 5. **Effect of microsome concentration on ouabain-sensitive Rb^+ influx and ouabain binding to kidney microsome-fused cells.** $^{86}Rb^+$ influx (*solid bars*) and [^{3}H]ouabain (*shaded bars*) binding were carried out as described in Fig. 4 and "Experimental Procedures," respectively.


500
400
300
200
100
0
Pumps / cell
0
16
62
250
500
800
Microsomal Protein Added
(mg x 10^{-2}) / ml cells

This system has the advantage that substrates normally present in the cytoplasm are now added to the extravesicular medium and can therefore be readily controlled. Figs. 6 and 7 show the results of experiments in which $^{86}Rb^+$ efflux and $^{22}Na^+$ uptake, respectively, were measured in IOV derived from control (mock-fused) and from microsome-fused dog erythrocytes. As shown, ATP addition has no effect on fluxes in IOV prepared from control cells, consistent with their lack of pumps. In contrast, with IOV prepared from fused cells, ATP addition increased both Rb^+ efflux (Fig. 6) and Na^+ influx (Fig. 7). The inclusion of 50 μM strophanthidin during some of the sodium uptake assays caused a 70% reduction in total $^{22}Na^+$ uptake (not shown), thus confirming that the ATP-dependent flux is, indeed, due to pump activity rather than to other ATP-dependent sodium leak phenomena which have been reported in dog erythrocytes (25). Since the percentage of IOV obtained from the cells after fusion was not notably different from that obtained from unfused cells (not shown), the initial rapid but limited ATP-dependent loss of $^{86}Rb^+$ from the former (Fig. 6) suggests the presence of a small population of vesicles with a relatively large number of pumps. The possibility that this reflects a skewed distribution of pumps in the fused cells is discussed below.

As for SR Ca-ATPase, we have estimated the efficiency of delivery of the kidney Na,K-ATPase into red cells. Thus, in the experiment shown in Fig. 4, for example, 1.33 mg of kidney microsomal membranes (specific activity, 6.5 μmol/mg/min at 37 °C) were added to 1 ml dog red cells, resulting in a ouabain-sensitive Rb^+ uptake of 1.1 mmol/(L cells x h). Assuming that two Rb^+ are pumped for each molecule of ATP hydrolyzed and that the rate observed at 2 mM RbCl is about half-maximal (26), the estimated delivery is 0.13% of input of Na,K-ATPase molecules. While efforts to increase the extent of exogenous pump delivery further using increasing amounts of microsomes were unsuccessful (see Fig. 5), greater incorporation could be achieved using microsomes of higher specific activity as mentioned above. These results suggest that the ability of intact red blood cells to incorporate new membrane, or to resist incurring lysis following the

FIG. 6. **ATP-dependent Rb^+ efflux from IOV derived from dog kidney microsome-fused dog erythrocytes.** The reaction was initiated by adding 0.1 volume of $^{86}Rb^+$-loaded (+0.5 mM Rb^+) IOV from microsome-fused or control (mock-fused) red cells to 0.9 volume of prewarmed medium (30 °C) containing choline chloride (45 mM), NaCl (5 mM), $MgSO_4$ (2 mM), Tris-glycylglycine, pH 7.4 (20 mM), EGTA (0.2 mM), and ATP (0.05 mM). Aliquots were removed for measurement of vesicle-associated radioactivity as described under "Experimental Procedures." *a*, mock-fused cells with (●) and without (O) ATP; *b*, fused cells with (▲) and without (Δ) ATP.


a
2.05
2.00
1.95
1.90
1.85
1.80
Log % Intravesicular Rubidium Remaining
0
60
120
180
240
Time (seconds)

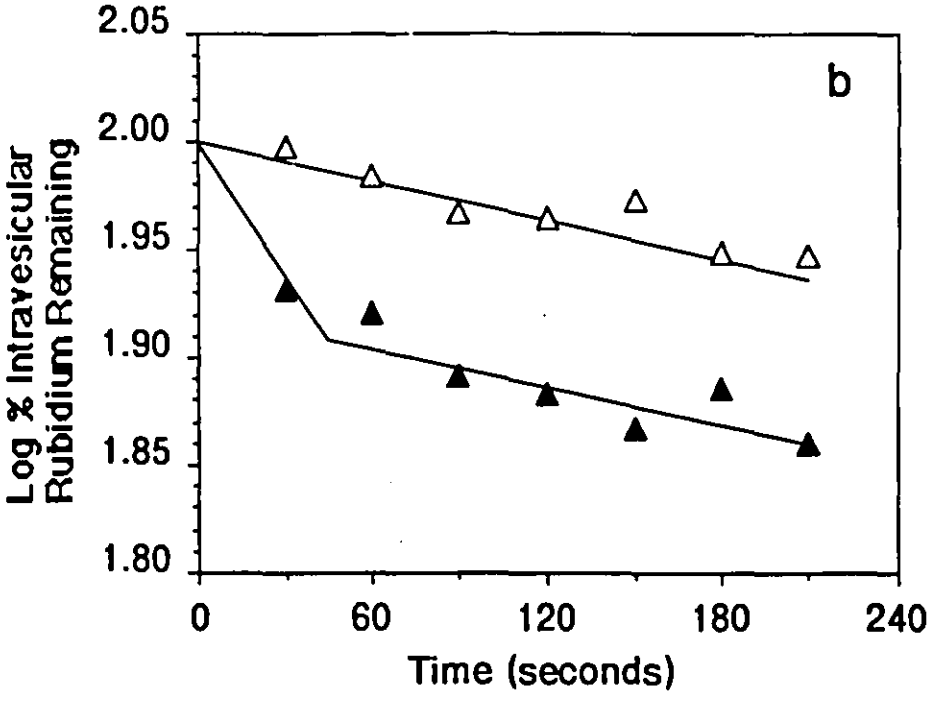
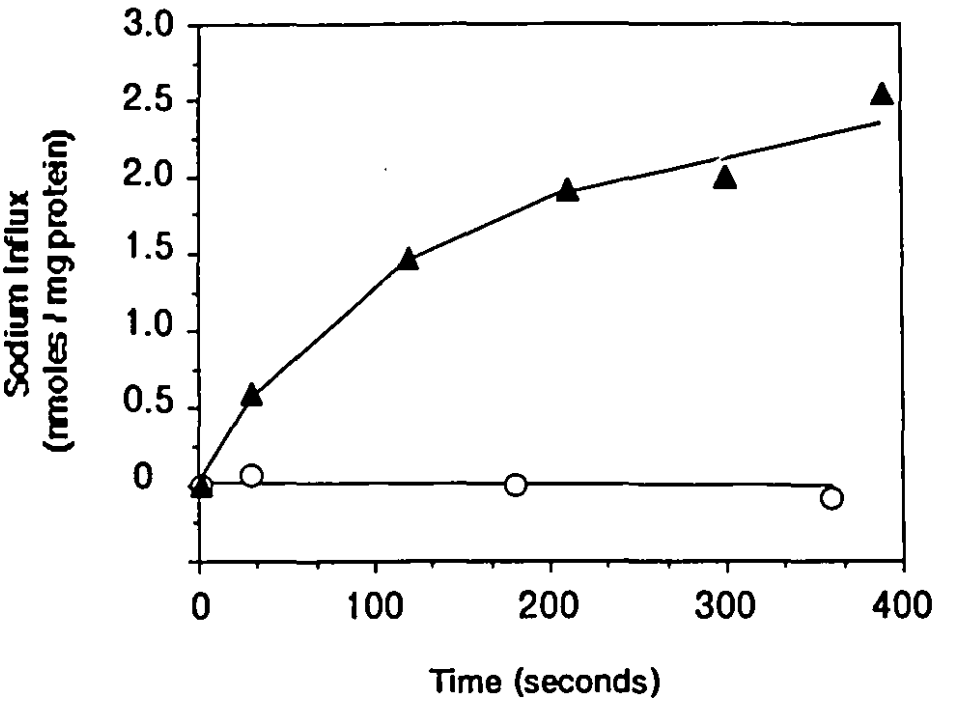

b
2.05
2.00
1.95
1.90
1.85
1.80
Log % Intravesicular Rubidium Remaining
0
60
120
180
240
Time (seconds)

FIG. 7. **ATP-dependent Na^+ influx into IOV derived from dog kidney microsome-fused dog erythrocytes.** KCl-loaded (0.2 mM) IOV (0.1 volume) derived from fused (▲) and mock-fused control (O) cells were added to 0.9 volumes warmed medium (37 °C) as in Fig. 6, except that the concentrations of NaCl and ATP were 2 and 0.1 mM, respectively.


Sodium Influx
(nmoles / mg protein)
3.0
2.5
2.0
1.5
1.0
0.5
0
0
100
200
300
400
Time (seconds)

incorporation of new membrane into their surfaces, may be rather limited (saturable) as in the case of Sendai virus fusion with erythrocyte ghosts (27).

Sheep and Human Red Cells. In other experiments, delivery of dog kidney Na,K-ATPase into sheep, human, and dog red cells and of rat enzyme in o sheep and human red cells was demonstrated (Table I). Although the specific activity of the rat microsomal preparations was always less (≈50%) than that of the dog preparations, resulting in lower activity of the newly delivered pumps, the rat enzyme has the advantage of being readily detected in the presence of pumps of nonrodent species as described below.

Delivery of rat kidney Na/K-ATPase into human erythrocytes: Distinguishing new rodent pumps by differential ouabain sensitivity

In order to compare "new" *versus* endogenous pump behavior in the same cell suspension, rat kidney pumps were incorporated into nonrodent species, namely sheep or human red cells. Since the rodent enzyme is three orders of magnitude less sensitive to ouabain than those of other species, it is possible to distinguish the endogenous and newly delivered rat kidney pumps on the basis of their sensitivity to low *versus* high concentrations of ouabain, respectively. This is shown in a representative experiment (Fig. 8) in which rat kidney pumps were delivered into human erythrocyte membranes. Although the activity of the new rat pumps is only 30% that of the endogenous pump activity, the inhibition profile is such that virtually all of the new pumps are active at 5 μM ouabain which completely inhibits the human pump. Similar results were obtained with sheep red cells (experiment not shown).

Intercellular Distribution of New Pumps.

To gain insight into the question of cell heterogeneity with respect to the level of incorporation of newly delivered pumps, pump-mediated Na^+ efflux kinetics were

examined in the microsome-fused cells. The relatively large luminal volumes of these cells facilitates resolution of the kinetic components. Since the experiments were carried out with dog cells, amiloride was used to minimize Na+ efflux via pathways distinct from the sodium pump, in particular the Na^+ fluxes due to volume regulatory Na/H exchange (28). Intracellular sodium was reduced to low levels (≈10 mM) in order to optimize the fractional loss of intracellular $^{22}Na^+$. The cells were also depleted of K^+ in order to maximize the affinity of the pump for Na^+ at its intracellular loading site(s) (11). Fig. 9*a* shows that the time course of ouabain-sensitive loss of $^{22}Na^+$ is biphasic, with ≈15% of the internal sodium being lost via ouabain-sensitive Na/K exchange, further loss being essentially ouabain-insensitive. In comparable experiments carried out with unfused high-K^+ sheep red cells with endogenous ouabain-sensitive Rb^+ influx into the total cell population similar to that of the fused cells used in the experiment shown in Fig. 9*a*, as well as unfused human red cells with somewhat higher activity, a similar early drop-off in efflux did not occur (not shown). These observations indicate that, in contrast to a relatively wide distribution of endogenous pumps of untreated cells, only a limited fraction of the microsome-fused cells acquired Na,K-ATPase molecules capable of mediating ouabain-sensitive $^{22}Na^+$ efflux. Consistent with the expectation that the higher the concentration of pumps in such a fraction, the earlier the drop-off in efflux, is the observation that a similar early depletion of a small fraction of the total intracellular Na^+ was not observed with fused dog cells with lower (≈50%) activity (not shown). Moreover, as shown in Fig. 4, using fused dog cells with normally high intracellular Na^+ and which had not been treated to reduce the intracellular Na^+ concentration, a similar rapid decrease in $^{86}Rb^+$ influx was not observed due, presumably, to the slower depletion of intracellular Na^+. The latter observation also supports the notion that the limiting substrate is intracellular Na^+ and not ATP.

TABLE I

Sodium pump activity of kidney microsome-fused red cells from various species

Fusions were carried out as described under "Experimental Procedures" and assays, as described in Fig. 4.

Cell type	Type of sodium pump		
	Endogenous	Exogenous (rat kidney)	Exogenous (dog kidney)
	mmol/(L cell x h)		
Dog	0.03[a]		2.15
Sheep (low K)	0.18	0.38	0.92
Sheep (high K)	0.61	0.26	0.72
Human	2.10	0.26	1.17

[a] Data represents ouabain-sensitive rubidium uptake.

FIG. 8. **Differential sensitivity to ouabain of endogenous and newly delivered Na,K-ATPase in rat kidney microsome-fused human red cells.** $^{86}Rb^{+}$ uptake assays were carried out with (O) control and rat kidney microsome-fused (●) human erythrocytes as described in Fig. 4, except that preincubation with varying concentrations of ouabain was carried out at 37 °C for 10 min and the reaction time was 45 min.

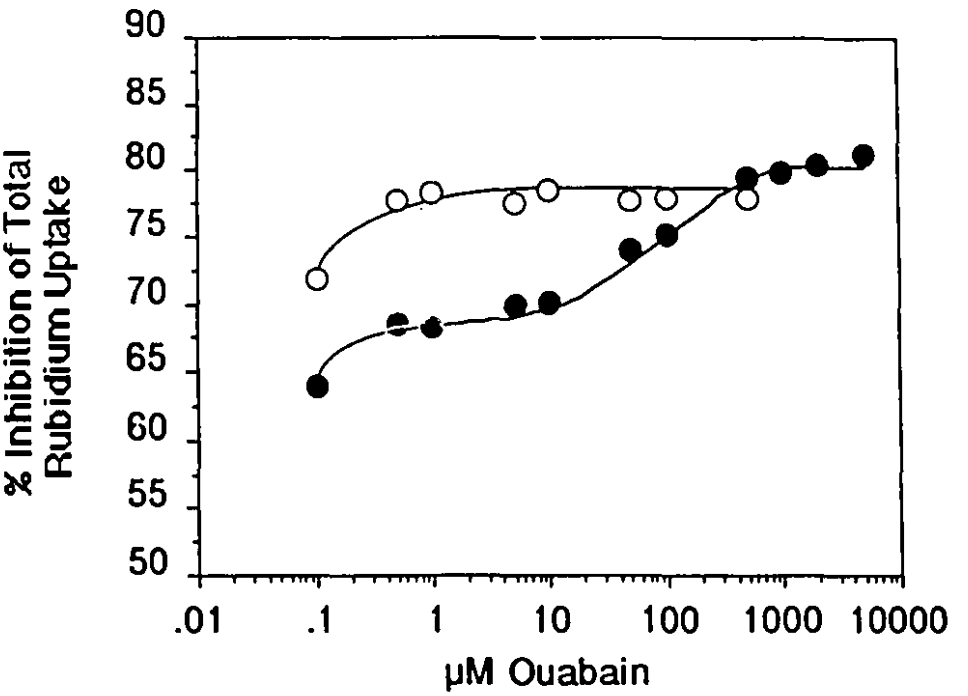
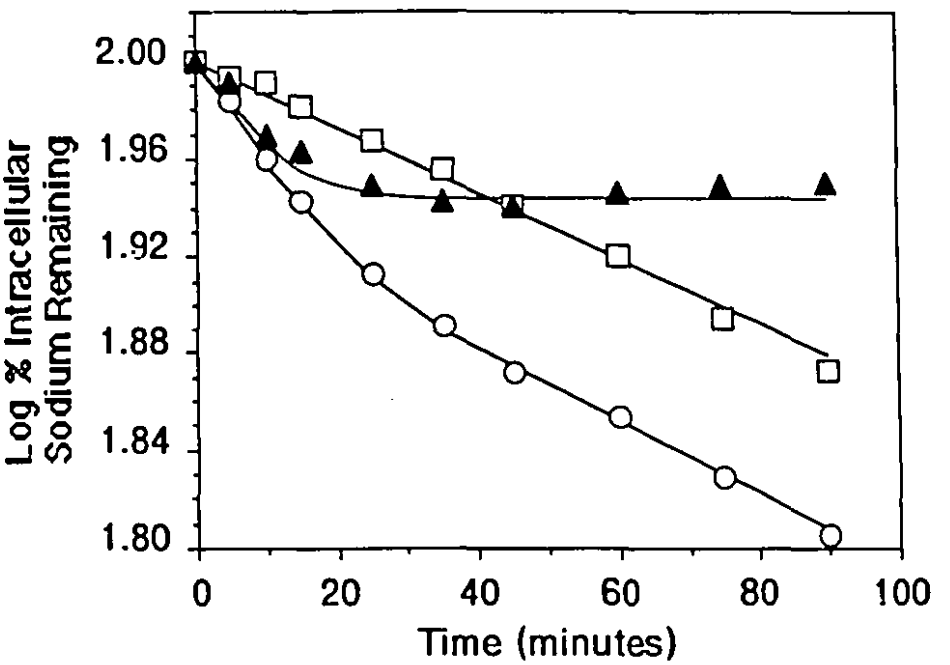
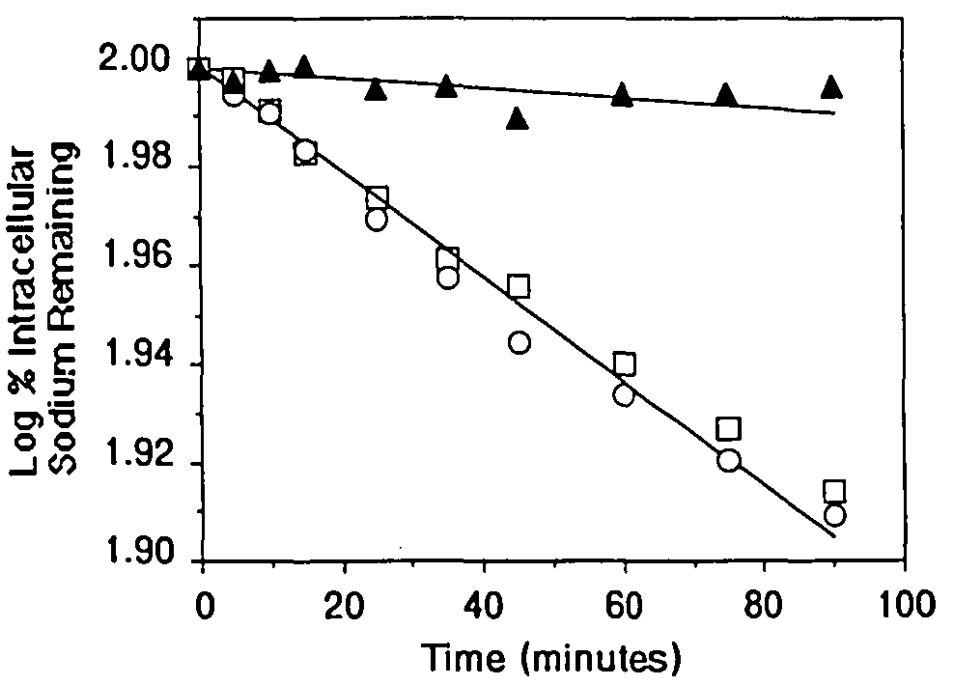

% Inhibition of Total Rubidium Uptake
90
85
80
75
70
65
60
55
50
.01
.1
1
10
100
1000
10000
µM Ouabain

FIG. 9. **Sodium efflux due to Na/K exchange and "uncoupled" (Na/O) sodium efflux from dog kidney microsome-fused dog erythrocytes.** Sodium efflux from dog kidney microsome-fused dog red cells in which the intracellular Na^+ and K^+ levels were adjusted to 10 and ≈0 mM, respectively, as described (12), was assayed at 37 °C as described by Dissing and Hoffman (11). Briefly, dog red cells (≈1 ml) which had been fused and ATP-repleted as described above were washed three times with 20-30 ml of loading solution comprising 150 mM choline chloride, 10 mM NaCl, 10 mM Tris phosphate, (pH 7.4), 10 mM glucose, 1 mM $MgCl_2$, and 68 mM sucrose. Following the last wash, this medium was supplemented with 0.5 mM adenine, 2 mM inosine, and 0.2 mM PCMBS. The cells were incubated in this medium at ≈8 °C for 36 h with gentle shaking, with two changes of the medium. The cells were then collected by centrifugation and loaded with $^{22}Na^+$ (100 μCi/ml packed cells) for 1-2 h at 4 °C. Following this treatment, they were diluted 10-fold in a similar buffer except PCMBS, adenine, and inosine were replaced by 4 mM dithiothreitol and 3 mM adenosine and incubated for 1 h at 37 °C. The cells were then washed four times and suspended at a final hematocrit of ≈1% in assay medium comprising, for Na/K exchange measurements, 150 mM choline chloride, 10 mM KCl, 10 mM Tris phosphate (pH 7.4), 10 mM glucose, 1 mM $MgCl_2$, and 68 mM sucrose. For measurements of Na/O flux, KCl was substituted by 10 mM choline chloride. Following the incubation, separation of cells from medium was carried out by diluting 0.2 ml cells into 0.8 ml ice-cold stop solution of the same composition as the medium used for assay and briefly centrifuging the cells through 0.2 ml of dibutylphthalate. Aliquots of the supernatant were taken for liquid scintillation counting and for hemoglobin determination, the latter to correct for any $^{22}Na^+$ loss due to cell lysis. In *a* the medium was free of Na^+ (Na/K exchange), and in *b* the medium was free of both Na^+ and K^+ (Na/O flux) with choline added as a substitute cation. During the course of the assay, the ouabain-insensitive loss of $^{22}Na^+$ followed a single exponential ($k = 1.4\ min^{-1}$) and was similar in the absence and presence of K^+. O, total efflux; □, efflux in the presence of 0.1 mM ouabain; and ▲, ouabain-sensitive $^{22}Na^+$ efflux.


Log % Intracellular Sodium Remaining
2.00
1.96
1.92
1.88
1.84
1.80
0
20
40
60
80
100
Time (minutes)


Log % Intracellular Sodium Remaining
2.00
1.98
1.96
1.94
1.92
1.90
0
20
40
60
80
100
Time (minutes)

Na/O Versus Na/K Exchange in Dog Kidney Na,K-ATPase

One of the interesting yet unresolved issues concerning red cell *versus* kidney sodium pump behavior is that a so-called "uncoupled" Na^+ efflux (Na/O flux) occurs in the absence of extracellular alkali cations, to an extent of about 15% of maximal Na/K exchange in the red cell, but only minimally (≈2%) in kidney enzyme reconstituted into proteoliposomes (29, 30). In the same experiment as that used to examine the kinetics of Na^+ efflux via Na/K exchange shown in Fig. 9*a*, Na^+ efflux into nominally K^+-free, Na^+-free medium was examined. As shown in Fig. 9*b*, this Na/O flux is barely detectable, that is ≤2% of the initial rate of Na/K exchange.

DISCUSSION

In studies concerned with the delivery of membrane proteins into recipient cells, various methodological approaches have been used including, for examples, the spontaneous insertion of cytochrome b_5 into erythrocytes (31), the electroinsertion of glycophorin and CD-4 receptors into erythrocytes (32), Sendai viral protein-mediated fusion of proteoliposomes into cultured cells (33, 34), Ca^{2+}-mediated fusion of proteoliposomes with erythrocytes (3, 34), and PEG-mediated cell-cell fusion (21, 35, 36), proteoliposome-cell fusion (37, 38), or microsome-cell fusion (39).

While a variety of criteria have been applied to assess protein delivery in these studies, functional incorporation of a transmembrane protein has been unequivocally demonstrated in only a few cases, and in fewer still have the relative levels of "correctly" and "incorrectly" oriented membrane-associated proteins been estimated. Studies in which protein delivery is evaluated solely by the detection of specific ligand binding cannot distinguish functional membrane proteins which have simply associated with cell surfaces from those which have adopted a native transmembrane disposition. In the case of non-solute-transporting proteins, criteria used to assess delivery have included detection of protein mobility (31), antibody-induced protein "patching" (31), and antibody-mediated cell

lysis (33, 40). These methods, however, may not be able to differentiate surface-adsorbed from incorporated protein. The transfer of the glucagon receptor from liver membranes to a foreign, adenylate cyclase-containing cell whereby transmembrane signalling has been observed (39) is an example of a system in which true functional incorporation has been clearly demonstrated.

Unambiguous demonstration of transmembrane integration is more straightforward for transport proteins which must be inserted in the target membrane with a native disposition in order to interact with specific ligands at the two membrane surfaces and to effect normal cation transport. Transfer of functional ion transport proteins into intact cell membranes has previously been reported using transporters purified and reconstituted into liposomes (3, 4). In the present study, functional incorporation has been unequivocally demonstrated using transport protein-rich microsomes which have not been delipidated or detergent-extracted. The translocation of cations energized by ATP accessible to the (originally) cytoplasmic sites of the pump molecules, and in the case of the Na,K-ATPase, the retention of Na/K exchange in IOV and the inhibition of activity by ligands (ouabain) known to perturb the enzymatic/translocation process distinguish properly delivered and transport-competent pumps from those that are simply adhering to the cells.

Our choice of the "native" Ca-ATPase from SR as a model of membrane pump delivery offered several advantages. First, the permeability of human red cells to Ca^{2+} is extremely low (41). Second, the Ca^{2+} flux mediated by exogenous inside-out oriented SR Ca-ATPase molecules can be readily distinguished from other fluxes or leaks by adding or omitting extracellular ATP. Furthermore, as shown in Fig. 3*b*, the inhibitory effect of intracellular ATP repletion on the steady-state increase in intracellular Ca^{2+}, an effect presumably mediated by endogenous red cell Ca-ATPase-mediated calcium efflux, is confirmation that the Ca^{2+} pump is functionally incorporated into the membrane of the erythrocyte such that it is delivering Ca^{2+} into the cytoplasmic space.

In an earlier report of delivery of SR Ca-ATPase into the red cell membrane (3), the authors used Ca^{2+} and other divalent cations to promote fusion of cardiolipin/phosphatidylethanolamine/phosphatidylcholine vesicles into which detergent-solubilized and purified Ca-ATPase had been reconstituted. The initial rate of Ca^{2+} uptake into cells treated with *N*-ethylmaleimide to inhibit Ca^{2+} efflux via endogenous plasma membrane Ca-ATPase was close to 100 μmol/(L cells x h). This value is of similar magnitude to those found here, without the use of *N*-ethylmaleimide (range: 35-160 μmol/(L cells x h)). However, in the previous experiments just cited (3), maximal intracellular $^{45}Ca^{2+}$ uptake was reached in about 30 min followed by a net loss. These fused cells appear to have had a considerable Ca^{2+} leak and/or the problem that reconstituted pumps are oriented, to varying proportions, in the inside-out as well as right-side-out orientation. With our system, Ca^{2+} uptake continued for more than 1 h, reaching a plateau after 1-2 h, with no evidence of a decline (not shown).

Under the conditions of these experiments, PEG is the fusion reagent of choice in that following removal by washing, it appears to have little, if any, detectable deleterious effects on red cell function as assessed by endogenous transport activity (see, for example, Table I) and overall cell metabolism as indicated by the ability to raise intracellular ATP levels upon incubation in appropriate repletion medium (see Figs. 3*b* and 4). In addition, the microsome-fused red cells can be stored in appropriate media for prolonged periods at 0° C with good retention of Na/K-ATPase function and minimal hemolysis.

In many studies, lectins have been reported to improve significantly association (42) as well as fusion (21, 38, 43, 44) of various membranes. In the present study, the lectin concanavalin A strongly increased the level of cell-associated Na,K-ATPase vesicles, without enhancing the number of cell membrane-incorporated pump molecules appearing after PEG treatment. This result is reminiscent of experiments showing that under conditions in which concanavalin A promoted agglutination of human red cells, it failed to enhance fusion efficiency (45). It should be noted that in the present case, even without

lectin, total cell-associated pumps are always in marked excess over functionally incorporated pumps (Fig. 5).

Our results provide not only unequivocal evidence for fusion of pumps into red cells, but information regarding the intercellular distribution of the new pumps. Thus, only a small fraction of cells (≈15%) exhibited rapid ouabain-sensitive Na^+ efflux at reduced internal Na^+ concentration. Consistent with the conclusion that only a small fraction of cells have pumps is the Rb^+ efflux kinetics observed in the experiment with IOV, where ATP addition caused a rapid loss of only 10% of the total intravesicular Rb^+ (Fig. 6). In contrast, with IOV from human red cells under comparable conditions and with a similar number of pumps/ml cells (46), the ATP-mediated loss was a much larger component (30%-50%) of the total intravesicular Rb+, but occurred at a rate less than one-fifth that observed with the IOV from the microsome-fused cells.

Based on the initial rate of $^{22}Na^+$ efflux of 5 mmol/(L cells x h) and assuming a turnover number of 30,000 Na^+ ions/min, the average number of pumps/cell is 225, which corresponds to 1500 pumps/cell in the estimated fraction (≈15%) of cells with pumps. Since the kidney microsomal vesicles have, on the average, 150 pumps/microsome (assuming an average microsome diameter of 0.2 μm (9) and ouabain-sensitive ATPase activity of 6 μmol/(mg x min), it might be concluded that the small fraction of cells receiving exogenous pumps have incorporated, on the average, 10 vesicles.

That the fusion process results in the delivery of pumps to only a small portion of cells is consistent with (i) large variances in the distribution of pumps/vesicle and/or in the distribution of fusions/cell and (ii) a high probability of having vesicles without pumps. These conditions are reasonable in view of the marked heterogeneity of kidney microsomal preparations with respect to size and pump density as well as the presence of contaminating membranes from organelles devoid of Na,K-ATPase (9) and the almost certain heterogeneity of peripheral red cells with respect to age-related structural and functional characteristics.

Aside from the red cell providing a compartment of sufficiently small surface:volume ratio to facilitate examination of the behavior of transport proteins delivered into them, this delivery system should be invaluable for obtaining new information about membrane transport systems. For example, it is clear that three tissue-specific isoforms of the catalytic subunit of the Na,K-ATPase have been identified, yet their functional significance and regulation remain to be determined. Even with tissues having the same catalytic isoform, for example the red cell and kidney (47), there are clear differences in their kinetic behavior, the basis of which has not been resolved thus far. A plausible explanation is that the enzyme may be regulated by specific lipid or protein component(s) of the membrane.

With our current system of examining the behavior of kidney pumps in their native state, *i.e.*, without detergent treatment and delivered "intact" into red cells, we have observed that the ratio of Na/0:Na/K fluxes mediated by the exogenous kidney enzyme molecules delivered into red cells is, indeed, very low, *i.e.*, ≤0.02 as reported previously for detergent-purified enzyme reconstituted into proteoliposomes (29, 30). Our failure to observe the substantially (≥5-fold) higher Na/0 activity reported for the red cell enzyme (11, 48-50), raises several questions. For example, it remains to be determined whether the almost negligible Na/0 flux of red cell-fused kidney pumps is due to failure of the "new" pumps to interact with a red cell-specific component normally responsible for enhancing this mode of transport to the Na,K-ATPase. Moreover, in the case of the recipient cells used in this study, it is plausible that the mature dog cell is not only devoid of endogenous pumps, but also the putative component responsible for enhancing Na/O flux. Alternatively, it is plausible that this difference in red cell and kidney pump behavior reflects distinct posttranslational modification(s). In the latter context, it must be remembered that the red cell sodium pump, like most of its intrinsic membrane proteins, is synthesized prior to the reticulocyte stage of cell development (51) and is largely removed during the subsequent maturation of the reticulocyte. The resulting mature cells of the

peripheral circulation retain pumps which have become, on the average, much "older" than those undergoing turnover as is the case of other cells including those of the kidney. Whether tissue-specific and/or time-dependent posttranslational modifications affect pump behavior and are the basis for the differences observed, remains to be determined. We are concurrently examining a number of criteria for determining whether the newly delivered pumps interact with the membrane in a manner similar to native red cell pumps.

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CHAPTER 3

FUNCTIONAL DIVERSITY OF TISSUE-SPECIFIC NA^+/K^+-PUMPS DELIVERED FROM EXOGENOUS SOURCES INTO ERYTHROCYTES

INTRODUCTION

In recent years considerable insight into the structure and tissue-specific expression of Na^+,K^+-ATPase isoforms has been obtained. However, the functional basis for the distinct forms is largely unknown. This paper describes experiments aimed to shed light on the tissue-specific kinetic behavior of the pump, in particular the activation by intracellular Na^+ and extracellular K^+. We have examined two issues: one deals with the question of whether pumps of the same isoform ($\alpha_1\beta_1$), but derived from different tissues or species, behave similarly; the other concerns the behavior of pumps from tissues of distinct isoform composition.

To address these questions, we developed a system for delivering Na^+/K^+-pumps of exogenous (microsomal) membrane sources into the structurally and metabolically simple mammalian erythrocyte using polyethylene glycol-mediated fusion (8). As discussed in that report, delivery of functional pumps was apparent in experiments showing that pumps of dog kidney microsomes fused into dog erythrocytes are fueled by intracellular ATP, and ATP added to inside-out vesicles prepared from the fused cells stimulates strophanthidin-sensitive Na^+ influx.

RESULTS AND DISCUSSION

Rat kidney pumps delivered into human erythrocytes: Comparative behavior of rat kidney and human erythrocyte pumps.

Previous studies have indicated that the isoform composition of the erythrocyte Na^+/K^+-pump, like that of the kidney, is $\alpha_1\beta_1$ (2,4). To compare the kinetic behavior of these two kinds of pumps, we have delivered the rat kidney enzyme into human erythrocytes. The activity of the exogenous kidney pumps was distinguished from that of endogenous erythrocyte pumps using low (5 μM) and high (5 mM) ouabain concentrations, respectively, as described earlier (8). The two pumps were compared with respect to the effect of extracellular K^+ (K_{ext}) on ouabain-sensitive K^+ influx using the congener $^{86}Rb^+$ as tracer. As shown in Fig. 1, kidney and erythrocyte pumps have indistinguishable behavior; for both, the apparent affinity for K_{ext} ($K_{K(ext)}$) was 0.16 mM.

[Extracellular Na^+ was maintained at a constant low (5 mM) concentration].

The effect of intracellular Na^+ concentration (Na_{in}) on ouabain-sensitive $^{22}Na^+$ efflux was tested using a modification (7) of the nystatin permeabilization method (1) to alter Na_{in} concentration ($[Na]_{in}$). These experiments were complicated by the fact that exogenous pumps are delivered into only a small fraction of the cells (8). As a result, the relatively high pump-mediated Na^+ efflux from the pump-rich cells decreases their $[Na]_{in}$ to concentrations lower than those of the whole cell population. Therefore, values for the apparent affinity for Na_{in} ($K_{Na(in)}$) obtained from plots of pump rate *versus* (measured) $[Na]_{in}$ must be larger than the 'true' value. Discrepancies between observed and 'true' values should, however, diminish as V_{max} decreases. Thus, in 7 independent experiments summarized in Fig. 2, values of $K_{Na(in)}$ of the exogenous kidney pumps decreased directly with V_{max}; $K_{Na(in)}$ for the erythrocyte pump was 2.8 mM, independent of V_{max}. At low V_{max}, the plot of $K_{Na(in)}$ values for the kidney pumps intersects that of the erythrocyte pumps. We conclude, therefore, that the Na^+/K^+-pumps of the rat kidney and the human erythrocyte have similar affinities for intracellular Na^+.

Comparison of rat axolemma and rat kidney pumps.

In experiments aimed to examine K_{ext} and Na_{in} activation of pumps of tissues of distinct isoform composition, rat kidney and axolemma microsomes were fused into dog erythrocytes which are almost devoid of pumps. Since the levels of exogenous pump activity were relatively low ($V_{max} \leq 1$ mmoles/L/hr; cf. activity of the rat kidney pump fused into human erythrocytes shown in Fig. 2), we assumed that deviations of $K_{Na(in)}$ from the 'true' $K_{Na(in)}$ were minimal. The results of these experiments indicate a 3-fold lower $K_{K(ext)}$ and a ≈3-fold higher $K_{Na(in)}$ for the axolemma compared to kidney pumps. Based on measurements of the proportion of total axolemma Na^+/K^+-ATPase which was relatively insensitive to ouabain, we concluded that the α_1 isoform constitutes ≈10% of the total axolemma Na^+/K^+-ATPase. From comparisons of the immunological reactivity (quantitative densitometry of Western blots) of rat axolemma, kidney and muscle, the latter comprising α_1 and α_2 isoforms quantified from ouabain-sensitivity profiles of EP

formation, we estimated that α_3 comprises ≥70% of the remaining ouabain-sensitive activity in axolemma.

CONCLUSION

In the present study, we show that pumps of the same isoform composition but derived from at least two distinct species (human *versus* rat) and tissues (erythrocyte *versus* kidney) have similar cation activation kinetics.

The kinetic differences between axolemma and kidney pumps probably reflect the distinct behavior of the α_1 and α_3 isoforms since α_3 is the predominant isoform in axolemma. These flux experiments are consistent with earlier studies of the cation activation kinetics of the membrane Na^+/K^+-ATPase of isoform-specific transfected HeLa cells. In those experiments, a 3-fold higher K_{Na} and a ≈1.5-fold decrease in K_K were observed (5). More recent studies concerned with the transport characteristics of these transfected cells have revealed similar differences, albeit of greater magnitude, *i.e.*, a >5-fold higher $K_{Na(in)}$ and a ≈3-fold lower $K_{K(ext)}$ for α_3 compared to α_1 or α_2.

In contrast to the aforementioned differences between axolemma and kidney pumps, studies of Na^+/K^+-ATPase activities of rat kidney, brain and pineal gland (predominantly $\alpha_3\beta_2$ pumps) led Shyjan *et al.* (9) to conclude that α_3 has a higher affinity for Na^+ than α_1 pumps, and similar affinities for K^+. Whether these dichotomies reflect an effect of the (distinct) β subunit, consistent with a kinetic effect of the β subunit described recently (6), remains to be determined. Even though β_2 is present in rat axolemma and pineal glands, we cannot rule out a difference in α-β pairing, for example; mainly $\alpha_3\beta_1$ in axolemma and $\alpha_3\beta_2$ in the pineal gland.

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FIG. 1 **Activation of endogenous human erythrocyte pumps and exogenous rat kidney pumps by $K_{ext.}$** Rat kidney microsomes were fused with human erythrocytes and assays were carried out using $^{86}Rb^+$ as a congener of K^+ as described previously (8). Endogenous human pump activity (open circles) was distinguished from exogenous rat pump activity (solid triangles) by the addition of 5 μM or 5 mM ouabain, respecti /ely, during a 5-minute preincubation.

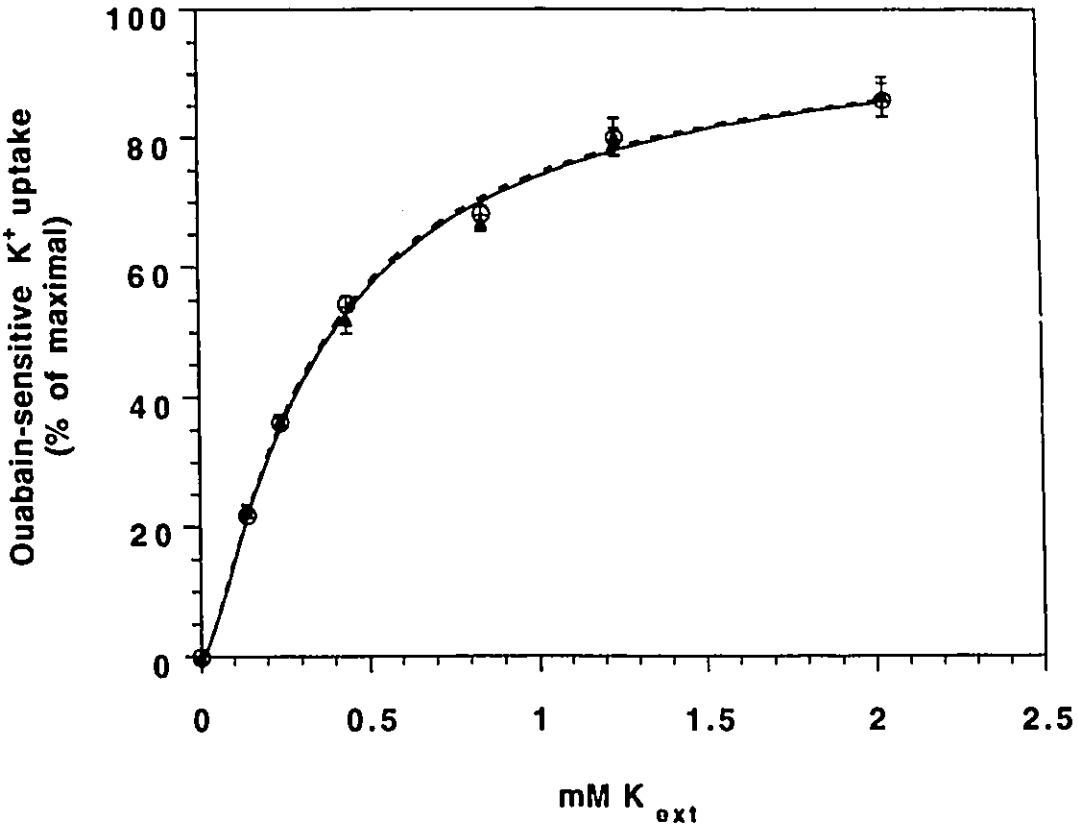

Ouabain-sensitive K+ uptake
(% of maximal)
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
mM K ext

FIG. 2. **Activation of endogenous human erythrocyte pumps and exogenous rat kidney pumps by Na_{in}: Relationships between $K_{Na(in)}$ and V_{max}.** The Na_{in} concentration of the fused cells was varied using nystatin with constant (10 mM) KCl and varying choline chloride to maintain osmolarity, and then equilibrated with $^{22}NaCl$ essentially as described by Mairbaurl and Hoffman (7). Endogenous pumps (open circles) and exogenous pumps (solid triangles) were distinguished as described in Fig. 1. $^{22}Na^+$ efflux assays were carried out as described previously (8). Kinetic parameters were determined as described in Table I.

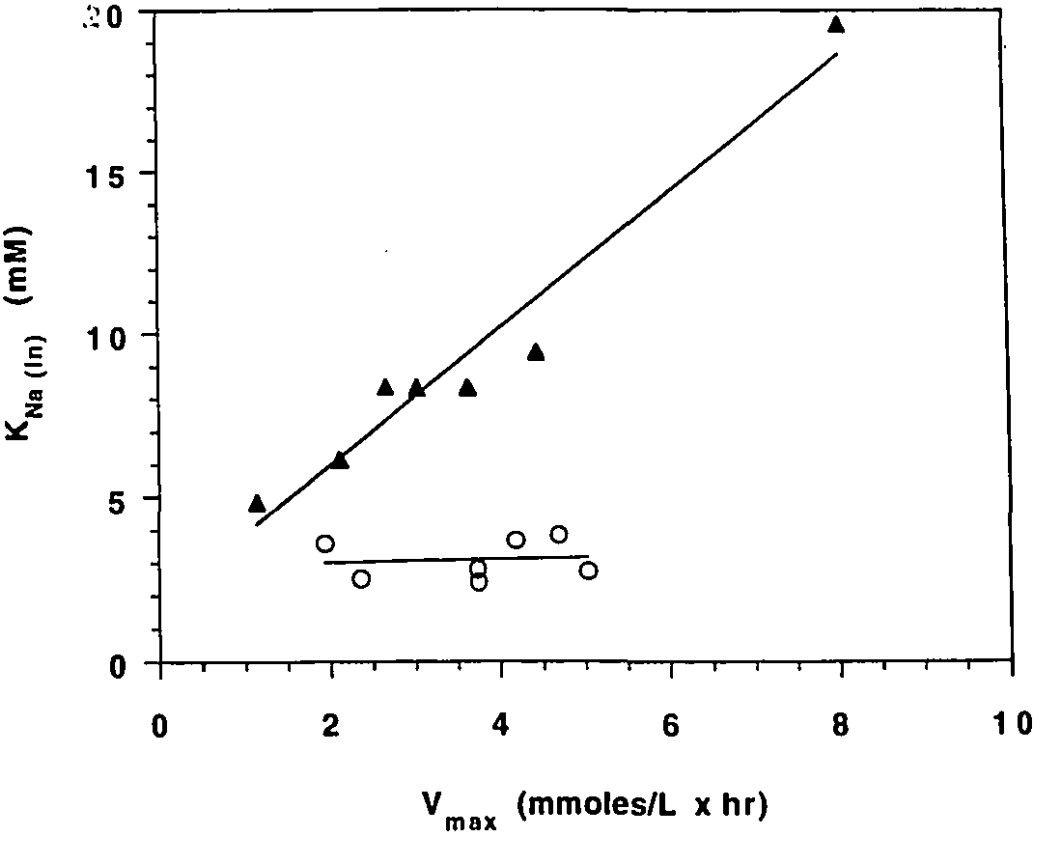

20
15
10
5
0
$K_{Na\ (in)}$ (mM)
0
2
4
6
8
10
V_{max} (mmoles/L x hr)

TABLE I

Intracellular Na^+ and extracellular K^+ dependence of rat kidney compared to axolemma Na^+/K^+-pumps

Kinetic constants were determined by the best fit parameters to non-linear regression plots using the non-cooperative substrate binding model of Garay and Garrahan (3). Values shown for $K_{K(ext)}$ and $K_{Na(in)}$ are the means ±S. D. of 4 and 2 separate experiments, respectively.

Fused Cell System	Apparent K_{cation}	
	$K_{K(ext)}$	$K_{Na(in)}$
Rat kidney/dog erythrocytes	0.37 ± 0.04	0.35 ± 0.09
Rat axolemma/dog erythrocytes	0.12 ± 0.04	1.10 ± 0.28

CHAPTER 4

TISSUE- AND ISOFORM-SPECIFIC KINETIC BEHAVIOR OF THE NA,K-ATPASE

ABSTRACT

The objective of this study has been to delineate the side-specific effects of Na^+ and K^+ on the transport kinetics of tissue-specific Na/K pumps. Two experimental systems have been used. In one, Na/K pumps of exogenous microsomal membrane sources (rat axolemma, kidney) were delivered by membrane fusion into dog erythrocytes, and in the other, the three isoforms of the catalytic subunit of the rat enzyme were individually transfected into HeLa cells as in previous studies (Jewell, E. A., and Lingrel, J. B. (1991) *J. Biol. Chem.* 266, pp.16925-16930), with the α_2 and α_3 isoforms rendered relatively resistant to ouabain by site-directed mutagenesis. Whereas the kidney microsomes comprise the α_1 catalytic isoform, the axolemma microsomes were predominantly α_3 (≈60%) with lesser amounts of α_2 (≈25%) and α_1 (≈15%) as measured by the ouabain-sensitive profile of phosphoenzyme as well as by immunoblotting with isoform-specific antibodies using membranes of known specific activity as standards (α_1 of kidney; α_1 and α_2 of muscle). Both systems were analyzed with respect to the effects of varying concentrations of cytoplasmic Na^+ and extracellular K^+ on pump-mediated $^{86}Rb^+(K^+)$ influx. With the individual isoform-transfected HeLa cells and monensin added to vary and control the intracellular Na^+ concentration, differences in apparent affinities of the α_3 isoform compared with the α_1 and α_2 isoforms were observed, *i.e.*, a ≈3-fold higher affinity for extracellular K^+ and ≈4-fold lower affinity for cytoplasmic Na^+. Thus, in the presence of 10 mM extracellular Na^+, apparent $K_{0.5}$ values for extracellular K^+ activation of $K^+(Rb^+)$ influxes were 0.22 ± 0.02 mM for α_1, 0.20 ± 0.02 mM for α_2, and 0.09 ± 0.01 mM for α_3. At high intracellular K^+ (≥100 mM) and saturating extracellular K^+ concentrations, apparent $K_{0.5}$ values for cytoplasmic Na^+ activation were 17.6 ± 1.1 mM for α_1, 19.7± 1.0 mM for α_2 and 63.5 ± 9.1 mM for α_3. The functional differences observed with the individual isoform-transfected cells were completely consistent with the kinetic differences observed with the axolemma and kidney pumps fused into erythrocytes. Axolemma pumps had a ≈3-fold lower $K_{0.5}$ for extracellular K^+ and a ≈2-fold higher $K_{0.5}$

for cytoplasmic Na^+. In the HeLa transfectants, differences in affinities for cytoplasmic Na^+ were associated with differences in the steady-state intracellular Na^+ concentration, *i.e.*, 27.5 mM in α_3-transfected cells compared with 15.7 mM and 19.7 mM in α_1- and α_2-transfected cells, respectively.

INTRODUCTION

The sodium-potassium ATPase (Na,K-ATPase or Na/K pump) is an ubiquitous membrane protein complex which couples the hydrolysis of ATP to the transport of Na^+ and K^+ ions against their respective electrochemical gradients. The complex comprises a 110 kDa catalytic α subunit and a smaller 35 kDa β subunit. Three isoforms of α have been identified, cloned and sequenced; three forms of β have also been sequenced, two (β_1 and β_2) in mammals and another called β_3, in amphibia (for reviews, see Refs. 1 and 2). These subunits are expressed in a tissue-specific as well as maturation-dependent manner (3). Most tissues express α_1 and β_1, consistent with the idea that the $\alpha_1\beta_1$ complex reflects the expression of the housekeeping genes. It is also clear that α_2 is found mainly in skeletal muscle, adult heart and nervous tissue, whereas α_3 is detected predominantly in excitable tissue. Except in tissues expressing only α_1 (kidney, for example), β_1 and β_2 are usually present in most tissues, although the nature of tissue-specific α-β pairing is an unresolved issue.

The primary structures of all the isoforms have been known for several years, and regions of certain distinct catalytic functions, in particular the ATP binding and phosphorylation sites, have been identified. However, issues regarding tissue- or isoform-specific functional differences have remained largely unresolved and controversial. In general, differences in apparent affinities for K^+ were either absent or small while studies of Na^+ effects indicated a somewhat higher apparent affinity (up to 2-fold) of the brain compared with kidney enzyme (for review, see Ref. 1). Although these results have been supported by more recent experiments with rat brain, pineal gland and kidney (4), they are at variance with those obtained using the individual isoforms expressed in cultured cells (5). In the latter study, the α_2 and α_3 isoforms of the rat enzyme were each rendered relatively ouabain-resistant (≈3 orders of magnitude more resistant to ouabain, as is the case for the native rat α_1 isoform, than the endogenous HeLa cell enzyme) by site-specific mutagenesis and then individually expressed in HeLa cells and selected for resistance to

ouabain. With membranes isolated from these cells, the authors observed a 3-fold lower apparent affinity for Na^+ and a small difference in affinity (1.5-fold higher) for K^+ of α_3 compared to α_1 and α_2.

In the present study, we have addressed the issue of isoform-specific differences in ligand interactions by studying the side-specific effects of the activating alkali cations on transport activity. By using these sided preparations, complications arising from concurrent changes in cation effects at stimulatory as well as inhibitory sites are minimized. Two systems of pump insertion into recipient cells have been used. One is the mammalian erythrocyte into which tissue-specific Na/K pumps from microsomal vesicles were incorporated by polyethylene glycol-mediated fusion (6). Thus, Na,K-ATPase enriched microsomes from rat kidney (α_1 isoform) and rat axolemma (≈60% α_3 with lesser amounts of α_2 and α_1) were incorporated into Na,K-ATPase-devoid mature dog erythrocytes. The advantage of the fused cell system is that the mammalian erythrocyte has long been the model of a simple, metabolically stable cell, and has been used extensively to characterize pump kinetics. Moreover, we have shown elsewhere that the behavior of a particular isoform is not significantly different from tissue to tissue (kidney *versus* erythrocyte) or even among different mammalian species (rat *versus* human). Thus, with rat kidney microsomes fused into human red cells, kinetic differences between the human erythrocyte ($\alpha_1\beta_1$ form; 7, 8) and rat kidney enzyme (also $\alpha_1\beta_1$) could not be detected (9). With the fused cells, we have assessed both the effects of varying extracellular K^+ (K_{ext}) and intracellular Na^+ (Na_{in}) on K^+($^{86}Rb^+$) influx.

The other system is the individual rat α subunit isoform-transfected HeLa cell which offers the unique opportunity of characterizing the behavior of each of the three α isoforms separately (5). With these cells, the kinetic behavior of transport-competent pumps expressed at the cell surface was also studied by measuring pump-mediated $^{86}Rb^+$(K^+) influx, using monensin to vary and equilibrate intracellular Na^+. We show that with both systems, essentially identical functional differences are observed, namely that the

α_3 isoform has a higher (3-fold) apparent affinity for K_{ext} and lower (≥3-fold) affinity for cytoplasmic Na^+.

EXPERIMENTAL PROCEDURES

Membrane preparations. Na,K-ATPase-enriched microsomes from rat kidney outer medulla were prepared as described by Jørgensen and Skou (11), and those from rat axolemma, according to Sweadner (12). The vesicles were stored in 250 mM sucrose, 25 mM imidazole and 1 mM EDTA-Tris, pH 7.2, either on ice for 2 weeks or frozen at -70 °C for several months without loss of activity. Protein was determined using a detergent-containing modification (13) of Lowry's original method (14). Total and latent enzyme activities were assayed as described by Forbush (15). The specific activities of both preparations were in the range of 2-4 μmoles ATP hydrolyzed/(mg protein·min); 80-90% of the activity of kidney microsomes, and 20-30% of that of the axolemma microsomes, was latent, representing sealed, right-side out microsomes. A plasma membrane-enriched fraction of rat muscle membranes was a kind gift from Dr. Amira Klip, Hospital for Sick Children, Toronto, and was also suspended in the above storage buffer and stored at -70 °C.

Microsome-fused erythrocytes. Blood was drawn from the jugular vein of healthy mongrel dogs into heparin. The red blood cells were washed three times by centrifugation and then suspended in buffered NaCl comprising 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 5 mM glucose. The fusion procedure and subsequent ATP repletion were carried out with fresh cells as described by Munzer *et al.* (6). The fused cells were then washed three times in wash buffer (as above, plus 2 mM $MgCl_2$ and 68 mM sucrose) and suspended at a hematocrit of 10-20% at 0 °C in the same medium as that used for the transport assay (see below).

HeLa cells. HeLa cells were transfected with different isoforms of the α catalytic subunit of the Na,K-ATPase as described previously (5). Wild-type cells were maintained

in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and transfected cells were grown in the same medium with 1 μM ouabain added (5).

Transport assays. Unidirectional Rb^+ influxes into microsome-fused cells were measured and calculated as described by Munzer *et al.* (6) using $^{86}Rb^+$ as a congener of K^+, with the following modifications. The assay medium contained 145 mM choline chloride, 5 mM NaCl, 10 mM P_i-Tris (pH 7.4), 5 mM glucose, 2 mM $MgCl_2$, 68 mM sucrose, 0.2 mM amiloride without or with 5 mM ouabain. To 200 μl of assay medium were added 37.5 μl of cells and, following a 5-min preincubation at 37 °C, the assay was initiated by adding 12.5 μl of medium of the same composition but with various concentrations of KCl as indicated and 0.25-0.5 μCi of $^{86}RbCl$. The reaction was carried out for 15 minutes at 37 °C during which time the rate of Rb^+ uptake remained practically constant (not shown). At the end of the reaction, aliquots were removed and separated from the medium by centrifugation through dibutylphthalate. Cell lysates were subsequently prepared for the determination of hemoglobin and radioactivity, and the uptake rates were expressed on the basis of the packed cell volume as described previously (6). For experiments in which extracellular Na^+ was varied, choline chloride was added to maintain isotonicity. Reversible nystatin permeabilization as described elsewhere (10) was used to vary intracellular Na^+ with choline chloride as substitute.

Transport assays in HeLa cells were carried out by measuring K^+ uptake, using $^{86}Rb^+$ as a congener, on cells grown to 40-60% confluence in 24-well Falcon tissue culture plates as follows. For assays with varying $[K]_{ext}$, cells were rinsed three times at 37 °C with a wash solution comprising 140 mM choline chloride, 10 mM NaCl, 4 mM P_i-Tris, pH 7.4, 5 mM glucose, and 0.5 mM $MgCl_2$ (solution A), with 4 μM monensin added. They were then preincubated for 5 minutes at 37 °C in 0.5 ml of the same solution, with the following additions: 10 μM bumetanide to decrease ouabain-insensitive Rb^+ uptake and either 0.1 mM or 10 mM ouabain (base-line activity). The medium was then aspirated and 0.2 ml fresh medium containing the same components (solution A plus monensin,

bumetanide and ouabain) plus various concentrations of KCl (0.05-1.20 mM) with $^{86}RbCl$ (0.25 μCi) as tracer were added. The transport assay was carried out for 10 minutes and then terminated by aspirating the medium and washing the cells three times with ice-cold solution A. The cells were then solubilized for ≈40 min with 0.5 ml 0.2 M NaOH. Aliquots were removed for the determination of radioactivity by liquid scintillation spectrometry and protein, according to the method of Lowry (14).

For assays of the effects of varying intracellular Na^+ (Na_{in}) concentration ($[Na]_{in}$), the procedure was similar except that the wash medium contained 12 μM monensin and the preincubation and assay media contained 4 mM KCl, 12 μM monensin and 2-70 mM NaCl with choline chloride added to maintain isotonicity. Determination of intracellular cations was carried out simultaneously on cells similarly treated except that they were grown in 110-mm tissue culture dishes (see below and legend to Fig. 5).

Enzyme assays. Assays of ATP hydrolysis and phosphoenzyme were carried out essentially as described by Blostein (16), with the following modifications. The assay medium for ATP hydrolysis was that described elsewhere (6) and comprised 120 mM NaCl, 30 mM KCl, 30 mM imidazole, pH 7.4, 5 mM $MgCl_2$, and 2 mM ATP. For the determination of phosphoenzyme, the membranes were first permeabilized and pre-treated with ouabain as follows: muscle membranes diluted to 0.25 mg/ml were permeabilized by preincubation for 10 min at room temperature in medium containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 50 mM choline chloride, 2.5 mM $MgCl_2$ and 0.33 mg/ml saponin; axolemma membranes were treated with SDS (0.65 mg/ml) in the presence of bovine serum albumin as described by Forbush (15). Aliquots (20 μl) of the permeabilized membranes were then diluted 1:1 with 1.5 mM Tris-P_i, pH 7.4, containing 1.5 mM $MgSO_4$ without or with ouabain, preincubated for 10 minutes at 37 °C and then chilled. Phosphorylation reactions were initiated by adding 60 µl of reaction medium containing (final concentrations) 0.5 μM [γ-^{32}P]ATP, 5 mM Tris-HCl, pH 7.4, and 50 mM NaCl to 40 μl membranes, both equilibrated for 2 min at room temperature, followed by a 5-s

reaction at room temperature. Values of total phosphorylation obtained at various ouabain concentrations, expressed as a percentage of that obtained in the absence of ouabain, were analyzed by fitting the data to a two-compartment (muscle membranes) or three-compartment (axolemma membranes) model using a non-linear least-square analysis of the sum of 2 or 3, respectively, general logistic functions (17) as described elsewhere (18).

SDS-Polyacrylamide gel electrophoresis and immunoblotting. Immunoblotting using isoform-specific antisera was carried out following SDS-polyacrylamide gel electrophoresis of rat kidney, axolemma, and muscle membranes using 7.5% gels as described by Laemmli (19). Proteins were transferred to nitrocellulose or Immobilon membranes which were then blocked overnight at 4 °C in 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 0.1% Tween-20 containing 5% skim milk powder. The immunologically reactive bands were developed first with α subunit-specific antibodies and then with peroxidase-conjugated secondary antibodies (provided with the Amersham Corp.ECL kit) which were either anti-rabbit IgG or anti-mouse IgG, depending on the primary antibody used (see below). Densitometry was carried out using a SciScan 5000 Scanner (United States Biochemical Corp.) and software. The antibody to the α_1 isoform was a mouse monoclonal IgG raised against the dog kidney enzyme; the α_3-specific antiserum was a rabbit polyclonal IgG raised against a synthetic peptide corresponding to amino acids 2-14 of the rat α_3 Na,K-ATPase subunit coupled to keyhole lympet hemocyanin. These antibodies were generous gifts from Dr. M. Caplan (Yale University). Antiserum specific for the α_2 subunit was a rabbit polyclonal IgG raised against a fusion protein (derived from the *Escherichia coli* trpE gene plus the α_2 rat Na,K-ATPase gene) and was purchased from UBI, Syracuse, NY.

Other analytical procedures. Intracellular Na^+ and K^+ analyses on cell extracts were carried out by flame photometry (Instrumentation Laboratory model 443 photometer) using 15 mM $LiNO_3$ as internal standard. Erythrocyte extracts (see above) were prepared by lysing the cells in at least 20 volumes of 15 mM $LiNO_3$, followed by a 2-min microfuge

spin to pellet the membranes. HeLa cell extracts were obtained as described by Haber *et al.* (36) except that the cells were washed at 0 °C with isotonic choline chloride. For erythrocytes, values of $[Na]_{in}$ and intracellular K^+ ($[K]_{in}$) were expressed on the basis of the packed cell volume determined from concomitant measurements of lysate hemoglobin divided by the mean cell hemoglobin concentration of the original cell suspension. For HeLa cells, the values were expressed either on the basis of the cell protein of the solubilized cells, or as a millimolar concentration estimated from the ratio of $[Na]_{in}$ to total ($[Na]_{in}$ plus $[K]_{in}$), assuming that the latter was close to isotonic (160 mM).

Intracellular ATP concentrations were measured in neutralized trichloroacetic acid extracts of the cells using the luciferin-luciferase method (20).

Kinetic analysis of data. The data for Na^+ and K^+ activation of $Rb^+(K^+)$ influxes were analyzed using the following Michaelis-Menten model for either (i) a non-cooperative two-site (extracellular K^+ activation) or three-site (cytoplasmic Na^+ activation) model as described by Garay and Garrahan (21), *i.e.*,

$$v = V_{max}/(1 + K_s/[S])^n \qquad \text{(Eq. 1)}$$

where n is the number of sites, and K_s is the apparent affinity, *i.e.*, $K_{K(ext)}$ for extracellular K^+ and $K_{Na(in)}$ for intracellular Na^+; or (ii) a cooperative model, *i.e.*,

$$v = V_{max}[S]^n/(K^n_{0.5(s)} + [S]^n) \qquad \text{(Eq. 2)}$$

where $K^n_{0.5(s)}$ is the $K_{0.5}$ value, *i.e.*, $K_{0.5(Kext)}$ for extracellular K^+ ($n = 2$) and $K_{0.5(Nain)}$ for cytoplasmic Na^+ ($n = 3$).

RESULTS

Our first approach towards elucidating the tissue-specific transport properties of the Na/K pump in sided preparations was to compare the transport characteristics of membranes derived from tissues of markedly distinct isoform composition. For this purpose, Na,K-ATPase-rich microsomes were isolated from rat kidney and axolemma and delivered into dog erythrocytes using polyethylene glycol as fusogen as described previously (6). Dog erythrocytes have the unique advantage that their endogenous ouabain-sensitive cation fluxes are negligible since they are almost devoid of Na,K-ATPase.

In the experiment shown in Fig. 1 the effect of varying $[K]_{ext}$ on Na/K pump activity (K^+ influx) of microsome-fused cells was measured using $^{86}Rb^+$ as a congener of K^+. The results shown are representative of 4 separate experiments in which kidney and axolemma microsomes were fused simultaneously into separate portions of the same washed dog erythrocytes. This was done in order to minimize experimental variability that might arise from using blood samples from different dogs or even different samples from the same dog. In order to optimize the proportion of $^{86}Rb^+$ taken up by the cells, extracellular Na^+ was reduced to 5 mM so that the concentration of K^+ could be varied within a relatively low concentration range (0.2 to 4 mM). $[Na]_{in}$, which was saturating since dog erythrocytes are high-Na^+ cells ($[Na]_{in}$ >100 mM), decreased <10% after 20 min under these conditions (not shown). As well, the intracellular ATP concentration measured in samples removed after the 1-hour repletion (6), and just prior to the flux assays, was close to saturation (0.5 - 1.0 mM; experiments not shown).

In the representative experiment shown in Fig. 1, the apparent affinity of the axolemma Na/K pumps for K_{ext} ($K_{K(ext)}$ = 0.12 mM) is nearly 3 times higher than that of kidney pumps ($K_{K(ext)}$ = 0.33 mM). These kinetic constants were based on a model (Equation 1) of identical non-interacting K^+ sites. $K_{0.5(Kext)}$ values for axolemma and kidney, based on a cooperative model (Equation 2) also differed by a factor of ≈3 (see legend to Fig. 1). Although the absolute values of these constants increased with increasing

extracellular Na^+ concentration, the ≈3-fold difference between kidney and axolemma enzymes persisted (not shown). Thus, in an experiment with 0.5, 5, 25 and 100 mM extracellular Na^+, the respective values of $K_{K(ext)}$ determined using Equation 1 were 0.06, 0.13, 0.33 and 0.71 mM for axolemma pumps, and 0.12, 0.39, 0.85 and 1.72 mM for kidney pumps.

In an analogous series of experiments, $[Na]_{in}$ was varied using nystatin, which allows equilibration of choline as well as alkali cations. In this way, the cells were loaded with a constant, relatively low amount of K^+ (20 mM) to ensure minimal changes in K_{ext} due to possible K^+ losses during the flux assay; choline chloride was added to maintain a constant (150 mM) intracellular chloride concentration. The choice of only a moderately low intracellular K^+ concentration was also based on preliminary experiments which showed that with K_{in} reduced to very low levels (≤2.0 mM), the apparent affinity for Na_{in} is increased to the extent that the pump is saturated at ≤10 mM Na_{in}, precluding accurate determinations of the apparent affinity for Na_{in} within a range of $[Na]_{in}$ that is technically feasible to achieve.

In the representative experiment shown in Fig. 2, the apparent $K_{Na(in)}$ or $K_{0.5(Nain)}$ of axolemma pumps (1.32 mM, Equation 1; 3.70 mM, Equation 2) is ≈2-fold higher than that of kidney pumps (0.41 mM, Equation 1; 2.14 mM, Equation 2). In this series of experiments, the activities of the exogenous pumps were relatively low [V_{max} ≤ 1 mmol/(L-cells·h)], which was fortuitously advantageous for the following reason. We showed elsewhere that exogenous pumps are delivered into only a small fraction of the cells (6). As a result, Na^+ is rapidly extruded from the pump-rich fused cells resulting in a lower $[Na]_{in}$ in those cells compared with $[Na]_{in}$ of the entire cell population. Therefore, values of $K_{Na(in)}$ obtained from plots of pump rate *versus* (measured) $[Na]_{in}$ are larger than the "true" values. However, such discrepancies diminish as V_{max} decreases, becoming negligible at V_{max} values ≤ 1 mmoles/(L-cells·h) (*cf.* Fig. 2, Ref. 9). Therefore, in the present experiments, any discrepancies between observed and true values of $K_{Na(in)}$ would

FIG. 1. **Activation by extracellular K^+ of rat kidney and rat axolemma pumps fused into dog erythrocytes.** Fused, ATP-repleted cells were preincubated for 5 min with or without ouabain after which $^{86}Rb^+(K^+)$ influx was assayed as described under "Experimental Procedures". Ouabain-sensitive K^+ influx values for the representative experiment shown are the means ± S.D. of triplicate determinations. The curves were drawn through Equation 1 (non-cooperative model) with $K_{K(ext)}$ values of 0.38 mM (kidney) and 0.12 mM (axolemma). Using the cooperative model (Equation 2), $K_{0.5(Kext)}$ values were 0.60 (kidney) and 0.22 (axolemma). ▲, kidney-; O, axolemma-fused cells.

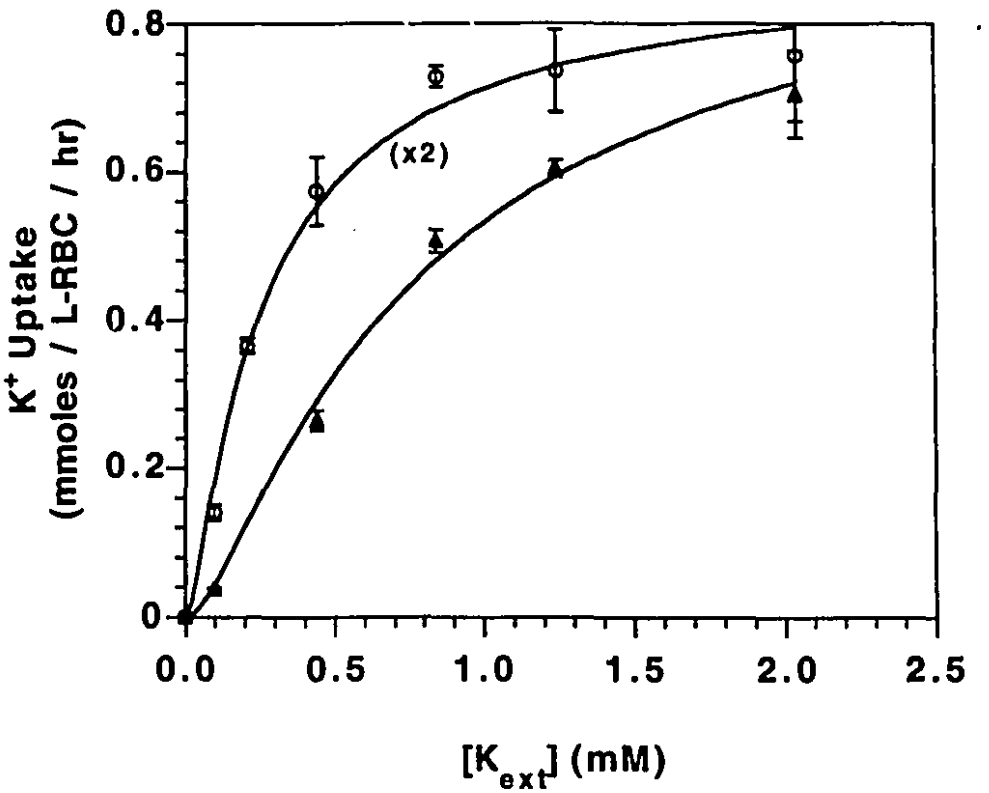
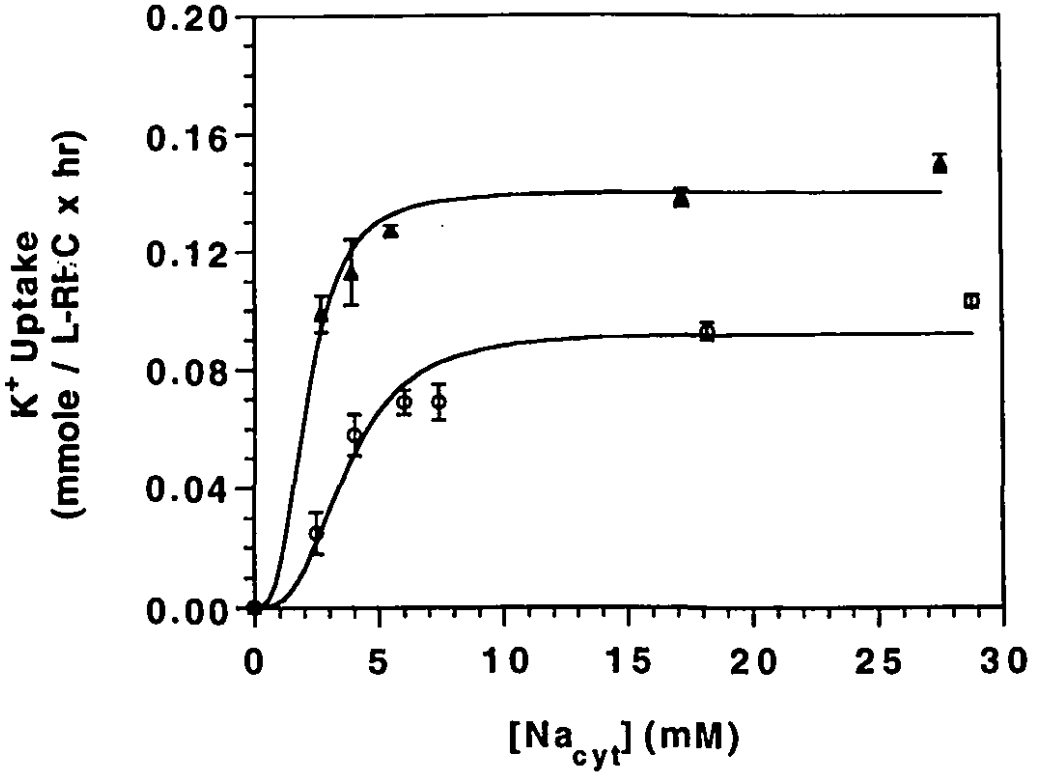

0.8
0.6
0.4
0.2
0
0.0
0.5
1.0
1.5
2.0
2.5
(x2)
K+ Uptake
(mmoles / L-RBC / hr)
[Kext] (mM)

FIG. 2. **Activation by intracellular Na^+ of rat kidney and rat axolemma pumps fused into dog erythrocytes.** Fused, ATP-repleted cells were loaded with 20 mM KCl and various amounts of NaCl (2.5 - 40 mM) and choline chloride to maintain constant tonicity (150 mM) using the nystatin permeabilization procedure, and assayed as described in Fig. 1 except that the concentrations of NaCl and KCl in the assay medium were 2.5 and 0.5 mM, respectively. Data points for the representative experiment shown are the means ± S.D. of triplicate determinations. The curves were drawn through Equation 1 with $K_{Na(in)}$ values of 0.41 mM for kidney and 1.32 mM for axolemma. Using Equation 2, $K_{0.5(Nain)}$ values were 2.1 mM for kidney and 3.7 mM for axolemma. ▲, kidney-; O, axolemma-fused cells.


0.20
0.16
0.12
0.08
0.04
0.00
K+ Uptake
(mmole / L-Rt:C x hr)
0
5
10
15
20
25
30
[Na cyt] (mM)

be small, particularly for axolemma-fused cells which had the lower activity. Accordingly, the true difference in $K_{Na(in)}$ between kidney and axolemma pumps may be, if anything, larger than that shown in Fig. 2. In fact, the $K_{Na(in)}$ of the kidney pumps may be overestimated due to the effect of amiloride added to inhibit Na/H exchange of dog erythrocytes (22). Thus, we observed a slight (15%) inhibition of Na,K-ATPase activity of kidney and axolemma at high Na^+ concentration, but a greater inhibition (≈45%) of the kidney but not axolemma enzyme at low Na^+ (preliminary experiments, not shown). This means that the true $K_{Na(in)}$ for the kidney pumps may be even lower than that observed.

To relate the foregoing kinetic differences between kidney and axolemma pumps to the isoform composition of these tissues, we quantified the amounts of the three isoforms in our axolemma preparations in the following manner. In one series of experiments (Fig. 3), the effect of varying ouabain concentration on the steady-state levels of Na^+-dependent phosphoenzyme, EP, was determined. The enzyme was first preincubated for 10 min at 37 °C with Mg^{2+}, P_i and various concentrations of ouabain after which a 5-s phosphorylation was carried out at room temperature. Since K^+ increases the rate of ouabain-enzyme dissociation and hence the IC_{50} for ouabain inhibition, these measurements of EP in K^+-free medium have the advantage of increasing the ouabain-sensitivity. As shown in Fig. 3, total ouabain-sensitive EP comprises three components. The largest fraction (component 1) has the highest affinity, consistent with the likelihood that it is the α_3 isoform (as discussed below). A small fraction (component 3) has low sensitivity and represents α_1, and the rest corresponds, presumably, to α_2. As shown in Table I, when the ouabain-sensitive EP profile is analyzed according to a three-site model, the fractional amounts of components 1, 2 and 3 were 65, 27 and 8% of the total, with IC_{50} values of 3.67×10^{-9} M, 2.36×10^{-7} M, and 3.49×10^{-5} M, respectively. (The fit of the data to a two-site model was less good). Similar axolemma microsomal preparations have been previously characterized by Urayama and Sweadner who reported low levels of α_1 (12) and 50-60% of the total activity as α_3 (23). The conclusion that α_3 is the component with highest affinity for ouabain is

consistent with evidence for two high-affinity components of rat brain Na,K-ATPase (see, for examples, Refs. 18 and 24) and, more importantly, with recent studies of ouabain binding to the isoforms individually transfected into baculovirus-infected insect cells (25) and NIH 3T3 cells (26). These authors showed that α_3 has a higher affinity for ouabain than α_2.

An alternative procedure for quantifying the proportion of different isoforms in axolemma was based on immunoblot analysis using membranes of known isoform composition and specific activity as "standards. For these experiments, we used antibodies specific for each of the three isoforms, with no cross-reactivity among the isoforms (preliminary tests, not shown). In one series of experiments, increasing amounts of kidney microsomes were used as α_1 standards, and samples of axolemma membranes of known total specific activity were subjected to SDS-polyacrylamide gel electrophoresis on the same gel and immunoblotted with anti-α_1 antibody. With the kidney enzyme, the intensity/unit enzyme of each band was obtained from the linear portion of plots of band intensity *versus* protein. From this value, the specific activity of α_1 and hence percentage α_1 in axolemma was estimated. A similar immunoblot comparison was carried out using plasma membranes of rat skeletal muscle which comprise only the α_1 and α_2 isoforms (27). From this estimate of the specific activity of α_1 in muscle taken together with the α_1/α_2 ratio obtained from the ouabain-sensitivity profile of EP in the muscle membranes (assayed as described for axolemma in Fig. 3, but with the data fitted to a two-site model, (not shown)), the specific activity of muscle α_2 was determined. These muscle membranes were then used as a 'standard' for estimating α_2 in immunoblots of axolemma using anti-α_2 specific antiserum. As shown in Table I, α_1 comprises 14 ± 11% and α_2, 25 ± 4% of the total axolemma activity. The remainder (61%) is, presumably, α_3. These values and those obtained from the ouabain-sensitive EP profile of the axolemma microsomes are summarized in Table I. The agreement between these two estimates of the proportions of the three isoforms gives

FIG. 3. **Effect of varying ouabain concentration on the phosphoenzyme (EP) levels of rat axolemma membranes.** Membranes, permeabilized with SDS, were preincubated for 10 min at 37 °C with Mg^{2+}, P_i and the indicated concentrations of ouabain and then phosphorylated for 5 s at room temperature with 50 mM NaCl and 0.5 μM [γ-^{32}P]ATP as described under "Experimental Procedures". The results were fitted to a three-component general logistic function as described under "Experimental Procedures". Values are the means ± S.D. of triplicate determinations (*error bars* are within the *symbols*) and the results shown are representative of 2 independent experiments. The IC_{50} values for components 1, 2 and 3 of the average of two experiments are 3.67×10^{-9} M, 2.36×10^{-7} M, and 3.49×10^{-5} M, respectively.

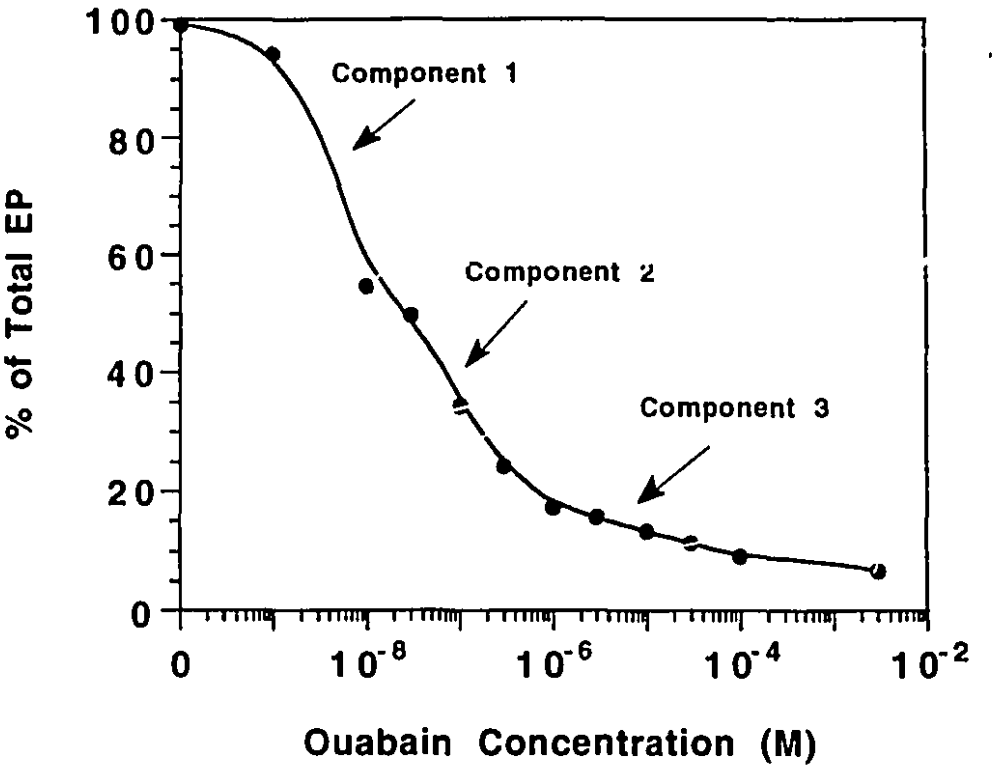

100
80
60
40
20
0
% of Total EP
Component 1
Component 2
Component 3
0
10-8
10-6
10-4
10-2
Ouabain Concentration (M)

TABLE I

Distribution of α_1, α_2 and α_3 isoforms in rat axolemma microsomes

In one series of experiments (A), the assay of phosphoenzyme (EP) sensitivity to varying ouabain concentrations was carried out as described in Fig. 2 and the data analyzed using a three-component model. In the other series of experiments (B), specific activities of α_1 and α_2 in axolemma were obtained from measurements of immunological reactivities using kidney microsomes of known specific Na,K-ATPase activity as a standard for α_1 and muscle plasma membranes as a standard for α_2 as described under "Results". In a representative experiment, the specific activity of α_1 in muscle was 0.25 μmoles/(mg·min), based on the immunological reactivity ratio of muscle/kidney and the known specific Na,K-ATPase activity of kidney membranes (2.2 μmol/(mg·min) in this case). This value multiplied by the EP the ratio of α_1/α_2 (1.17 according to the ouabain-sensitivity profile of muscle EP carried out as described for axolemma in Fig. 2, but using a two-component model) gave a specific activity of 0.29 μmol/(mg·min) for muscle α_2. Values are the averages ± S.D. of the number of experiments shown in parentheses. Activity of α_3 was calculated as the total specific activity of the preparation minus that of α_1 plus α_2.

Estimation Method	% of total axolemma Na,K-ATPase activity		
	α_1	α_2	α_3
A. EP sensitivity to ouabain	7.8 ± 0.6 (2)	27.1 ± 4.2 (2)	65.1 ± 3.7
B. Western blots	14.2 ± 11.1 (3)	24.8 ± 4.0 (2)	61.0 ± 8.3

credence to the conclusion that α_3 predominates (≈60% of the total enzyme) in our axolemma membrane preparations.

Comparison of pump activity of isoforms individually transfected into HeLa cells. The foregoing kinetic analysis using sided preparations of kidney and axolemma pumps revealed differences in relative affinities for both K_{ext} and Na_{in} which, in turn, are different from those reported in a number of comparative studies of Na,K-ATPase activity of permeable membranes isolated from varicus tissues (see, for examples, Refs. 28-33). In contrast, the results are qualitatively similar to those of a comparative analysis of the kinetics of Na,K-ATPase activation by Na^+ and K^+ using membranes derived from HeLa cells individually transfected with the three α isoforms (5), particularly with respect to the response to varying Na^+ concentration.

To further define the functional differences among the individual isoforms, ion flux measurements were carried out with the same transfected HeLa cell lines as those used in previous studies of Na,K-ATPase kinetics (5). Transport assays have advantages over Na,K-ATPase determinations using unsided membrane preparations in that they reflect the activity of pumps which are delivered to the plasma membrane in a transport-competent state; as well, their kinetic behavior is minimally complicated by competing cation effects. In these experiments, ouabain-sensitive $^{86}Rb^+(K^+)$ influx was measured in medium containing various concentrations of Na^+, with monensin added to equilibrate Na^+. Cells were washed and preincubated with monensin at 37 °C (*cf.* Ref. 34) in order to equilibrate Na^+ for sufficient time to allow any transient pH changes to subside prior to the initiation of the reaction with $^{86}Rb^+(K^+)$ as discussed by Stimers *et al.* (35). The use of monensin to alter cell Na^+ has been thoroughly investigated (36) and in the present studies, we did not observe any deleterious effect of monensin on either the non-pumped (ouabain-insensitive) Rb^+ fluxes or ATP levels, at least within the period of the experiments (preliminary experiments, not shown). In the experiments with varying K_{ext}, extracellular Na^+ was maintained at a relatively low concentration in order to maximize the fractional uptake of

$^{86}Rb^+(K^+)$ as in experiments with the microsome-fused erythrocytes. With 10 mM extracellular Na^+, $[Na]_{in}$ rose to 24 mM following the monensin pre-treatment and remained relatively constant throughout the transport assay.

In the representative experiment shown in Fig. 4, the curve was fit using the non-cooperative model (Equation 1). Since the internal Na^+ activation sites were not saturated at $[Na]_{in}$ ≈24 mM, particularly with regard to the α_3 isoform (see below), values are expressed as a percentage of the V_{max} predicted by the curve fit (see legend to Fig. 4). Mean values for $K_{K(ext)}$ obtained with replicate similar experiments are given in Table II; values of $K_{0.5}$ obtained by fitting the data to the cooperative model (Equation 2) are also shown. As indicated in Table 2, the apparent $K_{K(ext)}$ values are similar for α_1 and α_2, but 2.5-fold lower for α_3. It is unlikely that this difference in affinity is due to changing $[K]_{in}$ since $[K]_{in}$ was high (130-135 mM) and did not change significantly during the course of the assay of any of the transfected cell lines (data not shown). This higher apparent affinity for K_{ext} of α_3 compared to α_1 and α_2 is consistent with the results obtained with axolemma and kidney (Fig. 1). However, the difference between α_3 of axolemma and α_1 of kidney may be larger (≈4-fold) if one takes into account the relative proportion of α_3 in axolemma (≈60% of the total activity). Whether this difference in the two systems is meaningful remains to be determined. It is plausible, for example, that the difference reflects distinct effects of the membrane potential on the apparent affinity for K^+ in the different isoforms; in the erythrocyte, but not the HeLa cell, the membrane potential is low and "clamped" at the chloride equilibrium potential.

The kinetics of activation of $^{86}Rb^+(K^+)$ influx by cytoplasmic Na^+ were examined in a series of experiments in which the transfected cells were incubated with various concentrations of Na^+ and monensin. An alternative procedure whereby the extracellular Na^+ concentration is kept constant and intracellular Na^+ raised by varying the concentration of monensin (*cf.* Refs. 35, 36) has the disadvantage of limiting the lower range of $[Na]_{in}$ to concentrations above the endogenous levels. For these experiments, the extracellular K^+

concentration was 4 mM. This concentration was sufficient to saturate the extracellular K^+ activation sites, with no evidence of a decrease in activity even at the highest concentrations of added Na^+ (preliminary studies in the absence of monensin, not shown), consistent with earlier studies with red cells (37). For each experiment, cells were grown on duplicate 110-mm culture dishes as well as on 24-well (10-mm) plates. Cells on both were washed, preincubated and incubated under identical conditions except that $^{86}Rb^+$ was omitted from the culture dishes which were used for the determination of intracellular Na^+, K^+ and protein. For each condition, two plates were taken for assay, one at the beginning and one at the end of the 10-minute assay period. Average values ± S.D. (three experiments) of the means of the two measurements, are shown in Fig. 5. In this figure, $[Na]_{in}$ is expressed as the ratio of $Na^+/(Na^+ + K^+)$. (Assuming that the sum is 160 mM for isotonic conditions, this value can be multiplied by 160 to obtain $[Na]_{in}$ in mM). For each pair of plates, the average deviation from the mean of the two measurements was <10%, indicating relatively little change in $[Na]_{in}$ over the course of the assay (not shown).

As shown in Fig. 6, the apparent affinity of α_3 for Na_{in} is much lower than that of either α_1 or α_2. Since it was technically difficult to raise $[Na]_{in}$ above ≈45 mM ($Na^+/(Na^+ + K^+)$ = 0.30), data points for the α_3-cells were well below saturation. When the data were analyzed according to the two models described by Equations 1 and 2, the curves were almost superimposable. However, the best fit of the data points to the non-cooperative model predicted a V_{max} value almost an order of magnitude higher than that of either α_1 or α_2. This is difficult to reconcile with measurements of membrane Na,K-ATPase activity indicating generally similar specific activities of all the expressed isoforms under optimal conditions (Ref. 5 and unpublished observations). Although it is entirely plausible that the lower degree of pump saturation under steady-state conditions *in situ* (see Table III) does increase the number of pumps expressed at the cell surface, as is the case of HeLa cells cultured in K^+-depleted medium (38), the ≈10-fold higher V_{max} of α_3 is larger than might be expected (*cf.* Ref. 38). However, when the data were fitted to the cooperative model,

the predicted V_{max} of α_3 was only 2- to 3-fold higher than that of the other two isoforms (see legend to Fig. 6).

The mean values ± S.D. of kinetic constants obtained by fitting the data of replicate experiments such as those depicted in Figs. 4 and 6 to the non-cooperative and cooperative models are summarized in Table II. Whichever model is chosen, it is clear that the apparent affinity of α_3 for K_{ext} is several-fold higher than that of α_1 or α_2, and for Na_{cyt}, several-fold lower. With the data fitted to the cooperative model, values, in mM, for α_1, α_2 and α_3, respectively, were 0.33 ± 0.02, 0.30 ± 0.03 and 0.17 ± 0.02 for apparent $K_{0.5(Kext)}$ and 17.6 ± 1.1, 19.7 ± 1.0 and 63.5 ± 9.1 for apparent $K_{0.5(Nain)}$. The relatively high $K_{0.5(Nain)}$ values are not surprising since intracellular K^+ was high (135 mM) and decreased only 20% with increasing $[Na]_{in}$.

Steady-state intracellular Na^+ concentrations in isoform-transfected HeLa cells. Information concerning the physiological relevance of the much higher apparent affinity of α_3 compared to the other two isoforms was obtained from measurements of the intracellular cation composition of the cells at steady-state under normal culture conditions. As indicated in Table III, the intracellular Na^+ concentration of the α_3 transfectant is approximately twice as high as that of the other two transfectants. Similar results were obtained with analogous HeLa cells transfectants using a different (cytomegalovirus) promoter for plasmid construction (not shown). It is unlikely that this difference in the steady-state cation composition reflects different levels of pump expression since the apparent V_{max} for α_3 is, if anything, greater than that of the other two isoforms. These findings indicate that an important determinant of the resting $[Na]_{in}$ is the relative apparent affinities of the enzyme for intracellular Na^+ and K^+.

FIG. 4. **Activation by extracellular K^+ of the individual α_1- , α_2- and α_3 isoform-transfected HeLa cells.** Cells cultured in 24-well tissue culture plates were washed in Solution A (see "Experimental Procedures") containing 4 μM monensin. After preincubation for 5 min at 37 °C in the same medium but with 10 μM bumetanide added and with 0.1 mM or 10 mM ouabain, $^{86}Rb^+(K^+)$ influx was carried out for 10 minutes at 37 °C with various concentrations of K_{ext} as described under "Experimental Procedures". Values for the representative experiment shown are the means ± S.D. of triplicate determinations. The results were analyzed using a two-site non-cooperative model (Equation 1), which gave a better fit than the cooperative model (Equation 2). The mean values ± S.D. for the $K_{K(ext)}$ of replicate experiments are given in Table II. For α_1, α_2 and α_3, respectively, average values of V_{max} obtained from replicate experiments were 75, 64 and 7% of the average values of the maximum V_{max} obtained in replicate experiments with varying $[Na]_{in}$ carried out as described in Fig. 6 and analyzed according to the three-site cooperative model (Equation 2). Symbols for the isoform-transfected cells are: O, α_1; □, α_2; ▲, α_3.

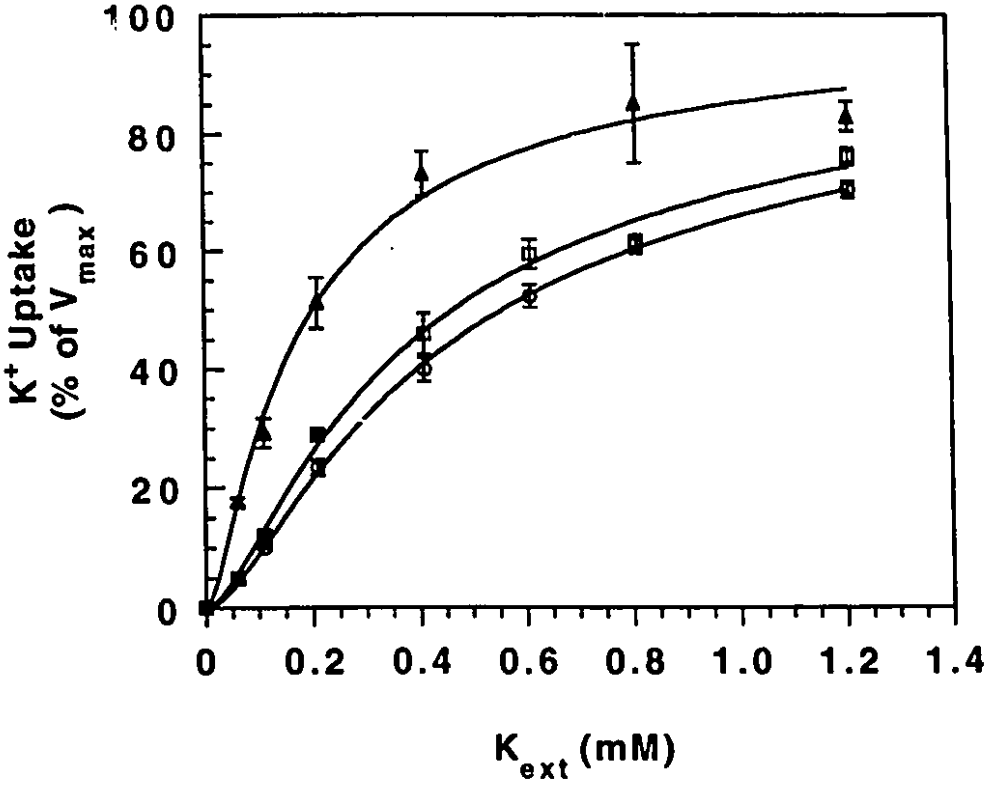

100
80
60
40
20
0
K+ Uptake
(% of Vmax)
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Kext (mM)

FIG. 5. **Intracellular Na^+ levels in transfected HeLa cells treated with various concentrations of Na^+ in the presence of monensin.** Cells were treated exactly as described for the flux assays (see Fig. 6) except that they were grown on-110 mm plates. At each Na^+ concentration, two plates were taken for analysis of Na^+, K^+ and protein, one at the beginning and one at the end of the period of the flux assay. Each data point is the average ± S.D. of three experiments; for each experiment, the mean of the measurements was obtained, one at the onset and one at the end of the 10-min flux period. Symbols for the mean values are O, α_1; □, α_2; ▲, α_3.

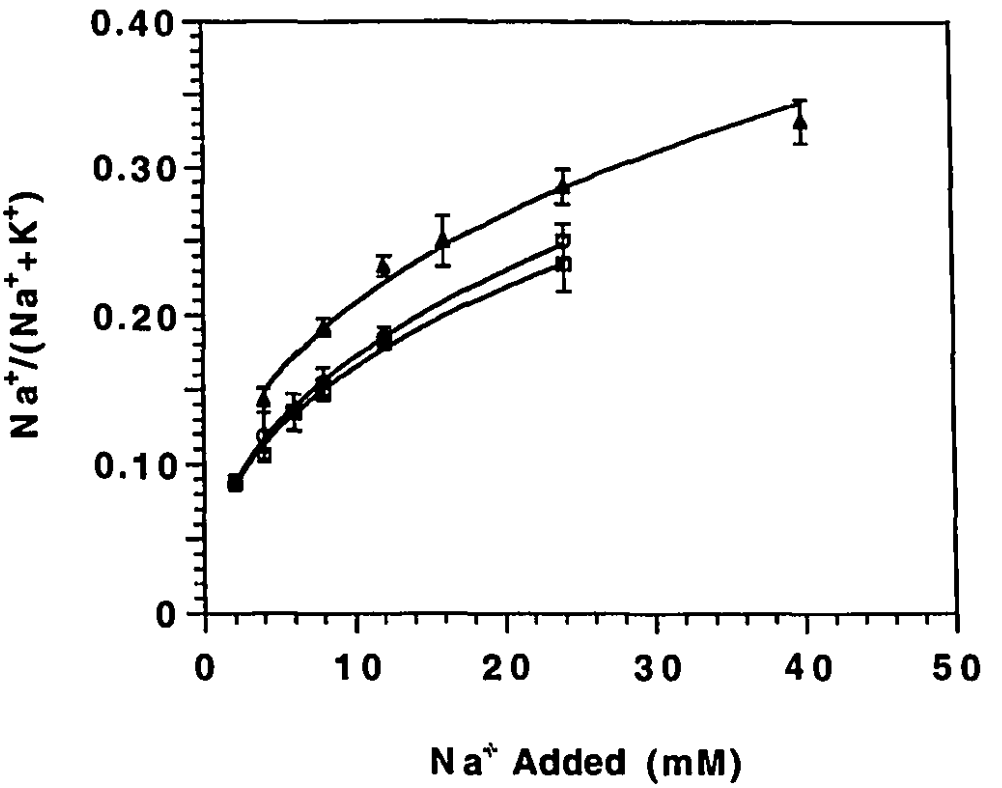

0.40
0.30
0.20
0.10
0
$Na^+/(Na^+ + K^+)$
0
10
20
30
40
50
Na^+ Added (mM)

FIG. 6. **Activation by cytoplasmic Na^+ of the individual α_1- , α_2- and α_3 isoform-transfected HeLa cells.** Cells were treated and assayed as in Fig. 4 except that the preincubation and assay media contained 12 μM monensin, 4 mM $^{86}Rb^+(K^+)$ and various amounts of NaCl as indicated. Values for the representative experiment shown are the means ± S.D. of triplicate determinations. The results were analyzed according to a three-site cooperative model (Equation 2). V_{max} values were 651, 644, and 1763 nmol/(mg protein·h) for α_1, α_2, and α_3, respectively, and the mean values ± S.D. for the kinetic constants, $K_{0.5(Nain)}$, of replicate experiments are given in Table II. When the results were analyzed according to a three-site non-cooperative model (Equation 1), V_{max} values were 1196, 1298, and 8,681 nmol/(mg protein·h) for α_1, α_2, and α_3, respectively. The mean values ± S.D. for the kinetic constants, $K_{Na(in)}$, of replicate experiments are given in Table II. Symbols for the isoform-transfected cells are: O, α_1; □, α_2; ▲, α_3.

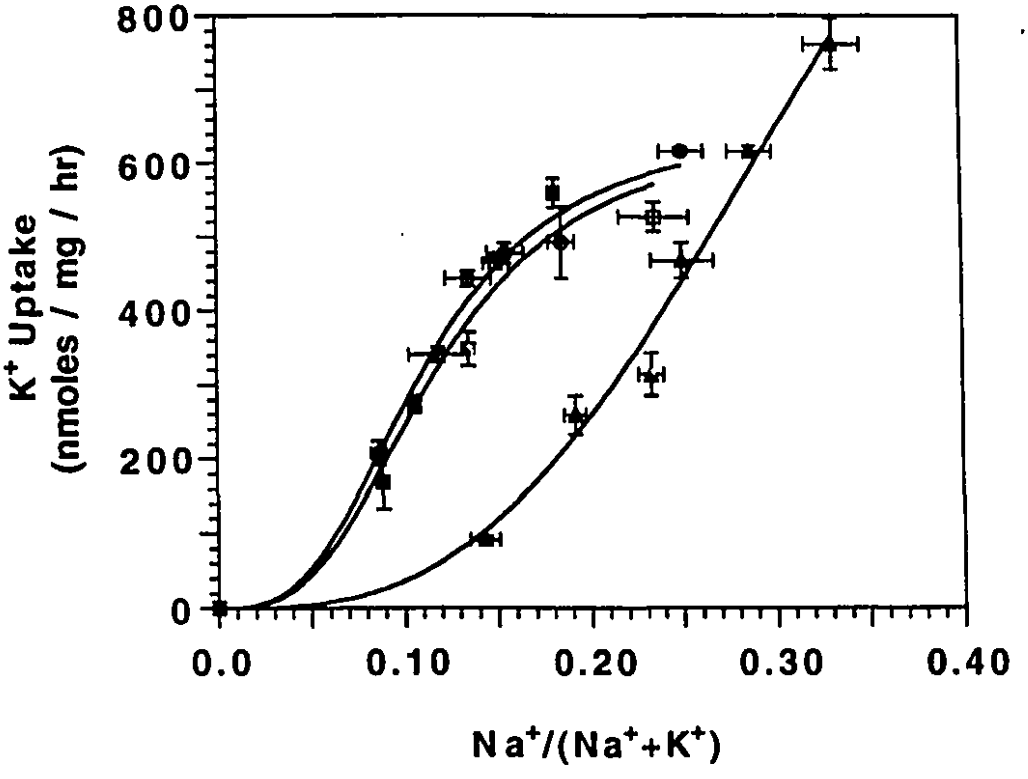

800
600
400
200
0
0.0
0.10
0.20
0.30
0.40
K+ Uptake
(nmoles / mg / hr)
Na+/(Na+ + K+)

TABLE II

Comparison of intracellular Na^+ and extracellular K^+ activation of Na/K pumps of rat α_1, α_2 and α_3 isoforms expressed in HeLa cells

Values for apparent kinetic constants for rat α subunit isoform-transfected HeLa cells were determined using the non-cooperative and cooperative models of ligand binding (Equations 1 and 2, respectively, under "Experimental Procedures"). Values shown are the averages ± S.D. of the number of experiments shown in parentheses.

Model	Isoform present	Kinetic constants	
		$K_{K(ext)}$	$K_{Na(in)}$
		mM	
Non-cooperative	α_1	0.22 ± 0.02 (3)	8.96 ± 1.28 (4)
(Equation 1)	α_2	0.20 ± 0.02 (3)	12.48 ± 1.60 (3)
	α_3	0.09 ± 0.01 (4)	78.72 ± 17.60 (6)
		$K_{0.5(Kext)}$	$K_{0.5(Nain)}$
Cooperative	α_1	0.33 ± 0.02 (3)	17.60 ± 1.12 (4)
(Equation 2)	α_2	0.30 ± 0.03 (3)	19.68 ± 0.96 (3)
	α_3	0.17 ± 0.02 (4)	63.52 ± 9.12 (6)

TABLE III

Intracellular Na^+ and K^+ levels in cultured HeLa cells

Cells grown on 110-mm tissue culture dishes were taken directly from culture in Dulbecco's modified Eagle's medium and analyzed with respect to their intracellular Na^+ and K^+ contents as described under "Experimental Procedures". Values shown are related to total cellular protein and represent the averages ± S.D. of the number of dishes shown in parentheses.

HeLa cell lin	Na^+	K^+	Calculated $[Na]_{in}$
	nmol/μg cell protein		*mM*
Wild type	74 ± 1	1210 ± 37	9.3 (4)
α_1	102 ± 11	989 ± 21	15.7 (3)
α_2	156 ± 5	1117 ± 87	19.7 (2)
α_3	230 ± 10	1106 ± 44	27.5 (3)

DISCUSSION

Two different cell systems have been used in the present experiments aimed to examine the side-specific effects of Na^+ and K^+ on tissue-specific expression of the Na,K-ATPase. Each has unique advantages. The microsome-fused erythrocyte system (6) is simple and its intracellular cation composition readily controlled; as well, the membrane potential of the erythrocyte is essentially "clamped" at the chloride equilibrium potential (≈-10 mV). The unique advantage of using HeLa cell lines transfected with the individual cDNAs of the three rat α subunit isoforms is that the only "variable" is the catalytic α isoform. Accordingly, the distinctive ion transport properties attributable to the individual α subunits, all paired with the same β_1 subunit, can be unequivocally assessed (5).

Both systems have provided essentially similar results regarding the relative affinities of the various α isoforms for activation by cytoplasmic Na^+ and extracellular K^+. Assuming that the pump behaviors observed in microsome-fused erythrocytes reflect those of native enzymes, the results reported here suggest that the transfected HeLa cell expresses Na,K-ATPase molecules that behave very much like those normally synthesized by various tissues *in vivo*. These similarities in the two cell systems also support the conclusion that the site-specific mutagenesis which conferred ouabain resistance does not alter the kinetic behavior of the isoforms.

In the present study, the comparative pump kinetics of the individual isoform-transfected HeLa cells are not only similar to those of native kidney and axolemma pumps fused into erythrocytes, but also to those obtained in previous studies of Na,K-ATPase of unsided membranes from the same HeLa transfectants (5). In the latter experiments, however, smaller differences in apparent affinities for Na^+ and K^+ were observed, probably due to complicating effects of concomitant changes in activating and inhibitory ions at the two sides of the membrane.

In comparing the pump properties of the different isoform-transfected HeLa cells and those of the "native" enzymes of our microsome-fused erythrocytes, it must be noted

that the HeLa cell expresses only the β_1 isoform. With regard to kidney microsomes, it has been well-documented that they contain $\alpha_1\beta_1$ pumps, which is also the combination present in the α_1-transfected HeLa cells. In rat brain, both β subunits have been demonstrated (40-42) and both, albeit in unknown proportion, are, presumably present in our axolemma preparations (unpublished results). Yet, in spite of the possibility that all $\alpha\beta$ combinations may exist in brain tissue, certain pairings appear to predominate. For example, in glia, α_2 has been codetected only with β_2 (43), while in large neurons of the brain stem, only β_1 has been detected along with α_1 and α_3 (41). These structural relationships, coupled with our finding that α_3 is the predominant isoform in axolemma, suggest that most of the activity observed in the present study is due to the same pump units ($\alpha_3\beta_1$) as those in the α_3-transfected HeLa cells.

Relevant to the foregoing considerations, a higher affinity for Na^+ of the pineal gland enzyme (predominantly $\alpha_3\beta_2$) was reported by Shyjan *et al.* (4) in their comparative study of the Na^+ and K^+ activation of kidney, brain, and pineal gland; a difference in affinity for K^+ could not be detected. It remains to be determined whether the dichotomy between these results and ours is due to the influence of the β subunit on the transport properties of the α subunit. Thus, in frog oocytes injected with mRNA's of various pump subunits, the apparent affinity of the pump for K_{ext} changed when the same α isoform was expressed with a different β isoform (44). More recently, Lutsenko and Kaplan (45), working with purified dog kidney Na,K-ATPase, demonstrated that the β subunit plays a direct role in K^+ binding and occlusion. It is therefore conceivable that differences among β subunit isoforms may result in different apparent $K_{0.5}$'s of pumps from various tissues. Another unresolved issue is the basis for the difference between the present results and those of others regarding Na^+ affinities of α_1 *versus* α_2 (31, 46).

The distinct K^+ sensitivities of the different α subunits may have interesting physiological significance. The extracellular ionic environment of the central nervous system is unique compared with most other tissues. Typical K^+ levels in the cerebrospinal

fluid are ~2.9 mM (49), roughly 60% of those found in blood plasma and the interstitial fluids of most other tissues. Maintenance of cation homeostasis in brain tissue is, in turn, critical to the maintenance of the transmembrane potential and hence to proper nerve function. There is evidence, based on the effects of strophanthidin (48) on the rate of K^+ removal from the extracellular spaces of neurons *in situ*, that one of the mechanisms contributing to this homeostasis is the Na,K-ATPase. As discussed by Syková (49), neuronal pumps activated by the influx of Na^+ during action potentials appear to be responsible for the active clearance of K^+ from this compartment, although it is likely that glial pumps also play a role in extracellular K^+ clearance (39). In cases where a rapid decrease in $[K]_{ext}$ leads to an "undershoot" (48), the α_3 isoform would be uniquely poised to continue pumping according to the demands of intracellular Na^+, without being significantly inhibited by this depletion of extracellular K^+.

With regard to the differences in apparent K_{Na} values of the α_1 and α_3 isoforms, Jewell and Lingrel (5) have previously hypothesized that α_1 represents a "housekeeping" form of the Na^+ pump which is capable of responding to typical physiological demands (5; *cf.* Ref. 31). From studies with cultured HeLa cells (38), it has been shown that this isoform operates near its apparent $K_{0.5}$ *in situ*, enabling it to respond efficiently to an increased Na^+ permeability. In neurons and other excitable tissues, however, it is possible that the large influxes of Na^+, such as those likely to occur during repeated action potentials, overwhelm the capacity of housekeeping pumps. In this situation, the α_3 isoform, which appears to be expressed predominantly in excitable tissues, may be activated to deal with an excess of intracellular Na^+. Thus, these pumps would be ideally suited to respond to this challenge in two ways: their low affinity for Na_{in} would endow them with a relatively large reserve capacity, while their high affinity for K_{ext} would allow them to continue to function even when $[K]_{ext}$ is depleted due to pump-mediated K_{ext} clearance (48). It is noteworthy that during pathological processes such as seizure, ischemia or excitotoxicity, intracellular Na^+ levels can be dramatically increased and result

in cellular death, effects that can also be produced by ouabain (*cf.* Ref. 50). Selective activation of the α_3 isoform during these processes is suggested by the results of recent studies using cultured neurons. In one study (51), selective inhibition by ouabain at low concentration markedly enhanced the susceptibility of these cells to damage by the Na^+ gained during excitotoxic stimulation. In another study, pump sensitivity to ouabain was shown to increase following a glutamate-induced rise in $[Na]_{in}$ (52) , suggesting activation of a ouabain-sensitive isoform (α_3) which is distinct from the "common-type" isoform (α_1).

In recent years considerable effort has been directed towards obtaining functional expression of the individual Na,K-ATPase isoforms in various cell systems including yeast (53), insect cells (25, 54) and amphibian oocytes (55-57). The latter has been amenable to measurements of electrogenic pump current, albeit often with certain limitations including non-specific background currents, considerable endogenous pump activity and an endogenous (distinct amphibian β_3) β subunit. The present experiments are unique in that they report, for the first time, the transport characteristics of pumps expressed in, or fused into, mammalian cells. With these sided preparations, differences in apparent affinities for cytoplasmic Na^+ and extracellular K^+ are larger than evident from studies with unsided preparations. These differences are clearly reflected in the different steady-state physiological behavior of the cells, namely the higher level of intracellular Na^+ in α_3-compared to the α_1- or α_2-transfected cells. It should be noted, however, that the difference in $K_{0.5}$ of the α_3-transfected cells is 4-fold larger, which means that the they are operating at a lower state of occupancy of their intracellular Na^+ sites than α_1- and α_2-transfected cells. It remains to be determined whether these α_3-transfected cells are more sensitive to insults which cause rapid increases in Na^+ permeability, whether they can sustain growth in K^+-depleted media and whether they respond to their lower level of saturation with Na^+ at intracellular sites by expressing more pumps on the surface compared to α_1- and α_2-transfectants.

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CHAPTER 5

GENERAL DISCUSSION

5.1. Ion-motive ATPases in microsome-fused erythrocytes

The development of a procedure for the functional delivery of transport proteins such as the Na,K- and Ca-ATPase into erythrocyte membranes represents a novel approach to the study of membrane transport processes. Unlike many protein delivery protocols in which it is not possible to distinguish between adherent proteins and those which are properly integrated in the membrane (see Chapter 2), the present studies have unequivocally demonstrated functional pump delivery into mammalian erythrocyte membranes. The main evidence supporting this claim is the side-specific dependence of active ion transport on ATP. Thus, Ca^{2+} uptake into SR vesicle preparations is normally fueled by extravesicular ATP. Following fusion of SR vesicles with human erythrocytes, Ca^{2+} uptake into the cells was dependent on ATP added to the extracellular medium. That no Ca^{2+} uptake was stimulated by exogenous ATP in control (mock-fused) cells indicates the functional integration of the SR Ca-ATPase into the microsome-fused cells. Similarly, because mature dog erythrocytes are normally devoid of endogenous Na,K-ATPase activity, dog kidney microsome-fused dog erythrocytes, but not mock-fused cells, demonstrated a substantial ouabain-dependent Rb^+ uptake in the presence of millimolar concentrations of intracellular ATP. Moreover, IOV's derived from the membranes of kidney microsome-fused dog erythrocytes showed an ATP-dependent Na^+ uptake and Rb^+ efflux that was not observed in IOV's derived from the membranes of control (mock-fused) dog erythrocytes. The cardiac glycoside dependence of the Na^+ flux confirms that it was mediated by the Na,K-ATPase. The successful use of the Na,K-ATPase, along with the Ca-ATPase as a prototype of this technology, demonstrates that this methodology is generally applicable to the delivery of ion-motive ATPases into erythrocytes of various mammalian species including humans, dogs, and sheep.

Microsome-fused erythrocytes have several advantages over other systems employed for membrane protein transport studies. One of the most significant is that mammalian erythrocytes comprise a single, large compartment, making them ideal vesicles

for transport kinetics studies. Unlike reconstituted proteoliposomes or other vesicular preparations (*e.g.*, synaptosomes) with a small surface-to-volume ratio, erythrocytes can sustain linear transport fluxes for many minutes (compare, for example, Fig's 1 and 2a in Chapter 2), which greatly facilitates kinetic analyses. Red cells also have other advantages, such as their ease of manipulation *in vitro* and their well characterized biological membrane (see Schatzmann, 1988). A particular benefit of the PEG fusion system is that pumps are delivered into vesicles without the use of detergents, thus avoiding possible alterations of kinetic behavior which have been reported following exposure of the Na,K-ATPase to detergents (see below).

Quantitative assessments of Na,K-pump delivery into erythrocytes provided some insight into the nature of the membrane fusion process. For example, the activity of functional ion-translocating pumps delivered into erythrocyte membranes, measured by ouabain-sensitive $^{86}Rb^{+}$ influx, reached its maximum level at a ratio of 2.5 mg of microsomal protein per ml of cells (Chapter 2, Fig. 5). In contrast, the number of cell-associated pumps, measured by ^{3}H-ouabain binding, continued to increase even at a ratio of 8 mg of microsomal protein per ml of erythrocytes. These observations suggest that the capacity of erythrocytes to bind microsomes is several-fold greater than their capacity to integrate them into their membrane bilayer (thus permitting ion transport by exogenous pumps). In fact, within the "subpopulation" of the total cells that incorporates microsomal pumps (see below), the estimated number of vesicles that successfully fuses with a given cell is, on average, ten (see Chapter 2). Assuming that the surface area of a typical dog erythrocyte is 117 μm^2 (Hoffman, 1986) and that the average surface area of a spherical microsome is approximately 0.031 μm^2 (based on a diameter of 0.2 μm; see Forbush, 1983), there is enough space for approximately 3000 vesicles to directly contact the surface of the cell. Thus, only a small fraction of the vesicles that can theoretically associate with an erythrocyte participate in membrane fusion.

Information regarding the intercellular distribution of newly delivered pumps was obtained from the time course of the Na^+ efflux from dog kidney microsome-fused dog erythrocytes (Chapter 2, Fig. 9a). These results indicated that only a small fraction of the total Na^+ loss was ouabain-sensitive. The rapid depletion of Na_{in}, which occurred in only approximately 15% of the total cells, implied the delivery of a relatively large number of pumps into a small population of cells. This is consistent with the findings described above, in which a single cell absorbs ten pump-rich microsomal vesicles. Perhaps this subpopulation of cells represents younger erythrocytes which, like reticulocytes (Xu *et al.*, 1992), may be more receptive to the incorporation of new material into their membranes.

Based on these results, preliminary experiments designed to improve pump delivery from dog kidney microsomes into dog erythrocytes were carried out (Appendix II). For example, improving the specific activity of microsomal membranes using gradient purification increased pump delivery up to 1.5-fold. However, since the modest improvement in delivery was offset by at least a 50% loss of the starting material, this approach was not routinely used. Surprisingly, SDS-purified Na,K-ATPase membranes, which had specific activities 3-5 times greater than those of normal microsomal preparations, were unable to support pump delivery into erythrocyte membranes. This observation suggests that membrane fusion by this method requires the presence of intact, vesicular structures.

Efforts to reduce the steric barriers to membrane approach by using enzymes to remove material from the erythrocyte glycocalyx had little or no effect on pump delivery. However, reducing the size of microsomes through mild sonication slightly increased pump delivery, implying the presence of a physical barrier to microsome-cell associations. Similarly, extraction of microsomes with high concentrations of KCl to remove peripheral membrane proteins produced an approximately 50% increase in pump delivery. Taken together, these results suggest that the success of membrane fusion in this system may depend in part on the physical structure of the external membrane surfaces.

Lectins, which can substantially improve membrane protein delivery into cells (see, for example, Szoka *et al.*, 1981), caused a dramatic increase in the number of ouabain binding sites associated with erythrocytes. However, they had no substantial effect on the level of functional pump delivery, suggesting again that erythrocytes have an intrinsic limitation in their capacity to fuse with exogenous membranes (see above and Chapter 2).

Attempts to disrupt the membrane cytoskeleton using either a 10-minute incubation at 48 °C (Brandts *et al.*, 1977; Lysko *et al.*, 1981) or a 60-minute incubation with 5 mM diamide (Deuticke *et al.*, 1983) had no discernible effect on pump delivery into dog erythrocytes. Although the efficacy of these treatments in disrupting the cytoskeleton was not verified, the results suggest that membrane fusion in this system is not significantly affected by the cytoskeleton. However, in the study by Xu *et al.* (1992), the higher incorporation of pumps into sheep reticulocytes compared to mature sheep erythrocytes could be due to the cytoskeleton. As discussed by Gratzerm (1981), there are discontinuities in the cytoskeleton of reticulocytes. It is conceivable that the density of membrane protein packing in these younger cells is less than that of mature erythrocytes, thereby increasing the exposure of the lipid bilayer and the likelihood of membrane fusion with microsomes.

In keeping with this hypothesis, the addition of cholesterol or exogenous lipids into erythrocyte membranes might be expected to improve pump delivery, possibly by increasing the amount of cellular lipid available to interact with the microsomes. Others have demonstrated that the degree of membrane fusion may be improved by adding appropriate exogenous lipids (see, for example, Martin and MacDonald, 1976; Stamatatos, *et al.*, 1988). Thus, erythrocyte membrane doping with cholesterol appeared in some cases to improve pump delivery (see Appendix I). In addition, pre-fusion doping of erythrocyte membranes with positively charged liposomes composed of 75% phosphatidylethanolamine, 10% phosphatidylcholine, and 15% 1,2-dioleoyloxy-3-(trimethylammonium)-propane (DOTAP) supported nearly the same level of pump

incorporation at 30% PEG as seen in undoped cells at 45% PEG (see Chapter 2). Unfortunately, cells doped with these lipids tended to be very fragile, leading to low recoveries of the starting material. The converse procedure, that of doping microsomal membranes with fusogenic lipids, resulted in an apparent lack of pump delivery. It is possible that the pre-fusion conditions were inappropriate, leading to substantial aggregation of the liposome-microsome mixture.

5.2. Erythrocyte *versus* kidney Na,K-pumps

Functional studies of erythrocyte and kidney Na,K-ATPases have suggested that there are specific differences in their kinetic behavior (for review, see Blostein, 1989). Thus, the Na/O flux of the kidney pumps reconstituted into proteoliposomes comprises only about 2% of the total Na/K flux (Karlish *et al.*, 1988; Cornelius, 1989), whereas in erythrocytes it comprises approximately 15% of the Na/K flux (Garrahan and Glynn, 1967b) and is accompanied by anion cotransport (Dissing and Hoffman, 1990). A further difference between kidney and erythrocyte enzymes has been revealed by investigations of EP intermediates carried out at 0 °C. These studies showed that the erythrocyte pump is found predominantly in the E_1P conformation (White and Blostein, 1982; Kenney and Kaplan, 1985), whereas the kidney pump exists predominantly in the E_2P conformation (White and Blostein, 1982). Comparative studies of Na,K-ATPase immunological reactivity (Inaba and Maeda, 1986; Blostein and Grafova, 1990) and mRNA expression (Dhir *et al.*, 1990) have confirmed that the kidney and the erythrocyte both contain the same α_1 catalytic subunit isoform. Messenger RNA from the β_1 isoform, which is present in kidney, was also observed in erythrocytes (Dhir *et al.*, 1990). However, since the level of expression was very low, it is possible that the α subunits of erythrocyte pumps are influenced by association with an as yet unidentified β subunit homologue. A more plausible explanation for the differences observed between the kidney and erythrocyte Na,K-ATPases, particularly those regarding the E_1P-E_2P equilibrium distribution, is that

the distinct membrane environments of the two tissues have differential effects on the rate constants of steps in the pump reaction cycle (see below). Alternatively, these discrepancies in pump behavior may be related to tissue-specific posttranslational modifications.

In order to gain further insight into the basis for the differences between kidney and erythrocyte Na,K-ATPases, studies of the activation of these two pumps by alkali cations were carried out. Thus, using rat kidney pumps delivered into human erythrocytes by the membrane fusion protocol, the behavior of both pumps in the same membrane environment was examined. The activities of exogenous rat and endogenous human pumps were distinguished on the basis of their differential sensitivity to ouabain (see Section 1.2.4.a.). Studies of activation by K_{ext} indicated that the $K_{K(ext)}$'s were virtually identical for the two kinds of pumps. Although the calculated $K_{Na(in)}$ initially seemed to be higher for erythrocyte pumps than for kidney pumps, these experiments were complicated by exogenous pump delivery into only a small fraction of the cells (see Chapter 3). The relatively high pump-mediated Na^+ efflux from this small fraction of pump-rich cells decreased the $[Na]_{in}$ below that of the total cell population, thereby artificially increasing the $K_{Na(in)}$ obtained from plots of pump rate versus (measured) $[Na]_{in}$. Thus, accounting for the discrepancies between the observed and "true" values of $K_{Na(in)}$, there was no significant difference in the Na^+ activation kinetics of rat kidney and human erythrocyte pumps.

One pump flux mode which appears to be unique to red cells is the anion-coupled Na/O flux. In a series of experiments using dog kidney pumps delivered into dog erythrocytes (see Chapter 2), the measured Na/O flux of the exogenous kidney pumps was only ≈2% of the total Na/K flux. Although similar to that reported for kidney pumps reconstituted into proteoliposomes (Karlish and Kaplan, 1985; Cornelius, 1989), this value is far below that expected for erythrocyte pumps (see above). However, since mature dog erythrocytes have nearly undetectable Na,K-pump activity, it is not known whether dog erythrocyte pumps are themselves capable of Na/O flux activity. One way in which this

could be examined is by studying dog reticulocytes, which have ample Na,K-ATPase activity (Maeda and Inaba, 1985). Another way of circumventing this problem would be to investigate the Na/O flux of rat kidney pumps delivered into human erythrocytes. Although experiments of this nature were attempted, the low levels of ouabain-sensitive Na^+ efflux compared to the ouabain-insensitive baseline flux precluded accurate estimates of the Na/O flux component. Nonetheless, because of its unique properties (see Section 1.2.3.b.), the basis for the erythrocyte's electroneutral anion-coupled efflux remains an interesting issue.

A different series of experiments designed to examine the possible role of the membrane environment on pump modulation involved fusing rat kidney pumps into LK sheep erythrocytes (see Appendix I). The findings clearly demonstrated that the L_p antigen in LK sheep erythrocytes is a distinct membrane component which can interact with and modulate both endogenous sheep and exogenous rat pumps. Xu *et al.* (1993) subsequently showed that the L-antigen is responsible for the profound inhibition of pump activity by K_{in}, not only for endogenous red cell pumps, but also for exogenous rat kidney pumps delivered into LK but not HK sheep erythrocytes. These studies support the use of the microsome-fused erythrocyte system as a means of examining the effects of the membrane environment on pump behavior.

5.3. Apparent cation affinities of Na,K-pump isoforms

In the first of two assay systems used to investigate the apparent cation affinities of different Na,K-ATPase catalytic α subunit isoforms, Na,K-pumps from either rat kidney or rat axolemma microsomes were delivered into dog erythrocytes via PEG-mediated membrane fusion. Since axolemma membranes contain a mixture of the catalytic α subunit isoforms (Urayama *et al.*, 1989), quantitative immunoblotting and ouabain sensitivity profiles of the steady-state levels of EP in these microsomes were used to assess the relative amounts of each isoform in this tissue. Both methods indicated that the α_3 isoform comprised approximately 60% of the total axolemmal Na,K-ATPase, with the remainder

consisting of the α_2 and α_1 isoforms. In contrast, only the α_1 isoform was detected in kidney membranes. While these findings suggest that the majority of pump activity in axolemma-fused dog erythrocytes is due to the α_3 isoform, it is also possible that the incorporation of functional pumps into these cells is not proportional to the levels of isoforms present in the original microsomes. As mentioned previously (see Appendix II), attempts to deliver pumps from SDS-purified Na,K-ATPase membranes into erythrocyte membranes were unsuccessful, implying that membrane fusion and subsequent pump delivery may require intact, vesicular structures. Since preliminary experiments (Thérien and Blostein, unpublished observations) show that the majority of the ouabain-sensitive Na,K-ATPase activity of rat axolemma microsomes (α_2/α_3) is activated by SDS, it is likely that these pumps are particularly enriched in the membranes of sealed axolemma vesicles. In comparison, the ouabain-resistant (α_1) ATPase activity of these microsomes is not significantly activated by SDS. Thus, assuming that only very small amounts of α_1 pumps were incorporated into the cell membranes, the proportion of pump activity in axolemma microsome-fused erythrocytes attributable to the α_3 isoform would increase from 60%, as estimated above, to at least 75% of the total activity.

These results from rat axolemma and kidney Na,K-pumps delivered into dog erythrocyte membranes contrast sharply with most previous reports of the apparent cation affinities of brain (or axolemma) and kidney Na,K-ATPases (see Chapter 1, Table II). Many of the earlier studies were carried out using whole brain homogenates, which contain a mixture of all three α subunit isoforms. This may, in some cases, explain why the observed differences in apparent affinities were small. However, there seems to be no obvious combination of brain α subunit isoforms capable of explaining the reversal in affinities which is reported here, *i.e.*, that the kidney α_1 isoform has a lower apparent affinity for Na^+ than the mixture of brain isoforms. Moreover, quantitative estimates of the relative amounts of isoforms in rat brain homogenates suggest that the α_1 isoform comprises ≤20% of the total Na,K-ATPase (Matsuda and Iwata, 1987; Gerbi *et al.*, 1993).

The other assay system used to investigate the apparent cation affinities of different Na,K-ATPase isoforms employed HeLa cells which had been individually transfected with the various rat α subunit isoforms (Jewell and Lingrel, 1991). One of the advantages of this expression system is that the kinetic behavior of each of the Na,K-ATPase isoforms can be examined separately in the same cellular environment, free of contaminating activity from other isoforms. In order to distinguish the activity of the transfected enzymes from that of the endogenous HeLa enzyme, the α_2 and α_3 isoforms were rendered as resistant to ouabain as the rat α_1 isoform through site-directed mutagenesis of residues in the first extracellular region of the pump (see Price and Lingrel, 1988). By culturing and assaying these transfectants in the presence of 1 μM ouabain, the endogenous ouabain-sensitive human pump activity is completely inhibited whereas that of the transfectants remains unaffected.

When ATPase assays in which one of the alkali cations was varied and the other held constant at a saturating level were carried out on membranes isolated from these cells, the activities of the α_1 and α_2 isoforms were nearly indistinguishable. In contrast, the α_3 isoform had a three-fold higher K_{Na} and a slightly lower K_K compared to the other two isoforms (Jewell and Lingrel, 1991). The corresponding cation activation studies on intact cells indicated that the α_3 isoform has an approximately three-fold lower $K_{K(ext)}$ as well as at least a three-fold higher $K_{0.5(Nain)}$ than the α_1 or α_2 isoforms. These findings indicate that the isoform-specific differences in apparent cation affinities obtained from studies with sided *versus* unsided assay preparations are generally similar. Quantitatively, however, there is a somewhat greater difference in the apparent affinities for K^+ between the α_3 and α_1 isoforms in the cation flux studies compared to the ATPase studies. This difference could be due to competition by increasing amounts of K^+ for the Na^+ activation sites in the unsided ATPase assay. Thus, in the ATPase assays, the activity of the α_3 isoform, due to its higher K_{Na}, could be more inhibited than that of the other isoforms as [K] increases, leading to an overestimate of its K_K and thereby reducing the difference between this value

and the K_K's of the other isoforms. It is also plausible that the apparent affinity difference between the two assay methods is due to subtle alterations in pump kinetic behavior caused by the effects of the membrane potential in the sided assay system (see below). Whether there are similar discrepancies in the K_{Na}'s is less apparent due to the variances in the estimates of these values. Also, as shown in Chapter 4 (Table II), the difference between the apparent affinities of the isoforms for Na^+ depends on the equation used to analyze the data. Thus, with the non-cooperative-sites model developed by Garay and Garrahan (1973), the difference in the $K_{Na(in)}$'s of the isoforms was nearly ten-fold compared with an approximately four-fold difference using a cooperative-sites model (see Lytton, 1985a). However, the predicted V_{max} values, as well as the general fit of the data to the latter model, were better, suggesting that it provides a more accurate description of Na^+ activation kinetics in this system.

The results from transport assays of HeLa cells are strikingly similar to those obtained using microsome-fused erythrocytes. Thus, rat axolemma pumps, when fused into dog erythrocyte membranes, have an approximately three-fold lower $K_{K(ext)}$ and an approximately three-fold higher $K_{Na(in)}$ than rat kidney pumps. Since it is likely that these kinetic constants for the axolemma-fused cells reflect a mixture of predominantly the α_3 isoform along with some of the α_1 and α_2 isoforms, the actual differences in apparent cation affinities between the kidney α_1 and axolemma α_3 isoforms may be larger than those observed. In the case of the $K_{K(ext)}$'s, however, the difference between the axolemma and kidney isoforms is, unexpectedly, even larger than that found with the transfected HeLa cells. Thus, in addition to the presence of more than one isoform in the axolemma preparations, there may be other differences between the two assay systems. Possible explanations for these discrepancies are discussed below.

Two factors which are sometimes cited to explain differences in Na,K-ATPase kinetic behavior observed in various studies are the choice of assay methodology and the method of tissue preparation. However, although the assay conditions of the ATPase

studies listed in Table II varied considerably with respect to the concentrations and ratios of Na^+ and K^+, the conclusion that the kidney isoform had a lower apparent affinity for Na^+ nearly always prevailed. Moreover, as discussed above, the results obtained from ATPase measurements and Rb^+ flux measurements of HeLa cells were quite similar, suggesting that, in most cases, competition between Na^+ and K^+ has a relatively minor influence on apparent cation affinity values. Similarly, the variety of detergents and chaotropic agents employed in the studies listed in Table II did not appear to alter the conclusion that the kidney isoform had a lower apparent affinity for Na^+ than those of the axolemma. While some investigators have suggested that membrane disruption (Lytton, 1985a; Brodsky and Guidotti, 1990) alters the apparent affinity for Na^+ of the Na,K-ATPase, the agreement between the results of the Rb^+ uptake assays shown in Chapter 4 with those of Jewell and Lingrel's (1991) ATP hydrolysis assays demonstrate that this is not the case in the transfected HeLa cell system. Alterations in the kinetic behavior of the Na,K-ATPase following detergent purification of membranes (Huang *et al.*, 1985; Feige *et al.*, 1988; Sweadner, 1994) and functional reconstitution of the Na,K-ATPase into proteoliposomes (Cornelius and Skou, 1991) have been reported. It is not known if detergents selectively affect specific isoforms or act equally on all of them.

Jewell and Lingrel (1991) have suggested that differential $\alpha\beta$ subunit pairing may explain the discrepancies between their findings of isoform-specific behavior compared to some of those in the literature. In particular, Shyjan *et al.* (1990a), using differential doses of ouabain to distinguish the activity of the ouabain-sensitive (α_3) component of rat pineal gland membranes, demonstrated that the $K_{0.5(Na)}$ of this isoform is approximately half that of the α_1 isoform from kidney. As mentioned in Section 1.2.4.d., rat pineal glands express the α_3 and β_2 isoforms, and possibly an unidentified β subunit isoform (Shyjan *et al.*, 1990a). HeLa cell transfectants, on the other hand, express the endogenous human β_1 isoform (Mercer *et al.*, 1986) which is, presumably, paired with the transfected rat α subunits. That the β subunit can influence the apparent affinity of pumps for K_{ext} has

recently been reported (Jaisser *et al.*, 1992; Lutsenko and Kaplan, 1993; Eakle *et al.*, 1994). It is not known what effects this subunit may have on the apparent affinity for Na_{in}. In addition, it is not known if differential glycosylation of the same β subunit isoform can influence apparent cation affinities.

The nature of the α/β subunit pairing in axolemma membranes has not yet been determined. Structural observations suggest that the majority of large neurons in the brain stem and spinal cord, from which axolemma membrane preparations are derived, express mainly the α_3 and β_1 isoforms (see Section 1.2.4.d.). If $\alpha_3\beta_1$ pumps are the major species delivered into the axolemma microsome-fused erythrocytes, it may explain why the relative values of the apparent cation affinities of the α_1 (kidney pumps) and α_3 (axolemma pumps) isoforms fused into these cells are similar to those of the HeLa transfectants. However, as described previously, the contrast between these relative cation affinities and those of microsomal axolemma and kidney Na,K-ATPases (Table II) is difficult to explain on the basis of the isoform distributions. Moreover, analyses of isoform pairing do not readily explain why Shyjan *et al.* (1990a) find that the α_3 isoform in the pineal gland, which is presumably paired with the β_2 isoform, has a lower $K_{0.5(Na)}$ than the enzymes from either brain or kidney membranes. This conclusion is qualitatively similar to those of the studies shown in Table II, wherein the majority of the α_3 isoform is most likely paired with the β_1 isoform. Given that brain membranes contain a mixture of all three α subunit isoforms, the findings in pineal glands imply that the actual $K_{0.5(Na)}$ of the α_3 isoform in brain membranes is even lower than that observed. The apparently similar behavior of the α_3 isoform presumably paired with either of the β subunit isoforms suggests that the β subunit alone is not sufficient to account for the discrepancies in apparent cation affinities between the microsome-fused erythrocytes/HeLa transfectants and the microsomal Na,K-ATPases shown in Table II.

An alternative explanation for these apparent cation affinity differences is based on the conformational equilibrium of the Na,K-ATPase. As discussed by Läuger (1991) and

Plesner (1986), the rate constants of individual reaction steps, even those far removed in the reaction cycle from the ligand binding event, can affect apparent affinity estimates. (The apparent affinity in this case is defined as the reciprocal of K_m, the substrate concentration that supports half of the maximal velocity.) As an example, Vilsen (1993) described moderate increases in the $K_{m(Na)}$ and $K_{m(K)}$ of a mutant rat α_1 subunit compared to the wild type enzyme (see Section 1.2.3.e.). This author also observed a 15-fold decrease in the $K_{m(ATP)}$. Since the mutated residue, Gln^{329}, lies within the fourth predicted transmembrane domain of the α subunit, it is probably not involved in ATP binding (see Fig.2). Nevertheless, it is clear from these results that the structural change associated with the mutation has dramatically influenced the enzyme's $K_{m(ATP)}$. Vilsen suggested that this change most likely reflects a shift in the steady-state equilibrium toward E_1, which has a much smaller $K_{m(ATP)}$ than E_2. Similarly, the increase in the $K_{m(K)}$ (decrease in apparent affinity) of the mutant enzyme may also be explained on the basis of a shift in the steady-state equilibrium toward E_1 (see also Eisner and Richards, 1981). However, the concomitant increase in the $K_{m(Na)}$ cannot be explained by this shift, since it should have increased the enzyme's probability of reacting with Na^+ and therefore its apparent affinity. Thus, Vilsen argues that this mutation, in addition to changing the conformational equilibrium, also affected the intrinsic cation binding affinity and therefore involves one of the amino acids in or very near the Na^+ binding site.

Matsuda and Iwata (1987, 1988) have carried out comparative studies of the steady-state EP distributions in rat kidney *versus* rat neural membrane preparations. Based on the sensitivity of the EP to dephosphorylation by K^+ or ADP, they concluded, as have others (Hara and Nakao, 1981; White and Blostein, 1982), that the kidney (α_1 isoform) Na,K-ATPase is mainly present as E_2P at 0 °C. In contrast, they found that the enzyme in medulla oblongata and spinal cord membranes (which, like axolemma membranes, contain very little of the α_1 isoform) exists predominantly as E_1P. On the basis of the previous kinetic

arguments, the neural enzyme would be expected to have a lower K_{Na} than the kidney enzyme, which agrees quite well with the results of the ATPase assays shown in Table II.

In fact, however, this interpretation may be complicated by the effects of temperature on the EP equilibria. White and Blostein (1982), as well as Kaplan and Kenney (1985), have shown that the human erythrocyte Na,K-ATPase is almost entirely present as E_1P at 0 °C. This lack of E_2P, along with a drastic reduction in the apparent turnover of the enzyme, suggested that the E_1P-E_2P transition is somehow inhibited at this temperature. In studies using kidney (Hara and Nakao, 1981; White and Blostein, 1982; Matsuda and Iwata, 1988) and brain (Plesner *et al.*, 1981; Matsuda and Iwata, 1988) preparations both E_1P and E_2P were seen at 0 °C, suggesting that the two forms of EP can interconvert. Nevertheless, due to the experimental difficulties of resolving EP species at 37 °C, it is not known if the steady-state EP ratios at 0 °C are equivalent to those at 37 °C.

A factor which has a significant effect on EP ratios is the membrane lipid environment. Studies of partially delipidated Na,K-ATPase preparations have indicated that K^+-sensitive dephosphorylation (*i.e.*, the E_1P-E_2P conformational transition) is very sensitive to lipid removal (Wheeler, 1975; Hegyvary *et al.*, 1980; Harris, 1985) and the concentration of membrane cholesterol (Yoda and Yoda, 1987b). Matsuda and Iwata (1986) have shown that the α and $\alpha(+)$ isoforms of rat brain respond differently to phospholipase C-induced delipidation, suggesting either that these isoforms differ in the way that they interact with their lipid environment, or perhaps that they exist in distinctly different lipid environments. More recently, Klodos *et al.* (1994) carried out a comparative study of brain and kidney EP's. In addition to confirming that the kidney enzyme is more sensitive to K^+-stimulated dephosphorylation, they found that changes in salt concentration induced unexpectedly heterogeneous responses in the EP's from both types of tissues. To explain their findings, they proposed a model in which the EP distribution is governed by membrane lipid phase changes.

These examples clearly illustrate that membrane lipids can exert a significant influence on the function of the Na,K-ATPase. Whether these factors can explain the discrepancies observed between microsomal ATPase studies (Table II) and the cation flux studies presented in Chapter 4 remains to be determined. For example, it would be interesting to determine the steady-state EP ratios of enzymes in the HeLa transfectants as well as in the microsome-fused erythrocytes. If enzyme conformation is a major determinant of apparent cation affinities in these systems, then one might expect to find a reversal of the steady-state EP ratios, such that the α_1 or kidney isoform is predominantly in the E_1P conformation and the α_3 or axolemma isoforms are predominantly in the E_2P conformations.

Another factor that could be involved in the differences between ATPase assays on unsided membranes and cation flux assays on intact cells is the membrane potential. As discussed previously (see Section 1.2.3.f.), studies indicate that the apparent affinity for K_{ext} (and Na_{ext}), but not Na_{in}, is significantly affected by transmembrane potentials (see for examples, Goldshleger *et al.*, 1987; Rakowski and Paxson, 1988; Vasilets and Schwarz, 1993; Stimers *et al.*, 1993). In addition, Rephaeli *et al.* (1986) demonstrated that a change in the electrical potential (equivalent to cytoplasmic depolarization) accelerates the E_1P-E_2P conformational transition of the kidney Na,K-ATPase reconstituted into proteoliposomes.

Of the two transport assay systems described in Chapter 4, one, the fused erythrocytes, probably has almost no membrane potential. As explained by Hoffman (1986), the membrane potential of erythrocytes is typically equivalent to the chloride equilibrium potential (≈ -10 mV) and is therefore unlikely to be affected by changes in cation concentrations. HeLa cells, on the other hand, may have larger membrane potentials, possibly as low as the K^+ equilibrium potential (≈ -80 mV). Due to the decrease in $[K]_{in}$ as $[Na]_{in}$ increases (up to a 20% change in $[K]_{in}$; see Chapter 4), these potentials could change slightly following treatment of the cells with monensin. However, the magnitude of

these changes (≈6 mV, based on the changes in cation concentrations determined in Chapter 4 and using the Nernst equation) is probably too small to have a noticeable effect on apparent cation affinities.

Since both the fused erythrocytes and the permeabilized axolemma and kidney membranes used for ATPase studies (Table II) have no appreciable membrane potentials, it is unlikely that the discrepancies between these studies can be attributed to membrane voltage effects on cation binding. Similarly, the agreement between HeLa cell ATPase assays on permeabilized membranes lacking potentials (Jewell and Lingrel, 1991) and cation flux studies on intact cells with potentials supports this conclusion. In spite of these findings, preliminary evidence from another system suggests that there may be isoform-specific responses to changes in the transmembrane electrical gradient. Thus, in BALB/c 3T3 cells, the α_3 isoform is activated by depolarization whereas the α_1 isoform is inhibited (Hara *et al.*, 1994). These effects could be significant *in vivo*, as discussed below. In addition, they may explain the larger difference in the $K_{K(ext)}$ between the α_1 and α_3 isoforms in microsome-fused erythrocytes compared to HeLa cell transfectants (see Chapter 4).

A number of reports (Lytton, 1985a; Brodsky and Guidotti, 1990; Sweadner, 1994) have suggested that there may be unidentified "factors", lost during the process of disrupting cells to isolate membrane preparations or during Na,K-ATPase purification, which modulate Na,K-ATPase behavior in intact cells. Although purely speculative at this time, it is possible that the mechanism(s) responsible for such modulation involve membrane lipid organization (see Klodos *et al.*, 1994). An alternative possibility, that of cell-specific or isoform-specific phosphorylation by protein kinases, must also be considered as a potential "purification-labile" modulatory factor (see Vasilets and Schwarz, 1992; Larsson *et al.*, 1994).

In spite of evidence (see Table II) supporting the conclusion that brain pumps have a higher apparent affinity for Na^+ than kidney pumps, results of transport assays in

cultured neurons are consistent with those presented in this thesis. Inoue and Matsui (1990) have shown that the excitatory amino acid glutamate, which activates large, channel-mediated Na^+ influxes, significantly stimulates those pumps in cultured rat central nervous system neurons that are sensitive to low ouabain concentrations. Similarly, Brines and Robbins (1992) found that inhibition of these ouabain-sensitive pumps by 1 μM ouabain during glutamate treatment resulted in profound cellular toxicity, whereas the majority of cells exposed to glutamate in ouabain-free media survived. (Presumably, this toxic effect was mediated by the rise in $[Ca]_{in}$ due to activation of the Na/Ca exchanger by the gain in $[Na]_{in}$.) Taken together, these studies suggest that it is the ouabain-sensitive Na,K-ATPase of neurons (probably the α_3 isoform; see Brines and Robbins, 1992) which is recruited in response to significantly elevated $[Na]_{in}$. In addition, results from cultured cells (Matsui *et al.*, 1994) as well as *in situ* neural sections (Sweadner *et al.*, 1990) indicate that the α_3 isoform is nearly inactive under resting cellular conditions. These observations parallel those reported for the HeLa cell transfectants (see Chapter 4, Fig. 4), whereby $[Na]_{in}$ activated the α_1 and α_2 isoforms to approximately 70% of their V_{max}'s, and yet activated the α_3 isoform to only ≈5% of its V_{max}. Thus a reasonable interpretation of the results from cultured neurons is that their ouabain-sensitive pumps have a relatively low affinity for $[Na]_{in}$, in keeping with the findings reported for both HeLa cells and microsome-fused erythrocytes.

The differences in apparent cation affinities among Na,K-ATPase isoforms suggest that the individual isoforms are tailored to carry out tissue-specific functions with regard to cation transport and regulation. For example, due to its ubiquitous presence and relatively high affinity for Na^+, Lytton (1985a) proposed that the α_1 isoform could serve as a "housekeeping pump", responding to the average cell's membrane leaks of Na^+ and thus maintaining the typically low $[Na]_{in}$ at homeostasis. The α_2 and α_3 isoforms, on the other hand, have been mainly detected in excitable tissues such as muscle and nerve. The specific localization of α_3 to neurons, and more recently, to the conduction system in the heart

(Zahler *et al.*, 1992), suggests that it may be especially important to cells which undergo substantial increases in membrane Na^+ permeability. (See Chapter 4 for a discussion of these issues with regard to K_{ext}.)

5.4. Conclusions

The membrane fusion methodology described in this thesis has facilitated the exploration of a number of issues regarding tissue-specific Na,K-ATPase behavior. One of its major advantages is that it is particularly well suited to cation flux studies of the Na,K- (and the Ca-) ATPase which permit the examination of certain aspects of enzyme behavior that cannot be studied in unsided preparations. In addition, unlike most enzyme reconstitution techniques, this method transfers pumps from one biological membrane into another without the use of detergents.

The conclusions drawn from the cation activation kinetics of tissue- and isoform-specific Na,K-pumps reported in this thesis (Chapter 4) differ from most of those previously published (see Table II). Results from rat kidney or axolemma microsome-fused dog erythrocytes show that axolemma pumps (mainly the α_3 isoform) have a three-fold higher $K_{Na(in)}$ and a three-fold lower $K_{K(ext)}$ than kidney pumps (the α_1 isoform). Similarly, studies using HeLa cells transfected with the three isoforms of the rat catalytic α subunit show that the α_3 isoform has at least a three-fold higher $K_{0.5(Nain)}$ and a nearly three-fold lower $K_{K(ext)}$ than either the α_1 or α_2 isoforms. Thus two different assay systems have provided very similar conclusions. In addition, the results comparing rat kidney and human erythrocyte pumps (both the α_1 isoform) in the human erythrocyte membrane environment indicate that the cation activation kinetics of these two kinds of pumps are nearly identical. Although one could argue that expressing pumps in different membrane environments, such as HeLa cells or erythrocytes, may alter their kinetic behaviors compared to those seen in their tissues of origin, the advantage of these

experimental systems is that the membrane environment is no longer a variable in comparative studies of isoforms.

In the foregoing section, a variety of factors that could potentially explain the discrepancies between these and previous studies was examined. They include differences in the ratios of isoforms, variations in the assay techniques and methods of and tissue preparation, the possible influence of the β subunit, the steady-state conformational equilibrium of the enzyme, and the role of the membrane environment and potential. Of these, it appears likely that the membrane (lipid) environment has the most significant influence in these experiments. As discussed above, there is ample evidence for its role in the modulation of the Na,K-ATPase. This may explain, for example, why the α_1 subunits of erythrocyte and kidney pumps behave differently depending on the tissue in which they are expressed (White and Blostein, 1982), in spite of the finding that they have the same responses to cations when in the human erythrocyte membrane (see Chapter 3). Moreover, given that the membranes of different tissues have their own unique lipid compositions, the notion of tissue-specific modulation of the Na,K-ATPase by membrane lipids seems quite plausible. In particular, the tissues of the nervous system display an abundance of unique lipids (*e.g.* sphingomyelin and glycosphingolipids) whose effects on this enzyme have not been thoroughly characterized. Further study will be required to determine if these lipids are capable of specific pump modulation (*e.g.* like fatty acids; see Section 1.2.5.d.) or if they have more general effects on membrane structure (*e.g.* fluidity; see Sutherland *et al.*, 1988; Klodos *et al.*, 1994).

Beyond the scope of the present experiments, other determining factors must be considered with respect to tissue- and isoform-specific apparent cation affinity differences. For example, variations in the primary amino acid sequences between isoforms could be the basis for structural differences which may in turn influence enzyme function. These variations, accounting for approximately 20% of the total number of amino acids (see Section 1.2.2.a.), need not necessarily occur at the cation binding sites in order to affect the

apparent affinities for cations (see Vilsen, 1993). Another factor which should be considered is the membrane potential. Although not an issue in studies using permeabilized membranes or erythrocytes, the findings of Hara *et al.* (1994) suggest that the different isoforms may have individual responses to changes in the transmembrane voltage. Finally, it remains to be determined if there are tissue-specific differences in Na,K-ATPase regulation. Observations from studies on the L-antigen of sheep erythrocytes and the membrane cytoskeleton (see Section 1.2.5.d.) exemplify how other membrane proteins can interact with and, in some cases, modulate the behavior of this enzyme. Moreover, studies investigating the regulation of the Na,K-ATPase by protein kinases are just beginning. It will be interesting to learn if the individual isoforms are differentially regulated by these and other components of cellular signal transduction systems.

CHAPTER 6

APPENDICES

APPENDIX I.

6.1. Rat kidney Na/K pumps incorporated into immature low K^+ sheep red cell membranes are stimulated by anti-L_p antibody

The assays summarized in the following appendix were carried out to see if rat kidney pumps delivered into LK sheep erythrocytes by the membrane fusion method described in Chapter 2 would be influenced by the L_p antigen of LK sheep erythrocytes (see Chapter 1, Section 1.2.5.d.). The working hypothesis, that the Lp antigen is a distinct molecular entity from the Na,K-ATPase that modulates its behavior, implied that this membrane regulator should be able to interact with and also modulate exogenous pumps delivered into LK erythrocytes. Indeed, this turned out to be the case as described below (for details, see Xu et al. (1992) Am. J. Physiol. 263: C1007-C1014).

ABSTRACT

A genetic dimorphism of sheep red cells characterized by differences in the intracellular K^+ concentration of mature red cells (low-K^+ or high-K^+ cells) reflects differences in their Na/K pumps and is known to be linked to the ML blood group system. We investigated the relationship of Na/K pumps in red cells from sheep of the low K^+ phenotype with an antigen, L_p, which is restricted to low-K^+ cells. Anti-L_p antibody stimulates the Na/K pumps in these cells, presumably be relieving inhibition of the pumps by L_p. The questions addressed were, is L_p a molecular entity distinct from pumps, and if so can it interact with pumps of exogenous origin. Rat kidney Na/K pumps were incorporated by fusion of microsomes into either low-K^+ or high-K^+ sheep red cells . The activity of the exogenous kidney pumps was distinguished from that of the endogenous red cell pumps by the low sensitivity of rodent pumps to ouabain. Anti-L_p stimulated by >50% rat kidney pumps incorporated into immature low-K^+ sheep cells. This indicates that L_p is a distinct molecular entity free to dissociate from endogenous pumps and inhibit exogenous pumps. Anti-L_p did not stimulate kidney pumps incorporated into mature low-K^+ cells, but did stimulate kidney pumps following in vitro maturation of microsome fused reticulocytes, probably reflecting restriction of lateral movement of pumps and antigens by the cytoskeleton in mature cells.

APPENDIX II

6.2. Summary of preliminary attempts to improve pump delivery from microsomes into erythrocytes

One of the limitations of the membrane fusion protocol described in Chapter 2 is that only a small fraction of the available microsomal activity becomes functionally incorporated into erythrocyte membranes (see Section 5.1.). Although not a problem in the majority of the studies presented here, the limit to pump incorporation does restrict the choice of microsomal preparations to those with high Na,K- (or Ca-) ATPase activities (*e.g.* greater than 1 μmole of ATP hydrolyzed/(min x mg-protein) measured as described in Chapter 2. In addition, the erythrocytes of certain species, such as dogs appear to require somewhat higher concentrations of PEG to achieve optimal pump deliveries. Unfortunately, this harsher treatment increases the fragility of the cells during and after the fusion process, resulting in significant losses of the starting material. In order to better understand the nature of the fusion process between cells and microsomes, as well as to improve the efficiency of pump delivery, various preliminary investigations were carried out. The findings are presented in the following table.

TABLE I

Modification	Goal	Delivery change (relative to control)	Observations
A. Microsome treatments			
Gradient purification using sucrose or metrizamide	Improve specific activity and remove unsealed vesicles	≈ 1.5-fold increase	Typically 50% loss of starting material on gradient
Extraction using high salt concentrations	Improve specific activity by removing loosely-associated protein	≈ 1.5-fold increase	
SDS purification to generate solubilized Na,K-ATPase	Maximize specific activity	No pump delivery	
Membrane doping with "fusogenic" lipids	Increase attractive forces between microsomes and cells; also decrease thermodynamic barriers to fusion	No pump delivery	

TABLE I (Continued)

B. Erythrocyte treatments

Treatment with trypsin or neuraminidase	Remove part of glycocalyx to reduce steric bulk	Same as control	
Treatment with 5 mM diamide[1]	Cross linking of cytoskeleton	Same as control	
Incubation at 48 °C for 10 min.[2]	Disruption of cytoskeleton	Same as control	
Membrane doping with "fusogenic" lipids	Increase lipid area of membranes	Significant improvement (could not compare to controls since less PEG used)	Cells very fragile and displayed continuous lysis during storage
Membrane doping with cholesterol	Increase lipid area of membranes	Up to 2.5-fold improvement in some experiments	
Treatment with lectins	Increase attraction between microsomes and cells	No significant improvement	Up to 15-fold increase in specific 3H-ouabain binding

[1] Deuticke et al., 1983.
[2] Brandts et al., 1977; Lysko et al., 1981.

APPENDIX III

6.3. Quantitation by immunoblotting of rat axolemma membrane Na,K-ATPase α subunit isoforms

In order to assess the behavior of the rat axolemmal Na,K-ATPase α_3 subunit isoform, it was first necessary to determine its relative abundance compared to the other isoforms present in this tissue. As described in Chapter 4 (see "Experimental Procedures" and Table I), one of the methods used to address this issue was quantitative immunoblotting of axolemmal membrane preparations. Using antibodies specific for the α_1 and α_2 subunit isoforms, it was possible to estimate their enzymatic activities in axolemma membranes by relating their immunological reactivities to those of the same isoforms in "standard" tissues (*i.e.* kidney and muscle) whose enzymatic activities were known. Data representative of the Western blots obtained as well as the calculations used to relate band intensities to enzymatic activities are shown.

TABLE II

Isoform	Kidney			Muscle		Axolemma		
	(Standard)							
α_1								
								
µg protein applied	0.1	0.2	0.4			1.0	2.0	
KIOD [1]	116	353	605			136	350	
KIOD/µg protein	1160	1765	1512			138	175	
Activity [2,3]		2.1					**0.23**	
	(Standard)							
α_1								
								
µg protein	0.3	0.4		4.0	10.0			
KIOD	201	292		431	818			
KIOD/µg protein	670	730		108	82			
% EP [4]		-			33.3			
Activity		2.1			**0.29**			
				(Standard)				
α_2								
								
µg protein				1.0	2.0	2.0	4.0	6.0
KIOD				305	745	318	1021	1206
KIOD/µg protein				305	373	159	255	201
% EP					66.7			
Activity					**0.59**		**0.36**	

[1] (see next page for explanations)

[1] absorbance units (optical density measurements of fluorographs)
[2] activity units from Na,K-ATPase ATP hydrolysis assays (μmole ATP hydrolyzed/ (mg protein x min))
[3] calculated activity units (numbers in **bold** type) derived from the average value of the KIOD/μg protein value of the "unknown" tissue divided by that of the "standard" tissue multiplied by the measured Na,K-ATPase activity of the "standard" tissue
[4] percent of total phosphoenzyme (see Chapter 4, Fig. 3)

Since the total Na,K-ATPase activity of the axolemma membranes depicted here was 2.2 units, the percentage of each axolemmal isoform can be calculated as follows:

α_1 0.23/2.2 = 11%

α_2 0.29/2.2 = 17%

α_3 100% - 11% -17% = 72%

CHAPTER 7

REFERENCES FOR INTRODUCTION AND GENERAL DISCUSSION

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