Bioscience Reports, Vol. 20, No. 2, 2000

REVIEW

Na^+, K^+-ATPase Isozyme Diversity; Comparative Biochemistry and Physiological Implications of Novel Functional Interactions

Ali Mobasheri,[1,5] Julio Avila,[2] Irene Cózar-Castellano,[2] Michael D. Brownleader,[1] Michael Trevan,[1] Martin J.O. Francis, Joseph F. Lamb,[4] and Pablo Martín-Vasallo[2]

Revised version received October 12, 1999; accepted October 26, 1999.

Na^+, K^+-ATPase is ubiquitously expressed in the plasma membrane of all animal cells where it serves as the principal regulator of intracellular ion homeostasis. Na^+, K^+-ATPase is responsible for generating and maintaining transmembrane ionic gradients that are of vital importance for cellular function and subservient activities such as volume regulation, pH maintenance, and generation of action potentials and secondary active transport. The diversity of Na^+, K^+-ATPase subunit isoforms and their complex spatial and temporal patterns of cellular expression suggest that Na^+, K^+-ATPase isozymes perform specialized physiological functions. Recent studies have shown that the α subunit isoforms possess considerably different kinetic properties and modes of regulation and the β subunit isoforms modulate the activity, expression and plasma membrane targeting of Na^+, K^+-ATPase isozymes. This review focuses on recent developments in Na^+, K^+-ATPase research, and in particular reports of expression of isoforms in various tissues and experiments aimed at elucidating the intrinsic structural features of isoforms important for Na^+, K^+-ATPase function.

KEYWORDS: Na^+, K^+-ATPase; subunit; isoform; isozyme; plasma membrane; membrane transport; ion homeostasis; cytoskeleton.

INTRODUCTION

The ability of living cells to respond to changes in their ionic environment and maintain a relatively constant internal milieu is one of their most fundamental properties. In response to external perturbations, cells will activate specific membrane bound transport systems in an effort to maintain overall homeostasis. The

[1]Dept of Veterinary Preclinical Sciences, University of Liverpool, Brownlow Hill and Crown Street, Liverpool L69 3BX.
[2]Laboratorio de Biología del Desarrollo, Departamento de Bioquímica y Biología Molecular, Universidad de La Laguna, La Laguna, Tenerife, Spain.
[3]Nuffield Department of Orthopaedic Surgery, Nuffield Orthopaedic Centre, University of Oxford, Headington, Oxford, United Kingdom.
[4]School of Biomedical Sciences, University of St. Andrews, St. Andrews, Fife, United Kingdom.
[5]To whom correspondence should be addressed.

0144-8463/00/0400-0051$18.00/0

coupling of metabolic energy to cellular function and the signaling between and within cells depends on the asymmetric distributions of ions across the cell membrane. All cells contain much more potassium and much less sodium than their surroundings and these cation concentration differences are tightly regulated (see Figure 1). The existence of a sodium transporter in the plasma membrane of animal cells was first suggested by Dean in 1941, who introduced the term ''pump'' to explain the asymmetric distributions of cations. Almost twenty years later, studies on squint giant axons established the existence of active Na^+ and K^+ transport across the axolemma (Caldwell *et al*., 1960). The existence of an ATP hydrolyzing enzyme that requires presence of sodium and potassium for activity was first demonstrated conclusively in crab nerve by Jens Christian Skou in 1957. Skou, a surgeon working in Aarhus, Denmark was studying local anaesthetics and inadvertently stumbled upon the Na^+, K^+-ATPase while studying other membrane associated enzymes such as acetylcholinesterase. At that time, it was well established that stimulation of nerves resulted in an influx of sodium ions into the axon (possibly through voltage activated sodium specific channels in the axonal plasma membrane) generating a nerve impulse. However, the mechanism of sodium efflux was not known. Skou's original and insightful approach to this problem led to the explicit identification of a magnesium dependent, sodium and potassium adenosine triphosphatase (Na^+, K^+-ATPase) as the sodium pump (Skou, 1957, Slou 1998a). His findings represented a milestone in the field of ion transport and in 1997, forty years after his pioneering experiments, he was awarded the Nobel Prize for his outstanding contribution to the field of Na^+, K^+-ATPase research (Skou, 1998b). A transcript of his Nobel lecture is published in *Bioscience Reports* (Volume 18, issue 4, pp. 155–169; Skou 1998b).

Na^+, K^+-ATPASE

Sodium Pump or Na^+, K^+-ATPase?

The sodium or Na^+/K^+ pump is the familiar name for Na^+, K^+-ATPase ($(Na^+ + K^+)$-stimulated adenosine triphosphatase; E.C. 3.6.1.37) an multi-subunit enzyme embedded in the plasma membrane (see Figs. 1, 2 and 3). It maintains the low intracellular Na^+:K^+ ratio vital for normal cellular activity and optimal cellular function. The Na^+, K^+-ATPase catalyzes the active uptake of K^+ and extrusion of Na^+ at the expense of hydrolyzing ATP generated from cellular glycolysis and oxidative phosphorylation thereby generating steep concentration gradients for these ions. The gradients are then harnessed by other membrane proteins for a variety of essential cellular functions, including electrical membrane potential changes mediated by ion channels, the active uptake of molecules like neurotransmitters, amino acids, sugars, nucleosides and the extrusion of Ca^{2+} (see Fig. 1). The Na^+, K^+-ATPase also regulates (directly or indirectly) many essential cellular functions, such as cell volume, heat production, and intracellular pH regulation (Rossier *et al*., 1987) in all animal cells.

Na^+, K^+-ATPase is a P-type ATPase

Na^+, K^+-ATPase is a member of a large family of P-type (phospho-intermediate type) ATPases that exist in prokaryotes and eukaryotes. P-type ATPases harness the

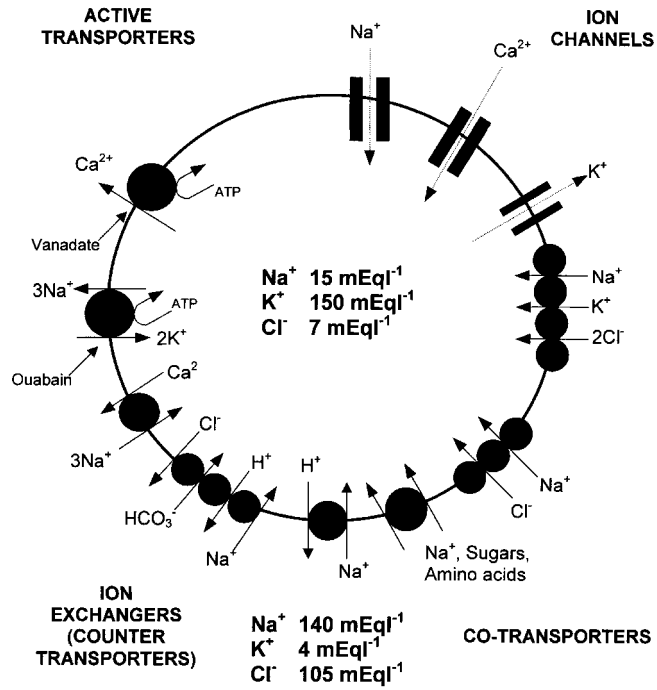

Fig. 1. The Na^+, K^+-ATPase in relation to other Na^+ dependent and Na^+ independent transport systems in a typical animal cell (adapted from Skou, 1998b). The Na^+, K^+-ATPase generates and maintains the cell membrane potential and the inward Na^+ gradient thus generated is utilized by other Na^+ dependent transport systems for uptake of sugars and amino acids and for the extrusion of calcium and protons. Fine regulation of intracellular ions such as Na^+, K^+ and Ca^{2+} is also important for cell volume regulation, cytoskeletal stability and organelle morphology. Chronic inhibition of the Na^+, K^+-ATPase by ouabain (or by ATP depletion induced by ischaemia) will influence associated transport pathways and result in accumulation of intracellular Na^+, K^+ and Ca^{2+} (Jamme *et al.*, 1997). The movement of osmotically obliged water into the cells results in cell swelling or oedema (Basavappa *et al.*, 1998) and ultimately in necrotic cell death.

energy derived from the hydrolysis of the terminal pyrophosphate bond of ATP to drive the transport of cations such as Na^+, K^+, H^+, Ca^{2+}, Cu^{2+} and Cd^{2+} (Fagan and Saier, 1994). All P-type ATPases undergo conformational changes as part of their reaction cycle (described in detail in Fig. 2) and also share homologous membrane bound catalytic α subunits that contain binding sites for ATP, the designated cation(s) and very often a specific inhibitor. In generally, P-type ATPases are inhibited by vanadate, a transition state analog of phosphate and a compound that helps distinguish many of the P-type ATPases from unrelated V-type and F-type proton ATPases. Other members of the "P" type ATPase family include the plasma membrane and organellar Ca^{2+}-ATPase (such as PMCA and SERCA; Carafoli, 1991),

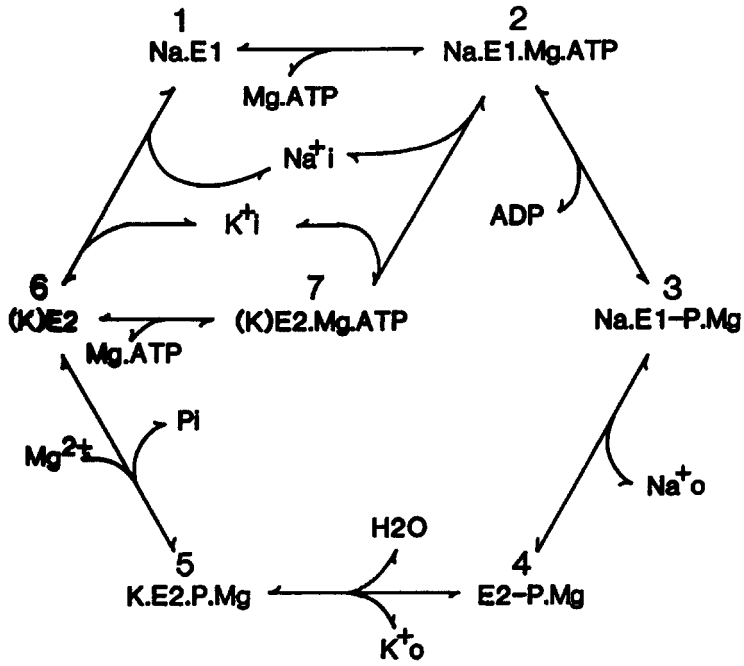

Fig. 2. Reaction cycle of the Na^+, K^+-ATPase showing conformational changes during the transport cycle. This simplified scheme shows how the Na^+, K^+-ATPase couples the energy of ATP hydrolysis to the movement of ions across the plasma membrane. Hydrolysis of ATP by the Na^+, K^+-ATPase is accompanied by sequential protein conformational changes that translocate the ions (Jørgensen, 1986). This figure shows that binding of Na^+ at the intracellular face of the enzyme accelerates the transfer of the terminal phosphate of ATP to an aspartate side chain in the active site, and this brings about a conformational change that delivers Na^+ to the outside of the cell. Binding of K^+ then causes a conformational change that accelerates the hydrolysis of the covalently bound phosphate, and the K^+ is transported to the inside of the cell as the enzyme returns to its initial conformation. The principal enzyme conformations are known as E1 and E2.

the recently characterized non-gastric H^+, K^+-ATPase, plasma membrane H^+-ATPase of plants and fungi, Ca^{2+}-ATPase of plants and animals (Serrano, 1989), gastric and non-gastric H^+, K^+-ATPase (Ganser and Forte, 1973; Kaunitz and Sachs, 1986 (Lorenzton, 1988), K^+-ATPase of bacterial plasma membrane (Helmer *et al.*, 1982), aminophospholipid-transporting ATPase (Auland *et al.*, 1994; Tang *et al.*, 1996) and Cl^--translocating ATPase (Gerencser and Purushotham (1996).

P-type ATPase Superfamily

By careful analyses of amino acid sequences of forty seven P-type ATPases from eukaryotes and prokaryotes, Fagan and Saier (1994) were able to reaffirm findings made by Serrano (1988) that all the P-type ATPases were found to have evolved from a common ancestral protein and, additionally, group these enzymes in

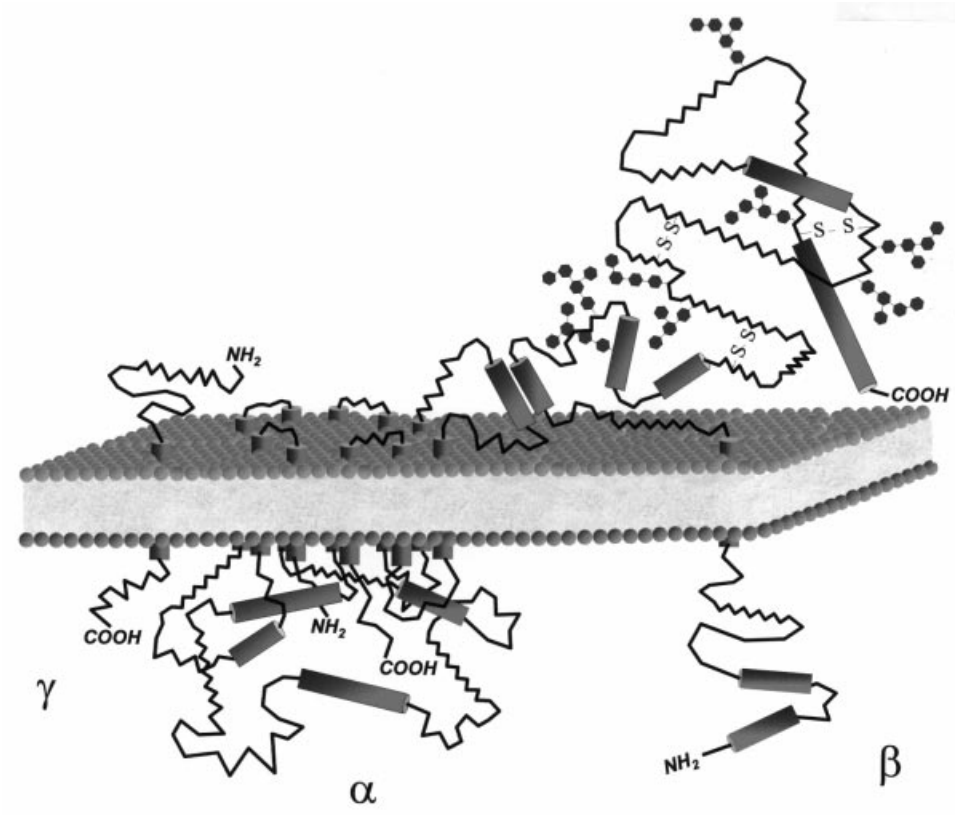

Fig. 3. Putative three-dimensional model of the topological structure of the Na^+, K^+-ATPase. This model of the Na^+, K^+-ATPase depicts the catalytic α subunit spanning the plasma membrane 10 times and places both the NH_2 and COOH termini in the cytosol. The β subunit spans the plasma membrane once and its NH_2 terminus is in the cytosol. The β subunit contains 3 disulfide bridges and may have three to eight potential sites for *N*-linked glycosylation (depending on the specific isoform). Areas with predicted α-helices and β-pleated sheets are shown as cylinders and zig-zags respectively. The γ subunit also spans the plasma membrane once and its COOH terminus is in the cytosol.

a single family divisible into four principal clusters that correlated with both their ion specificities and biological origins. Cluster 1 contained the Ca^{2+}-ATPase, cluster 2 contained the Na^+- and gastric H^+-ATPases, cluster 3 comprised the plasma membrane H^+-translocating ATPases of plants, fungi and lower eukaryotes and cluster 4 comprised all of the bacterial P-type ATPases (specific for K^+, CD^{2+}, Cu^{2+} and an unknown cation). They undertook a comprehensive phylogenetic study of P-type ATPases to define their evolutionary relationships in order to shed light on structure-function relationships of the different structural domains. Sequence comparisons can highlight conserved residues that may contribute to the structure and/or function of the enzyme family. These investigators further divided ATPases into four structural domains. They deduced that the *N*-terminal segments 1 (comprising the *N* termini of the ATPases, their 4 putative *N*-terminal transmembrane helices, and all inclusive cytoplasmic and extracellular loops) and the centrally-located large cytoplasmic

domain, which contains the phosphorylation domain and putative ATP binding sites (segment 2) evolved from a single primordial ATPase which existed prior to the divergence of eukaryotes from prokaryotes. The *C*-terminal segments 3 (containing the remaining *C*-terminal region) appeared to be specific to the eukaryotic P-type ATPases suggesting that the *C*-terminal domain, with the least degree of amino acid sequence conservation, may therefore, have arisen from eukaryotes diverged from prokaryotes.

The P-type ATPase classification proposed by Fagan and Saier (1994) has been superseded by a more precise taxonomic system (Moller *et al*., 1996; Axelsen and Palmgren, 1998). These investigators had the undoubted benefits of recently discovered gene sequences of several genomes available to them. There are currently 5 families of P-type ATPases that are categorized as Type I-V ATPases (Axelsen and Palmgren, 1998). Type I ATPases are heavy metal transmembrane pumps that transport cations such as Cu^+, Cd^{2+} and Ag^+. These Type I pumps comprise a putative *N*-terminal heavy metal binding site, they have small molecular weights in the region of 70–85 kD and are predominantly found in bacteria (Moller *et al*., 1996). However, a putative Cu^+-transporting ATPases in man has been identified whose deficiency is linked to progression of Wilson's and Menke's diseases (Vulpe *et al*., 1993, Bull *et al*., 1993). Prokaryotic ATPases usually have lower molecular weights ($M_r \sim 70$ kD) than those present in eukaryotes ($M_r \sim 100$–140 kD). Mg^{2+}-ATPase from *S. typhiurium* (Smith *et al*., 1993) and a putative Ca^{2+}-transporting ATPase from the cyanobacterium, *Synechococcus* (Kanamura *et al*., 1993) are, however, exceptions to the rule. Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, Na^+, K^+-ATPases and H^+, K^+-ATPases constitute Type IIA ATPases whereas Type IIB ATPases comprise plasma membrane Ca^{2+}-ATPase and H^+-ATPases from plants and fungi. Type IIB ATPases such as Ca^{2+}-ATPase and H^+-ATPase contain C-terminal hydrophilic domains that can interact with calmodulin or other modulators, respectively. This form of regulatory control is absent in the Type IIA ATPases. ATPases such as Ca^{2+}-ATPase and Na^+, K^+-ATPase, H^+, K^+-ATPase and Kdp K^+-ATPase are also associated with one or two smaller subunits. Kdp (type IA) ATPase comprises three integral membrane proteins that are believed to be encoded for by three structural genes of the kdpABC operon. Hesse *et al*. (1984) have isolated and sequenced the three structural genes that encode the three proteins of the Kdp-ATPase. KdpB is the catalytic subunit, KdpA is involved in binding of K^+, and KdpC appears to stabilize the complex between KdpB and KdpA (Buurman *et al*., 1995). The β subunit of the Na^+, K^+- and H^+, K^+-ATPases stabilize the catalytic α-subunit (Moller *et al*., 1996) and is believed to have evolved from the KdpC subunit. Interestingly, H^+ (Type IIIA)- and Ca^{2+} (Type IIA and Type IIB)-ATPases appear to have discarded this subunit. Na^+, K^+-ATPase is closely related to the H^+, K^+-ATPases, each ATPase demonstrating unambiguous and distinct ion specificities. Upon closer inspection, several amino acid residues are thought to contribute to the specificity of the ion binding site (Moller *et al*., 1996) and unsurprisingly, amino acids determining Na^+ specificity are conserved in all Na^+, K^+-ATPases but may not be essential for the activity of H^+, K^+-ATPases. Those amino acids contributing to H^+ specificity may therefore be invariant in all H^+, K^+-ATPases (Axelsen and Palmgren, 1998). Type III ATPases comprise plasma membrane H^+-ATPases and Type IV ATPases are a

novel class of prokaryotic P-type ATPase that have recently been linked to the translation of aminophospholipids such as phosphatidylserine and phosphatidylethanolamine across the lipid bilayer and are thought to contribute to lipid asymmetry (Auland *et al*., 1994; Tang *et al*., 1996). The amphipathic nature of phosphatidylserine distinguishes this class of ATPase from the vast majority of P-type ATPases that transport simple cations. Preliminary sequence alignment analyses indicates that the glutamate, arginine and threonine residues found in specific transmembrane helices, particularly in Ca^{2+}-ATPase and inextricably associated with cation transport are replaced by bulky hydrophobic amino acids that are thought to interact with the hydrophobic components of the phospholipid substrates. The first putative anion-transporting ATPase has recently been identified in the *Aplysia californica* foregut (Gerencser, 1996; Gerencser and Purushotham, 1996). Studies with liposome-reconstituted anion transporter ATPase indicate that an electrogenic ATP-driven Cl^--pump is involved in active Cl^--transport in the basolateral membrane of the epithelial absorptive cells. Since Type V ATPases comprise ATPases with no assigned substrate specificities, these anion-transporting ATPases fall within this sub-class. Whether any of the P-type anion pumps belong to Type V ATPases await cloning of the respective genes. The evolution of the substrate specificities of P-type ATPases and their classification is constantly under scrutiny and is beyond the scope of this review. Excellent contemporary reviews by Moller *et al*. (1996) and Axelsen and Palmgren (1998) provide further in-depth reading around this subject area.

All P-type ATPases sequenced to date share the following structural and kinetic features: they all form a covalent, aspartate phosphorylated intermediate during their catalytic reaction cycle; they all have an absolute dependence on ATP for the specific binding of the ion(s) they transport. Phosphorylation of the asparatate (D) residue occurs within an invariant sequence motif-DKTGT(I,L)T (Axelsen and Palmgren, 1998). Further analysis of the overall homology between membrane bound ATPases has been provided by Walderhaug *et al*. (1985). These workers analyzed the residues around the phosphorylation site of the Na^+, K^+-ATPase from the outer medulla of the dog kidney, the hog gastric H^+, K^+-ATPase, the corn root plasma membrane H^+-ATPase and the H^+-ATPase of the prokaryote, *Acholeplasma laidlawii*. The phosphorylation site of the Na^+, K^+-ATPase is the β-carboxyl group of an aspartyl residue (Nishigaki *et al*., 1974). Walderhaug *et al*. (1985) found that the active site of phosphorylation within the Na^+, K^+-ATPase, H^+, K^+-ATPase and the higher plant plasma membrane H^+-ATPase consisted of the sequence C-(S/T)—D-K. This is almost identical to the conserved sequence found within the Ca^+-ATPase of the sarcoplasmic reticulum. Other conserved sequences include the long "hinge" sequence found in the junction region (VAVTGDGVNDSPALKKADIGVAM); this sequence representing that found in the Na^+, K^+-ATPase (Moller *et al*., 1996).

Kinetic analyses of the Na^+, K^+-ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase have identified a four-step reaction mechanism, referred to as the Post-Albers scheme for the Na^+, K^+-ATPase [Albers 1967; Post *et al*., 1972) and involving the following reaction sequence $E1 \rightarrow E1P \rightarrow E2P \rightarrow E2 \rightarrow 1$ (de Meis, 1981; Glynn 1984, see Fig. 2). As a consequence of this phosphorylated aspartate intermediate, all P-type ATPases are inhibited by micromolar quantities of orthovanadate. P-type

ATPases cycle between two conformations during catalysis, referred to as E1 and E2 (Fig. 2). In addition, each P-type ATPase possesses a large subunit comprising distinct conserved domains, believed to be responsible for ATP binding and hydrolysis. In fact, all P-type ATPases contains common sequences implicated in (i) the coupling of ATP hydrolysis to transport, (ii) the formation of a phosphorylated aspartate intermediate and (iii) ATP binding. All P-type ATPases contain a cytosolic-protruding hydrophilic head that contains the phosphorylation- and ATP-binding site and a smaller highly variable *N*-terminal cytosolic domain, interspersed with a variable number of transmembrane helical channels. Prokaryotic Type I ATPases are believed to contain around 6 transmembrane helices whereas the Type II ATPases contain only 4 transmembrane helices in the *N*-terminal region. However, there can be up to 6 transmembrane helices in the *C*-terminal region of Type II eukaryotic ATPases compared to 2 helices in Type I ATPases. The smaller molecular weight of prokaryotic ATPases is essentially attributed to the smaller *C*-terminal membrane-associated domain.

Mammalian P type ATPases are involved in the exchange of cations; the Na^+, K^+-ATPase exchanges 3 Na^+ ions for 2 K^+ ions during an active cycle. In contrast plant and fungal plasma membrane H^+-pumps do not exchange ions. Serrano (1989) suggested that cation transport is a primitive evolutionary feature of P-type ATPases and that proton pumps originated in anaerobic primordial cells that needed to extrude protons. A major source of protons in primordial cells was likely to be the external medium and these cells developed a proton ATPase to counteract the high acidity of the external medium. Cells surrounded by a semi-permeable plasma membrane will swell and burst in a hypo-osmotic extracellular environment unless there is a rigid cell wall to prevent membrane rupture or that there are ion pumps that can counteract the passive inward leak of hydrated ions. Consequently, ATPases in primordial cells may have evolved to play a role in cell volume regulation (Robinson, 1990). The recent discovery of aquaporin water channels in the plasma membrane of plant, animal and yeast cells suggests that P-type ATPases may only contribute partially to the maintenance of water homoeostasis (for reviews see Chrispeels and Agre, 1994, Knepper, 1994).

The H^+, K^+-ATPase operates against a formidable transmembrane H^+-gradient that is estimated to be about 10^6 fold. The resultant thermodynamic constraint placed upon the enzyme has limited stoichiometry for its turnover to 1 mol of H^+-translocated in exchange for 1 mol of K^+ at the expense of 1 mol of ATP (Shull and Lingrel, 1986). Shull and Lingrel (1986) have isolated and sequenced the cDNA of the rat gastric mucosa H^+, K^+-ATPase with an oligonucleotide corresponding to conserved amino acid sequences of related cation transporting ATPases and cross hybridization with the sheep kidney Na^+, K^+-ATPase α-subunit cDNA. The overall homology between H^+, K^+-ATPase and Na^+, K^+-ATPase is about 62%. The regions of greatest homology are located around the phosphorylation site (Asp^{385}) and within the domains that are proposed to be the transmembrane energy transduction region and the nucleotide-binding site. The region that differed significantly was the *N*-terminal hydrophilic domain that is postulated to be responsible for cation discrimination.

The H^+, K^+-ATPase contains three possible glycosylation sites at Asn^{224}, Asn^{492} and Asn^{729}. Shull *et al.* (1985) found that these sites are conserved within the N^+, K^+-ATPase that contains a total of five *N*-linked glycosylation sites. Jackson *et al.* (1983) identified a fluorescein-5′-isothiocyanate lysine residue that is protected by ATP in the H^+, K^+-ATPase and subsequently confirmed by Farley and Faller (1985) to be Lys^{517}. Tamural *et al.* (1989) showed that the conserved Lys^{497} found in the Ca^{2+}-ATPase, α-subunit of the Na^+, K^+-ATPase and plant plasma membrane H^+-ATPase was modified by pyridoxal 5′-phosphate. Consequently, Lys^{497} may have an important role in cation-transporting ATPases.

The non-conserved regions of the P-type ATPases have evolved specialized functions and are believed to contribute to ion specificity, regulation and intracellular targeting. Many P-type ATPases have extended *C*-terminal auto-inhibitory regions. Proteolytic cleavage of this *C*-terminal region or removal by molecular genetic methods force the enzyme into a high affinity state for its substrates (Palmgren *et al.*, 1991; Palmgren and Christensen, 1993). Examples include the animal plasma membrane Ca^{2+}-ATPase (Zurini *et al.*, 1984) and the plant plasma membrane H^+-ATPase (Palmgren *et al.*, 1991).

P-type ATPase Interchangeability and Role in Vectorial Transport

In some instances, a subunit component of a particular P-type ATPase system may assemble with a subunit component of another P-type ATPase *in vivo* (Codina *et al.*, 1998). This suggests that there may be limited interchangeability of components of P-type ATPases *in vivo*. In epithelial cells members of the P-type ATPase superfamily generate the cation gradients responsible for vectorial fluid and solute transport across epithelia (Caplan, 1997a). In order to perform this specialized function, each P-type ATPase is restricted to a specific membrane domain (i.e., basolateral or apical). In non-epithelial cells the P-type ion pumps play a significant role in ion homoeostasis and are thus vital for optimal cellular metabolism.

Molecular Identification of the Na^+, K^+-ATPase

A breakthrough in the understanding of the molecular composition of the Na^+, K^+-ATPase came in the early 1980's. Collins and co-workers (1983) determined the sequence of tryptic peptides of α and β subunits of Na^+, K^+-ATPase isolated from kidney. The sequence information was necessary for subsequent cloning of the full-length cDNAs corresponding to the α and β isoforms. The complete amino acid sequence for the α and β subunit isoforms of the Na^+, K^+-ATPase was determined from corresponding cDNAs in sheep kidney (Shull *et al.*, 1985; Shull *et al.*, 1986a), pig kidney (Ovchinnikov *et al.*, 1985; 1987), and *Torpedo Californica* electric organ (Kawakami *et al.*, 1985; Noguchi *et al.*, 1986; Noguchi *et al.*, 1987). Molecular identification and sequencing of the γ subunit gene was not completed until 1993 by Mercer and colleagues.

Na^+, K^+-ATPase Structure

The Na^+, K^+-ATPase is composed of 3 polypeptide subunits (α, β, γ); a multipass transmembrane α subunit (M_r = 100–112 kDa) containing the binding sites for Na^+, K^+, ATP and cardiac glycosides, a class of naturally existing steroid compounds which serve as specific inhibitors of the Na^+, K^+-ATPase. The β subunit is often referred to as the "regulatory" subunit and is understood to be required for the biogenesis and activity of the enzyme complex that is believed to be a heterodimeric protomer (α-β)2 (Brotherus *et al.*, 1983; Fambrough *et al.*, 1994). The molecular weight of the β subunit depends on the size of its attached *N*-linked sugars (M_r = 35 kDa approximately; glycoprotein M_r = 45–55 kDa). The recently described γ subunit is a transmembrane protein (M_r = 6.5–10 kDa) is found to be associated with functional Na^+, K^+-ATPase (Mercer *et al.*, 1993). Since the description of the γ subunit, no official revision of subunit stoichiometry has been put forward but the γ subunit probably exists in roughly equal quantity to the α and β subunits in tissues where it is expressed. The α and β subunits are both essential for enzymatic activity (McDonough *et al.*, 1990a; 1990b). The α subunit contains the phosphorylation, nucleotide binding and cation binding sites and is often referred to as the catalytic subunit. The topological model of the pump is still very controversial (Lingrel and Kuntzweiler, 1994; Shainskaya and Karlish, 1994; Mohraz *et al.*, 1994). However, it is established that the amino and carboxy termini of the α subunit are intracellular and there is a large central intracellular domain (containing the phosphorylation, nucleotide binding and cation binding sites), anchored by 10 transmembrane domains. The existing models predict that very little of the α subunit is exposed on the extracellular face. In contrast the bulk of the β subunit is exposed to the outside of the cell where it could mask the α subunit (Arystarkhova *et al.*, 1995). The extracellular domain of the β subunit has three asparagine-linked (*N*-linked) carbohydrate groups and three disulfide bridges. Only a small portion of the β subunit is intracellular connected to a predominantly extracellular domain. Like the β subunit, the γ subunit spans the plasma membrane once but unlike the β subunit the amino terminus of the γ subunit is extracellular. A putative 3-dimensional model of the α, β and γ subunits of the Na^+, K^+-ATPase is shown in Fig. 3. However, due to the lack of a crystal structure for the Na^+, K^+-ATPase very little is known about the precise molecular structure and 3-dimensional organization of the enzyme in the plasma membrane.

Na^+, K^+-ATPase Ionic Stoichiometry

The Na^+, K^+-ATPase functions in almost all animals as the principal regulator of intracellular Na^+ and K^+ concentrations using ATP as an energy source. During each active cycle 3 Na^+ ions are pumped out of the cell in exchange for 2 incoming K^+ ions for every ATP molecule consumed which implies that the enzyme complex contains multiple bindings sites for Na^+ and K^+ (Sweadner, 1989, 1995). Thus, Na^+, K^+-ATPase function leads to intracellular accumulation of K^+ that is essential for the activity of many intracellular enzymes. The intracellular concentration of K^+ must be maintained at around 120–140 mM by active pumping from the extracellular environment where the K^+ concentration is around 5 mM, against a steep

concentration gradient. Thus the Na^+, K^+-ATPase maintains the low intracellular Na^+:K^+ ratio in face of an inward concentration gradient for Na^+ and an outward gradient for K^+. The Na^+, K^+-ATPase is most abundantly expressed in excitable tissues such as the brain, skeletal muscle, cardiac muscle and epithelia and moderately expressed in other tissue and cell types.

NA^+, K^+-ATPASE ISOFORMS; DISTRIBUTION AND PHYSIOLOGICAL FUNCTIONS

Before the α and β subunits were cloned in the mid 1980's a number of laboratories had identified two molecular forms of α subunits designated α and α^+ based on their different mobilities in SDS-polyacrylamide gels (Sweadner, 1979) and differential sensitivity to cardiac glycosides in cardiomyocytes (Adams *et al*., 1982). Biochemical studies employing SDS-PAGE showed that the α^+ form of the Na^+, K^+-ATPase in adipocytes was responsive to insulin whereas the α form was not sensitive to this hormone (Lytton, 1985; Lytton *et al*., 1985). Physiological studies on the mode of action of the cardiotonic steroid ouabain in the myocardium clearly established that two distinctly different binding sites exist for ouabain in ventricular myocytes; a high affinity ouabain binding site and a low affinity ouabain binding site responsible for the predominant inotropic effect of ouabain. Thus, it was clear at the time that several distinct forms of the α subunit existed. The controversy and confusion surrounding the α and α^+ forms and the low and high ouabain affinity sites was finally laid to rest when Shull and associates (1986b) cloned three distinct forms of the rat Na^+, K^+-ATPase α subunit now termed $\alpha1$, $\alpha2$ and $\alpha3$. It became evident that the $\alpha1$ and "α" isoforms were in fact one and the same whereas the α^+ form was identified as $\alpha2$ and $\alpha3$. The $\alpha3$ isoform was a novel isoform that had not been described previously (Hara *et al*., 1988). It is now accepted that multiple genes encode the Na^+, K^+-ATPase α subunit (Shull and Lingrel, 1987; Lingrel *et al*., 1990; Shamraj and Lingrel, 1994). Figure 4 depicts the tissue distribution of mRNAs encoding the Na^+, K^+-ATPase in various rat tissues. Three different isoforms of the catalytic α subunit have been identified at the genetic (Shull *et al*., 1986b; Kent *et al*., 1987) and protein level using immunological techniques (Felsenfeld and Sweadner, 1988, Urayama *et al*., 1989; Shyjan and Levenson, 1989; extensively reviewed by Sweadner, 1989). Western blots in Fig. 5 compare the expression of Na^+, K^+-ATPase α isoforms in several human and rat tissues. A putative fourth α subunit isoform has also been identified at the transcriptional level in mammalian testis (Shamraj and Lingrel, 1994) (Fig. 4A/B). Table 2 shows the similarities and differences between the four α subunit isoforms identified to date. The primary amino acid sequences of the $\alpha1$, $\alpha2$, and $\alpha3$ isoforms were initially deduced from cDNAs (Shull *et al*., 1985). The α isoforms have distinct kinetic properties that have been studied extensively by Lingrel and colleagues. Jewell and Lingrel (1991) compared the substrate dependence properties of the rat Na^+, K^+-ATPase the $\alpha1$, $\alpha2$ and $\alpha3$ isoforms expressed in human HeLa cells. They generated ouabain resistant forms of the rat Na^+, K^+-ATPase $\alpha2$ and $\alpha3$ isoforms by site-directed mutagenesis. HeLa cells were transfected with the $\alpha2$ and $\alpha3$ constructs together with the rat $\alpha1$ construct. The endogenous Na^+, K^+-ATPase containing human $\alpha1$

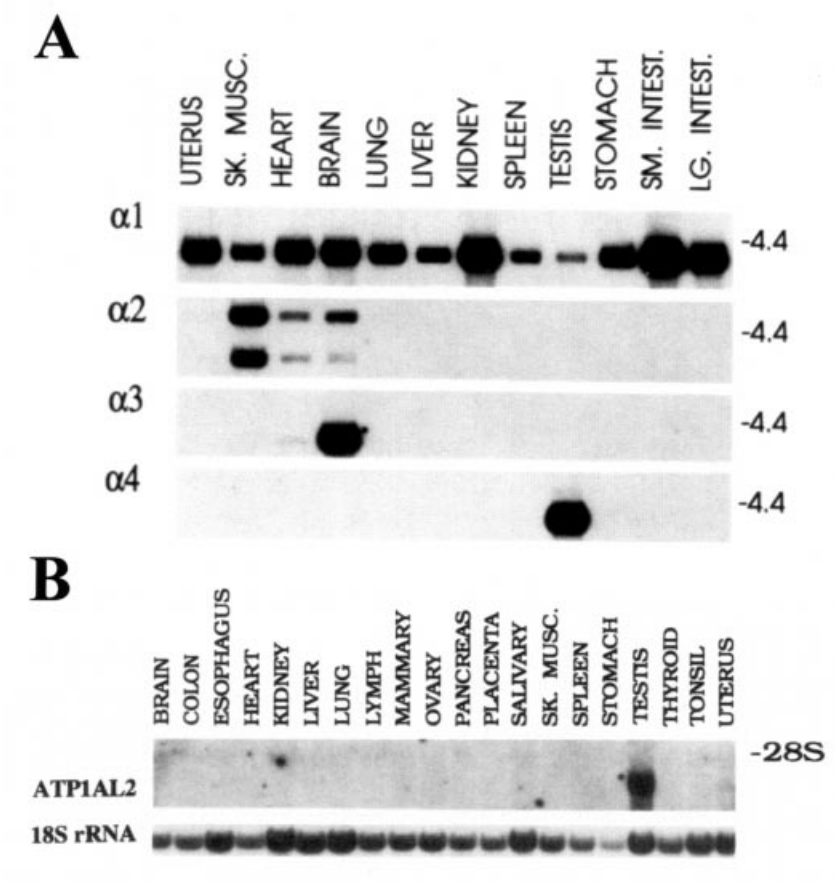

Fig. 4. *A*. The tissue distribution of the mRNAs encoding the rat Na^+, K^+-ATPase $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\alpha 4$ isoforms by Northern blot analysis. Abbreviations as follows: SK. MUSC., skeletal muscle; SM. INTEST., small intestine; LG. INTEST., large intestine. The experimental conditions in this paper were optimized for the rapid detection (48 hr) of the predominant αmRNAs, which does not allow for the detection of low abundance (low copy number) message. 4.4 indicates kilobase pairs. *B*. Northern analysis of the tissue distribution of the human *ATP1AL2* (putative fourth human α-like gene) mRNAs. Of the twenty one human tissues examined, only the testis showed positive expression of the *ATP1AL2* gene in the form of 3.9-kb mRNA. The 7 day autoradiographic exposure rules out the possibility of low copy number expression in tissues other than testis (reproduced from Shamraj and Lingrel, 1994, *Proceedings of the National Academy of Sciences, U.S.A.* 91, 12952–12956 by kind permission of J. B. Lingrel and by copyright permission of the National Academy of Sciences, U.S.A.). Copyright © (1994) National Academy of Sciences, U.S.A.

subunits was inhibited in the presence of 1 μM ouabain and the remaining ATPase activity in the system (corresponding to the transfected $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoforms) was studied. They found the affinity of the $\alpha 3$ isoform for Na^+ to be two to three times lower than the $\alpha 1$ and $\alpha 2$ isoforms, but that the $\alpha 3$ isoform had a slightly

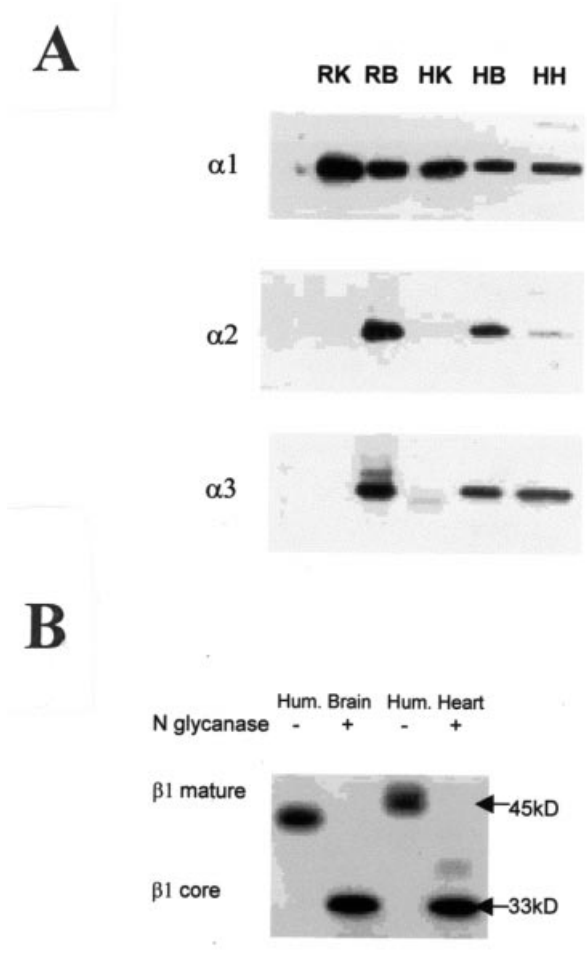

Fig. 5. *A*. Immunodetection of Na^+, K^+-ATPase α (α1, α2 and α3) isoforms in human and rat tissues by Western blotting using monoclonal isoform specific antibodies. Crude homogenates of rat kidney (RK), rat brain (RB), human kidney (HK), human brain (HB) and human heart (HH) were resolved by SDS-PAGE and trans-blotted into nitrocellulose membranes. The major α isoform in the kidney is α1 whereas all three α isoforms are present in heart and brain (α2 expression is lower in human heart compared to α1 and α3). *B*. Identification of Na^+, K^+-ATPase β isoforms in human brain and heart. Both β1 and β2 are present in human brain. However, the β1 isoform is the predominant β isoform in the heart (A) since only faint β2 signals can be obtained from the various ventricular and atrial regions of the heart (B) (reproduced from Wang *et al*., The *Journal of Clinical Investigation*, 1966 vol. 98, pp. 1650–1658 with kind permission of A. A. McDonough and by copyright permission of The American Society for Clinical Investigation and The Rockefeller University Press).

higher apparent affinity for ATP. Three different isoforms of the regulatory β subunit have been identified at the molecular genetic (Kawakami *et al*., 1986; Martin-Vasallo *et al*., 1989; Malik *et al*., 1996) and protein (Arystarkhova and Sweadner, 1997) using immunological techniques. The β subunit is important for the biogenesis of the enzyme complex and one of its isoforms ($\beta 2$) plays an important adhesion role in the central nervous system during development.

Tissue Specific Distribution of Isoforms

Thus far 4 α, 3 β and 1 γ isoform subunit have been identified in mammals (Martin-Vassalo *et al*., 1989; Shamraj and Lingrel, 1994; Malik *et al*., 1996; Mercer *et al*., 1993). However, other isoforms may exist and the search for additional α, β and γ subunits is already underway. The search is on for novel isoform subunits that may confer unique kinetic properties on the enzyme complex. Several lines of evidence have suggested the potential existence of additional β isoforms. Molecular studies on fish Na^+, K^+-ATPase suggest that up to seven distinct forms of the β subunit may exist (Cutler *et al*., 1997).

The $\alpha 1$ isoform serves as the ''housekeeping'' isoform, as judged by its abundance and ubiquitous cellular distribution. The remaining isoforms exhibit a more restricted tissue specific and developmental pattern of expression (see Fig. 4); the $\alpha 2$ isoform is expressed most abundantly in cardiac muscle, skeletal muscle, adipose tissue and glial cells in the brain (Sweadner *et al*., 1994; Peng *et al*., 1997). The expression of the $\alpha 2$ isoform appears to be insulin sensitive (Russo and Sweadner, 1993). The $\alpha 3$ isoform is found in high concentrations in neurons of the central nervous system (Sweadner, 1995; Peng *et al*., 1997) and cardiac muscle (Jewell *et al*., 1992; Swadner *et al*., 1994). In addition, $\alpha 3$ has been found to be expressed in lower quantities in ovary (Pathak *et al*., 1994), cartilage (Mobasheri *et al*., 1997a, b) and bone (Mobasheri *et al*., 1996). The $\alpha 4$ isoform appears to be specific to the testis (Shamraj and Lingrel, 1994). The $\alpha 1$ isoform is ubiquitously expressed (except in reticulocytes, Stengelin and Hoffman, 1997) whereas $\alpha 2$ appears to be concentrated in the nervous system (Martin-Vasallo *et al*., 1989; Lecuona *et al*., 1996; Peng *et al*., 1997). messenger RNA encoding the $\beta 1$ isoform is ubiquoitously expressed in all tissues examined (Martin–Vasallo *et al*., 1989) (see Fig. 6). The $\beta 2$ isoform appears to function as an adhesion molecule on glial cells (AMOG) specifically involved in mediating interactions between neurons and glia (Gloor *et al*., 1990). The $\beta 3$ isoform is the most recently described member of the β isoform gene family and is expressed predominantly in the testis but also in the brain, kidney, lung, spleen, liver and intestines (Malik *et al*., 1996; Peng *et al*., 1997; Arystarkhova and Sweadner, 1997) (see Fig. 6).

Functional Studies: Artificial Expression of Isoforms in Heterologous Cells

It is often not possible to study the kinetic properties of individual mammalian α isoforms in their counterpart cells. Host mammalian cells will invariably express endogenous Na^+, K^+-ATPase pumps making subsequent data analysis unrealistic.

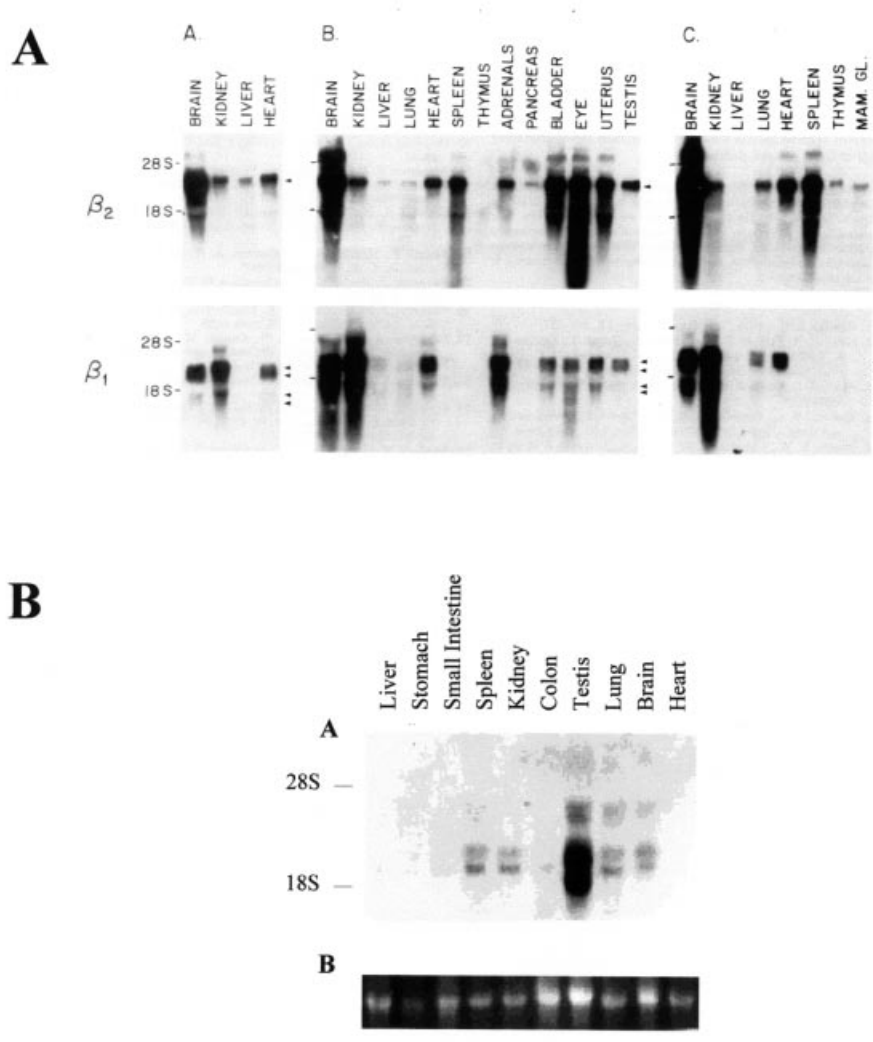

Fig. 6. *A*. Expression of the Na^+, K^+-ATPase $\beta 1$ and $\beta 2$ subunit mRNAs in 2-day-old, 2-week-old and adult rat tissues. The brain and kidney are the richest tissue sources of $\beta 1$ and $\beta 2$ mRNA. Note the low levels of $\beta 1$ and $\beta 2$ in 2-week old rat lung and liver. In the adult rat liver and spleen no β isoform mRNA is detectable (reproduced from Martin-Vasallo *et al*., *The Journal of Biological Chemistry*, 1989, vol. 264, pp. 4613–4618 by kind permission of P. Martin-Vasallo and by copyright permission of The American Society for Biochemistry and Molecular Biology). Copyright © (1989) The American Society for Biochemistry and Molecular Biology. *B*. Expression of the $\beta 3$ subunit isoforms in rat tissues. The $\beta 3$ subunit gene codes for two transcripts ~1.6 and ~1.8 kb in size. The $\beta 3$ isoform is most abundant in the testis and present in lower levels in brain, kidney, spleen, lung, stomach, colon and liver. No transcripts were detectable in the heart (reproduced from Malik *et al*., The *Journal of Biological Chemistry*,, 1996, vol. 271, pp. 22754–22758 by kind permission of R. Levenson and by copyright permission of The American Society for Biochemistry and Molecular Biology). Copyright © (1996) The American Society for Biochemistry and Molecular Biology.

Furthermore, the presence of multiple isoforms of α and β subunits of the Na^+, K^+-ATPase in most mammalian tissues has hindered the understanding of the roles of the individual isoforms in directing Na^+, K^+-ATPase function. Therefore, sf-9 insect cells systems have been used in conjunction with baculovirus vectors harboring full length and Na^+, K^+-ATPase rat or human constructs to successfully probe Na^+, K^+-ATPase isozyme function (Blanco *et al.*, 1993; De Tomaso *et al.*, 1993). Results obtained using this approach have convincingly demonstrated that the baculovirus system is suitable for the expression of the Na^+, K^+-ATPase isoforms and should provide a useful method for the characterization of the enzymatic properties of each isoform. Mercer's laboratory has led the field using this approach and has studied the kinetic properties of $\alpha1\beta1$, $\alpha1\beta2$, $\alpha2\beta1$, $\alpha2\beta2$, $\alpha3\beta1$ and $\alpha3\beta2$ isozymes. The $\beta1$ and $\beta2$ isoforms have been shown to assemble with the $\alpha2$ subunit to produce catalytically competent Na^+, K^+-ATPase molecules activated by the cations, stimulated by ATP and inhibited by ouabain (Blanco *et al.*, 1995a). The analysis of the kinetic parameters of the $\alpha2\beta1$ and $\alpha2\beta2$ isoforms has revealed equivalent sensitivity to ouabain and similar turnover numbers and apparent affinities for K^+ and ATP. The dependence on Na^+ has been shown to be different; $\alpha2\beta2$ displays a higher affinity for Na^+ than $\alpha2\beta1$. When compared to the rat $\alpha1\beta1$ isozyme expressed in sf-9 cells, the $\alpha2\beta1$ and $\alpha2\beta2$ isozymes have a lower affinity for K^+ and a higher affinity for Na^+ and ATP. Significantly, the $\alpha2\beta1$ and $\alpha2\beta2$ isozymes are 250 times more sensitive to ouabain than the rat $\alpha1\beta1$ which is generally considered to be resistant to ouabain (Jewell and Lingrel, 1991; Sweadner, 1995).

Co-expression of the rat $\alpha3$, $\beta1$ and $\beta2$ isoforms has also resulted in catalytically competent $\alpha3\beta1$ and $\alpha3\beta2$ isozymes (Blanco *et al.*, 1995b). Again as was the case for the $\alpha2\beta1$ and $\alpha2\beta2$ isozymes, similar turnover numbers and apparent affinities for cations and ATP have been documented for the $\alpha3\beta1$ and $\alpha3\beta2$ isozymes. Remarkably, the accompanying β isoform does not drastically affect the properties of the $\alpha3\beta1$ and $\alpha3\beta2$ isozymes suggesting that the kinetic properties are largely dictated by the catalytic α isoform, within the α-β dimer pair. As expected, the presence of the ouabain sensitive $\alpha3$ isoform results in high ouabain sensitivity by both $\alpha3\beta1$ and $\alpha3\beta2$ isozymes. Taken together these results strongly suggest that the most pronounced differences in kinetic differences in Na^+, K^+-ATPase function are a result of variations in α isoform composition. Apart from subtle differences in reactivity toward Na^+, K^+ and ATP, the most significant differences in Na^+, K^+-ATPase isozymes correspond to reactivity towards the cardiac glycoside ouabain. Isozymes containing $\alpha3$ ($\alpha3\beta1$ and $\alpha3\beta2$) display a high sensitivity to ouabain; isozymes containing $\alpha1$ ($\alpha1\beta1$) demonstrate a low sensitivity to the cardiotonic steroid and isozymes containing the $\alpha2$ isoform ($\alpha2\beta1$ and $\alpha2\beta2$) display an intermediate ouabain sensitivity.

More recent studies performed in insect cells have revealed information about the role of the $\beta3$ isoform subunit in the enzyme complex. Co-expressin of human $\alpha1$ and $\beta3$ proteins in baculovirus-infected Sf–9 cells resulted in the formation of a catalytically competent $\alpha1\beta3$ complex that exhibits enzymatic properties which are characteristic of Na^+, K^+-ATPase (Yu *et al.*, 1997). The recombinant $\alpha1\beta3$ isozyme thus formed displayed significantly lower sensitivity to ouabain than native $\alpha1\beta1$. Table 1 is a comprehensive summary of the expression-function relationship of Na^+,

Table 1. The Expression-Function Relationships of Na^+, K^+-ATPase Isoenzymes

Tissue/cell type	Isozyme combination(s)	Physiological function	Reference
Kidney (renal tubule cells)	$\alpha1\beta1$	Provide the driving force for Na^+ reabsorption in the kidney	Farman, 1996, Arystarkhova and Sweadner, 1997
Heart (cardiac myocytes)	$\alpha1\beta1$, $\alpha1\beta2$, $\alpha1\beta3$, $\alpha2\beta1$, $\alpha2\beta2$, $\alpha2\beta3$, $\alpha3\beta2$, $\alpha3\beta3$	Excitability. The α subunit is a target for digitalis glycosides. Regulation of Na^+/Ca^{2+} exchange	Nagakawa *et al*., 1990; Shamraj *et al*., 1991; Wang *et al*., 1996; Arystarkhova and Sweadner, 1997
Gut	$\alpha1\beta1$	Na^+ absorption	Marxer *et al*., 1989
Lung (alveolar epithelial cells)	$\alpha1\beta1$, $\alpha2\beta1$, $\alpha1\beta3$, $\alpha2\beta3$	Vectorial Na^+ transport and lung fluid reabsorption (after birth and stimulation with catecholamines)	Ridge *et al*., 1997; Sznajder *et al*., 1995; Zhang *et al*., 1997; Arystarkhova and Sweadner, 1997; Bertorello *et al*., 1999
Liver (hepatocytes)	$\alpha1\beta1$, $\alpha1\beta3$	Bile production. Part of the mitogenic program initiating hepatic regeneration	Schenk and Leffert, 1983; Lu and Leffert, 1991; Sun and Ball, 1992; Arystarkhova and Sweadner, 1997
Brain (neurons)	$\alpha1\beta1$, $\alpha1\beta2$, $\alpha1\beta3$, $\alpha3\beta1$, $\alpha3\beta2$	Generation of membrane potential and electrochemical gradients for neuronal excitability	Lecuona *et al*., 1996; Peng *et al*., 1997
Brain (glia)	$\alpha1\beta1$, $\alpha1\beta2$, $\alpha1\beta3$, $\alpha2\beta1$, $\alpha2\beta2$	K^+ re-uptake after depolarization	Lecuona *et al*., 1996; Peng *et al*., 1997
Eye (ciliary epithelium)	$\alpha1\beta1$, $\alpha1\beta2$, $\alpha2\beta1$, $\alpha2\beta2$, $\alpha3\beta1$, $\alpha3\beta2$	Ion transport. Plays an important role in the formation of aqueous humor. Regulation of Na^+ dependent transport systems	Cole, 1961; Martin-Vasallo *et al*., 1989; Geering, 1990
Cartilage (chondrocytes)	$\alpha1\beta1$, $\alpha1\beta2$, $\alpha1\beta3$, $\alpha2\beta1$, $\alpha2\beta2$, $\alpha2\beta3$, $\alpha3\beta1$, $\alpha3\beta2$, $\alpha3\beta3$	Maintenance of low intracellular Na^+:K^+ ratio	Mobasheri *et al*., 1998; Trujillo *et al*., 1999
Bone (osteoblasts)	$\alpha1\beta1$, $\alpha1\beta2$	Maintenance of intracellular K^+ and Ca^{2+} during bone formation	Mobasheri *et al*., 1996; Mobasheri *et al*., submitted
Bone (osteoclasts)	$\alpha1\beta1$	Polarization necessary for acid secretion and H^+ extrusion during bone resorption	Baron *et al*., 1986
Vascular Endothelial cells	$\alpha1\beta1$, $\alpha1\beta2$, $\alpha3\beta1$, $\alpha3\beta2$	Regulation of vascular tone and vasoconstriction	Mayol *et al*., 1998
Skeletal Muscle	$\alpha1\beta1$, $\alpha1\beta2$, $\alpha1\beta3$, $\alpha2\beta1$, $\alpha2\beta2$, $\alpha2\beta3$	K^+ uptake	Lytton *et al*., 1985; Arystarkhova and Sweadner, 1997; Thompson and McDonough, 1996
Smooth Muscle	$\alpha1\beta1$, $\alpha2\beta1$	Maintenance of intracellular Na^+ following activation of stretch activated channels by mechanical strain	Yamamoto *et al*., 1994; Songu-Mize *et al*., 1996; Liu *et al*., 1998
Testis	$\alpha1\beta1$, $\alpha1\beta2$, $\alpha1\beta3$, $\alpha4\beta3$	Regulation of Lumenal K^+ concentration	Shamraj and Lingrel, 1994; Mobasheri *et al*., unpublished observations
Prostate (prostatic epithelial cells)	$\alpha1\beta1$, $\alpha1\beta2$	Regulation of lumenal Na^+ and K^+ concentrations	Mobasheri *et al*., submitted

K^+-ATPase isozymes identified in various mammalian tissues. The $\alpha 1\beta 1$ complexes are the most ubiquitous and are the only complexes that contribute significantly to Na^+ reabsorption in the kidney (Farman, 1996; Geering, 1997).

Physiological Significance of Multiple α Isoforms

Expression of multiple Na^+, K^+-ATPase isoforms with different apparent affinities for Na^+ may be physiologically important in controlling intracellular Na^+ levels. Variations in intracellular Na^+ levels may have effects on other cellular functions such as regulation of intracellular free Ca^{2+}; slight increases in cytoplasmic Na^+ favours sequestration of free Ca^{2+} in intracellular stores. This increase in $[Ca^{2+}]$ available for mobilization is important in the regulation of contraction in skeletal/cardiac cells and excitability in neuronal cells (Blaustein, 1993; Juhaszova and Blaustein, 1997a, b). The subcellular localization of Na^+, K^+-ATPase isoforms is also important and may confer unique cell-type specific physiological properties (see Fig. 7). The lower apparent affinity of $\alpha 3\beta 1$ for Na^+ and K^+ may be an evolutionary adaptation to the physiological requirements of excitable tissues. This prospect supports the thesis that in excitable tissues, isozymes containing the $\alpha 3$ isoform may be activated as a last resort "reserve pump" after major variations in the concentration of cations (such as those resulting from continuous firing of action potentials) (Munzer *et al.*, 1994). This notion also suits the recently proposed hypothesis that $\alpha 3\beta 2$ and $\alpha 3\beta 1$ containing Na^+, K^+-ATPase pumps in cartilaginous tissues may enable cells to recover from major ionic fluctuations (mainly Na^+) that routinely occur under mechanical joint loading (Mobasheri *et al.*, 1998; Trujillo *et al.*, 1999; Mobasheri, 1999). These unique kinetic characteristics of the Na^+, K^+-ATPase may help achieve the desired physiological ionic milieu required for different tissues and different functions. The differences in ouabain sensitivity must be important in the cellular adaptation to variable ionic and osmotic environments.

Chromosomal dispersion of the α subunit genes and the tissue and cell specific pattern of α subunit expression suggests that the isoforms encoded by the genes have properties selected in response to different physiological demands. The tissue distribution and developmental regulation of the α isoforms is achieved by transcription factors that bind to negative or positive elements in the α isoform genes (Pathak *et al.*, 1994; Yu *et al.*, 1996; Ikeda and Kawakami, 1996; He *et al.*, 1996). The existence of negative elements (i.e. silencing factors such as neural-restrictive silencer element) and positive elements in different tissues suggests that expression of the Na^+, K^+-ATPase is tightly coupled to physiological demand.

Functional Changes Following Point Mutations of the α and β Isoform Subunits

An active area of Na^+, K^+-ATPase research is concerned with the identification of key amino acid residues in the α subunit involved in cation, ATP and ouabain binding. In the proposed model for the mechanism of activity of Na^+, K^+-ATPase several fundamental steps may be differentiated for the transport of Na^+ and K^+ ions: binding of the cations to the enzyme, conformational changes in the enzyme

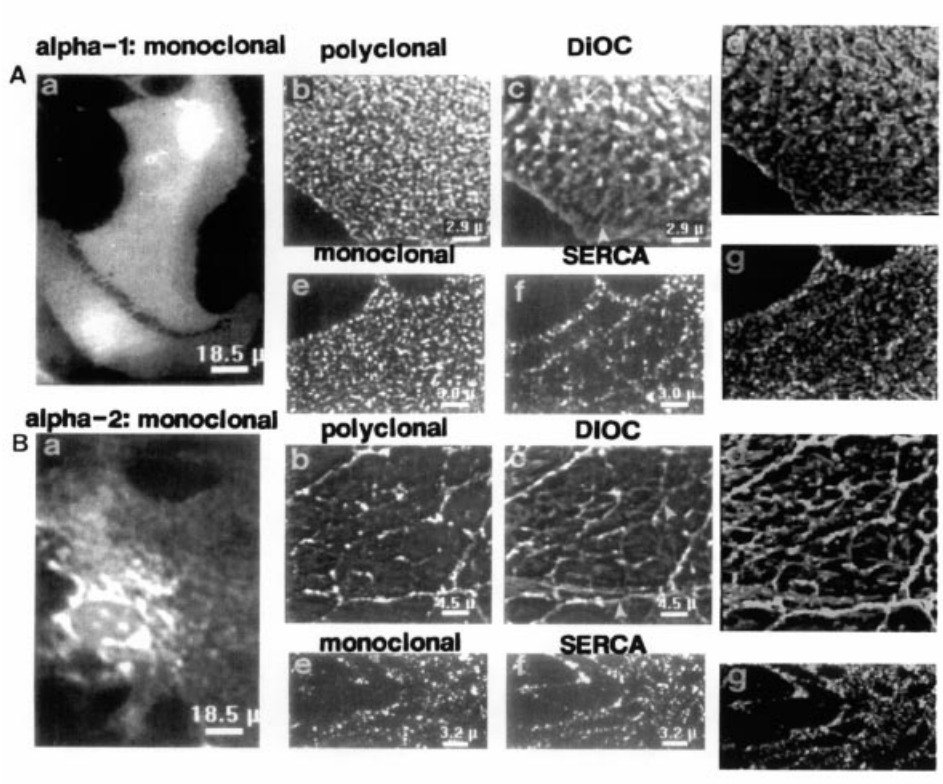

Fig. 7. Localization of Na^+, K^+-ATPase α isoforms in primary cultured astrocytes (A) and neurons (B). *A*. The major α isoforms expressed in astrocytes are $\alpha 1$ and $\alpha 2$ as demonstrated by polyclonal and monoclonal isoform specific antibodies. Astrocytes were also labelled with SERCA antibodies (sarcoplasmic reticulum (SR) and endoplasmic reticulum (ER) Ca^{2+}-ATPase) and DiOC, which preferentially stains mitochondria, and the ER. The $\alpha 1$ isoform is ubiquitously expressed in astrocytes whereas $\alpha 2$ displays a reticular pattern of expression co-localizing with the ER. The staining produced with polyclonal and monoclonal $\alpha 2$ specific antibodies significantly overlaps with underlying ER producing overlapping staining in the merged images of SERCA and $\alpha 2$ staining. In contrast $\alpha 1$ does not colocalize with the ER. *B*. In neurons $\alpha 1$, $\alpha 2$ and $\alpha 3$ are all present however $\alpha 3$ and $\alpha 1$ are most abundant. The subcellular distribution of the $\alpha 1$ and $\alpha 3$ isoforms in neurons is significantly different; intense $\alpha 3$ labelling of cell bodies and processes contrasts with $\alpha 1$ labelling of cell bodies (A). Focal "hot spots" of $\alpha 3$ staining are observed in axons and dendrites (arrows in Ba) in addition to non-uniform staining on the soma (Ba). Tangential sections through the soma demonstrate $\alpha 3$ staining in neuronal plasma membranes (Bb and Bc), which significantly contrasts with intracellular and reticular DiOC staining (reproduced from Juhaszova and Blaustein, 1997, *Proceedings of the National Academy of Sciences, U.S.A.* vol. 94, pp. 1800–1805 by kind permission of M. P. Blaustein and by copyright permission of the National Academy of Sciences, U.S.A.).

complex and the phosphorylation of the enzyme. Knowledge of the molecular interactions of different regions of the enzyme complex in each of the above steps is of great importance for understanding how the Na^+, K^+-ATPase operates.

Thus far, the data has been obtained from experiments in which the α subunit has been altered by site directed mutagenesis. Mutant α subunit cDNA constructs

in which key amino acid residues are mutated are expressed together with β subunit cDNA constructs in yeast cells, in *Xenopus laevis* oocytes or in cultured cells (COS or HeLa). Examining various enzymatic parameters of the mutated Na^+, K^+-ATPase compared with the non mutated Na^+, K^+-ATPase yields valuable information about the involvement of a specific amino acid residue in different steps in the transport of ions. The results obtained using this approach indicate that the interaction of both ions with the α subunit protein is complex and amino acids from different regions of the molecule participate in ion translocation. Only Ser^{775} interacts in a direct fashion with the K^+ (Argüello and Lingrel 1995; Blostein *et al*., 1997). Other amino acids participate in the binding of high affinity of the K^+ or Na^+ ion to the α subunit (see table 2), but their direct interaction with either ion has not been demonstrated. It is important to emphasize that the most important amino acid residues involved in high affinity ion binding are found in the transmembrane domains 4, 5 and 6 of the α subunit. This notion is concordant with the ''Post–Albers'' model that postulates that the ions are hidden in the interior of the protein to be transported to the other side of the membrane). It seems clear that certain amino acid residues are directly and exclusively involved in cation binding, but there are exceptions such as Asn^{776}, Asp^{805} and Asp^{808} in which the change for another amino acid residue in these positions affects the affinity of both ions for the protein (Pedersen *et al*., 1997; Pedersen *et al*., 1998). These residues may move between both ions during conformational changes that take place in the α subunit during ion transport.

Another important aspect of the Na^+, K^+-ATPase is the phosphorylation of the enzyme following the binding of an ATP molecule. Phosphorylation of the α subunit during transport of Na^+ and K^+ is closely coupled to conformational changes in the protein and to the different affinities of each conformation for each of the cations. Mutations in the residues involved in the affinity of the pump for the cations modifies the equilibrium between the two confirmations producing a secondary effect in the affinity of the α subunit for ADP and ATP and therefore in the phosphorylation of the protein. Furthermore, amino acids have been identified in the cytoplasmic side between transmembrane domains M4 and M5 that are directly involved in the

Table 2. Te α Subunit of the Na^+, K^+-ATPase Exists as Four Isoform Variants. The Na^+, K^+-ATPase Isoform mRNAs Are Tissue-Specific and Developmentally Regulated (Orlowski and Lingrel, 1988; Lecuona *et al*., 1996). The Table Below Shows the Similarities and Differences Between the Four α Subunit Isoforms Identified to Date. Also Shown is the Ouabain Sensitivity and Tissue Distribution of the Isozyme Proteins.

α isoform mRNA	Amino acid length	Homology to α1	Tissue distribution	Ouabain sensitivity	Molecular mass (M_r)
α1	1018	—	ubiquitous	low	112, 573
α2	1015	76% nucleotide 86% amino acid	brain skeletal and cardiac muscle, adipose tissue	high	111,736
α3	1013	76% nucleotide 85% amino acid	brain, cardiac muscle	high	111,727
α4	1021	76% nucleotide, 76% amino acid	testis	not determined	112,904

catalytic hydrolysis of ATP. On the one hand, Asp^{369} has been identified as the receptor of the phosphate group and is believed to be essential for the functionality of the active site of ATP hydrolysis (Kuntzweiler *et al*., 1995). On the other hand the binding of ATP to the active sites is influenced by mutations in two residues: Lys^{501} and Asp^{586} (Farley *et al*., 1997). Recently it has been demonstrated that the first of these participates in binding of ATP however, there is no evidence to support the hypothesis that this function is done through a direct interaction with the γ group of the nucleotide (Farley *et al*., 1997). In a similar fashion Asp^{586} could establish coordination with the divalent cation Mg^{2+} whose presence is essential for phosphorylation against the preliminary evidence which showed a direct interaction with hydroxyl groups of the ribose nucleotide. Finally, the inhibitory interaction of the pump with cardiac glycosides such as ouabain involves interactions with a considerably large number of amino acid residues in the α subunit protein. These amino acid residues distributed mainly in the extracytoplasmic side (M1–M2 and M5–M6) or in the transmembrane regions (M4, M6 and M10) (Burns *et al*., 1996; Croyle *et al*., 1997; Vasilets *et al*., 1998). The participation of the amino acids of the transmembrane regions in ouabain binding could indicate that the hydrophobic regions of this molecule are inserted in the membrane or that a cleft may exist allowing interaction between the drug and the transmembrane amino acids. Table 3 summarizes the results of site-directed mutagenesis experiments performed on the α subunit.

NOVEL FUNCTIONAL INTERACTIONS

Recent studies have demonstrated that the Na^+, K^+-ATPase is not merely a passive resident in the plasma membrane exclusively involved in cation transport and ion homoeostasis. Evidence suggests that components of the Na^+, K^+-ATPase may participate in other activities such as the development of the vertebrate embryo, neuronal guidance and development of the central nervous system, non-classical protein export, maintenance of cellular morphology and cell adhesion. The γ subunit for example may contribute to the generation of cation channel activity. In the sections that follow some of these recent developments are highlighted and discussed with particular reference to their physiological significance.

Interaction Between the Na^+, K^+-ATPase and the Cellular Cytoskeleton

The actin-based cytoskeleton, consisting of actin filaments and associated proteins is a dynamic structure that plays an active role in a multitude of cellular functions and events including stability of cell shape, the onset of motility and the control of hormone action. In the context of this review the actin cytoskeleton also plays a crucial role in regulating the distribution of integral membrane proteins such as the band-3 related anion exchanger (Drenckhahn *et al*., 1985). Actin and various actin-binding proteins bind to a variety of other transmembrane proteins including voltage sensitive Na^+ channels (Edelstein *et al*., 1998). Other Na^+ transporters that are directly or indirectly influenced by the transmembrane Na^+ and K^+ fluxes are also linked to cytoskeletal structures. These include the volume regulating $Na^+/K^+/2Cl^-$ cotransporter (Jorgensen *et al*., 1984) and the pH regulating Na^+/H^+ antiporter

Table 3. Summary of Site Directed Mutagenesis Studies on Key Amino Acid Residues in the α Subunit of the Na^+, K^+-ATPase

Amino acid	Region	Organism	Expression system	Functional consequences	References
$Leu^{793} \rightarrow Pro$	M5–M6	Sheep $\alpha 1$	HeLa cells	Resistance to ouabain	Burns *et al.* (1996)
$Glu^{334} \rightarrow Ala$	M4	Torpedo $\alpha 1$	Oocytes	Resistance to ouabain	Vasilets *et al.* (1998)
$Gin^{111} \rightarrow Arg$	M1–M2				
$Asp^{121} \rightarrow Gly$	M1–M2				
$Asn^{122} \rightarrow Asp$	M1–M2				
$Thr^{797} \rightarrow Ala$	M6	Sheep $\alpha 1$	HeLa cells	Resistance to ouabain	Croyle *et al.* (1997)
$Leu^{330} \rightarrow Gin$	M4				
$Ala^{331} \rightarrow Gly$	M4				
$Thr^{338} \rightarrow Ala/Asn$	M4				
$Phe^{982} \rightarrow Ser$	M10				
$Lys^{501} \rightarrow Arg/Glu$	M4–M5	Sheep $\alpha 1$	Yeast	ATP binding	Farley *et al.* (1997)
$Asp^{369} \rightarrow Ala$	M4–M5	Sheep $\alpha 1$	NIH 3T3	Receptor of phosphate	Kuntzweiler *et al.* (1995)
$Asp^{586} \rightarrow Glu/Asn$	M4–M5	Sheep $\alpha 1$	Yeast	Binding of Mg^{++} at the phosphorylation site	Farley *et al.* (1997)
$Leu^{332} \rightarrow Ala$	M4	Rat $\alpha 1$	Cos cells	Conformational change $E1P \leftrightarrow E2P$	Vilsen (1997)
$Glu^{327} \rightarrow Ala$	M4	Pig $\alpha 1$	Yeast	High-affinity occlusion of K^+	Jorgensen *et al* (1998)
$Glu^{327} \rightarrow Gin/Leu$		Rat $\alpha 2$	HeLa cells		Tepperman *et al.* (1997)
$Ser^{775} \rightarrow Ala$	M5	Sheep $\alpha 1$	HeLa cells	Direct K^+-ligating residue	Argüello and Lingrel (1995) Blostein *et al.* (1997)
$Gly^{330} \rightarrow Ala$	M4	Rat $\alpha 1$	Cos cells	Binding of K^+	Vilsen (1997)
$Pro^{780} \rightarrow Ala$	M5				
$Asp^{804} \rightarrow Asn/Glu$	M6	Rat $\alpha 1$	Yeast	Alternate interactions of Na^+ and K^+	Pedersen *et al.* (1998)
$Asp^{808} \rightarrow Asn/Glu$					
$Glu^{779} \rightarrow Ala$	M5	Rat $\alpha 1$	HeLa cells	Abolition of ion transport voltage dependence	Argüello *et al.* (1996)
$Asn^{778} \rightarrow Ala/Gln$	M5	Pig $\alpha 1$	Yeast	Binding of both Na^+ and K^+	Pedersen *et al.* (1998)
$Glu^{781} \rightarrow Ala$	M5	Rat $\alpha 1$	COS cells	Binding of intracellular Na^+	Vilsen (1995a)
$The^{809} \rightarrow Ala$	M6				
$Asn^{326} \rightarrow Leu$	M4	Rat $\alpha 1$	COS cells	Binding of intracellular Na^+	Vilsen (1995b)
$Thr^{774} \rightarrow Ala/Ser$	M5	Pig $\alpha 1$	Yeast	Binding of intracellular Na^+	Pedersen *et al.* (1998)

(Watson *et al.*, 1992). In epithelial cells amiloride sensitive Na^+ channels (ENaC) are also complexed to ankyrin, fodrin and actin itself (Smith *et al.*, 1991). Actin filaments have been shown to co-localize with amiloride sensitive Na^+ channels and modulate channel activity in epithelial cells (Cantiello *et al.*, 1991).

The Na^+, K^+-ATPase is by no means an exception; the actin cytoskeleton interacts with the Na^+, K^+-ATPase via the protein ankyrin (Nelson and Veshnock, 1987a, b; Morrow *et al.*, 1989; Nelson *et al.*, 1991). In epithelial cells of the renal tubule (and in cultured MDCK cells) the actin cytoskeleton is responsible for the polarization of the Na^+, K^+-ATPase to the basolateral membrane, which is crucial for the integrity of Na^+ reabsorption in the kidney (see Fig. 8A). In other types of ion transporting epithelia, for example the secretory epithelium of the prostrate gland, the Na^+, K^+-ATPase is targeted to the apical membrane (see Fig. 8B). Correct targeting and assembly is essential for the normal physiological function of epithelia. The protein sequence of the Na^+, K^+-ATPase α subunit contains sorting information that dictates the trafficking pathway of newly synthesized Na^+, K^+-ATPase protein and the ultimate cellular localization of functional enzyme (Caplan, 1997a, b). In the

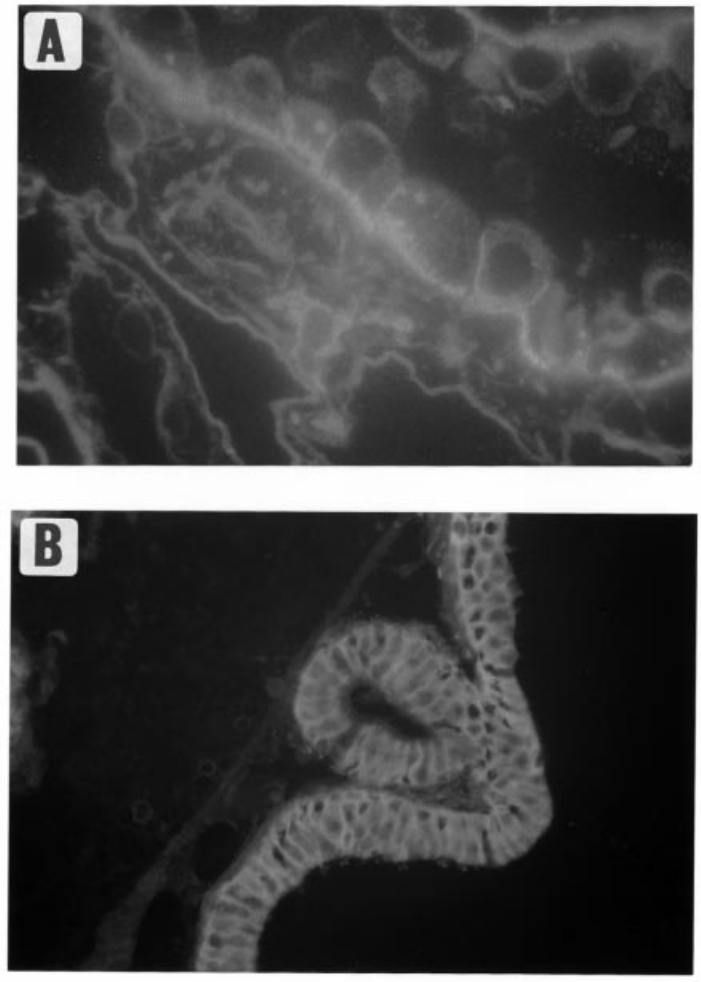

Fig. 8. Basolateral and lateral polarization of the α1 subunit of Na^+, K^+-ATPase in selected absorptive and secretory epithelia as demonstrated by immunofluorescence microscopy. *A*. In this high power immunofluorescence micrograph the Na^+, K^+-ATPase is predominantly localized in the basolateral membrane of the rat renal tubule where it provides the driving force for Na^+ reabsorption. Here the α1 subunit is localized using a specific polyclonal antibody and secondary rhodamine-conjugated antibody. *B*. In this low power immunofluorescence micrograph the secretory epithelium of the rat prostate gland is probed with another specific polyclonal antibody raised against the α1 subunit and a secondary fluorescein conjugated antibody. The Na^+, K^+-ATPase is predominantly polarized to lateral membranes of prostatic epithelial cells.

Table 4. Comparison of Conserved Actin- and ATP-Binding Domains of Mammalian Na^+, K^+-ATPase α Subunits with Those of the Heavy Head of Myosin II. *Position where substitution affects actin binding (adapted from Cantiello, 1995).

	Starting residue	ATP binding site	Similarity/ identity
		**	
Myosin	698	LEGIRICRKGFPSRI–LYADF	
Rat α1	882	LLGIRETNDD–RWINDVE-DSY	58.8/35.2
Rat α2	882	LVGIRLNNDD–RTVNDLE-DSY	62.5/37.5
Sheep α	876	LIGIRVDWDD–RWINDVE-DSY	70.5/43.7

analysis of disorders involving Na^+, K^+-ATPase, interest in interactions of the enzyme system with the cytoskeleton which is essential for sorting, correct targeting and stable expression has recently gained significant impetus (Beck and Nelson, 1996). Evidence suggests that in an experimental model of hypertension, genetic alterations to the cytoskeletal protein adductin is associated with increased Na^+, K^+-ATPase expression and activity in the kidneys of Milan hypertensive rats (Ferrandi *et al.*, 1996). The mutant adductin could interfere with the normal interaction between the spectrin-actin-ankyrin cytoskeleton and thus influencing the distribution and enhanced synthesis of Na^+, K^+-ATPase pumps that result in abnormally increased Na^+ reabsorption in kidneys of Milan hypertensive rats.

Most of the experimental data for interactions between cytoskeletal components and the Na^+, K^+-ATPase suggest an indirect interaction via ankyrin and spectrin. For example studies in the choroid plexus have demonstrated the preferential localization of the fodrin-ankyrin cytoskeleton with apical Na^+, K^+-ATPase (Alper *et al.*, 1994). However, there is evidence for a direct interaction between actin filaments and the Na^+, K^+-ATPase (Cantiello, 1995). Incubation of purified rat kidney Na^+, K^+-ATPase with unpolymerized actin increases ouabain sensitive ATP hydrolysis by 74%. Furthermore inmmunoblotting monomeric actin to purified Na^+, K^+-ATPase suggests that a direct interaction may take place between these two proteins. This notion is supported by sequence analysis, which has revealed two putative actin-binding domains in the α subunit of the Na^+, K^+-ATPase (see Table 4). The interaction between actin and the Na^+, K^+-ATPase may share similarities to that of another ATPase, myosin II. Actin triggers the myosin-mediated hydrolysis of ATP, in this case mediated by actin filaments, which is coupled to mechanochemical transduction in muscle. The actin-binding sequence of myosin (Leu-Glu-Gly-Ile-Arg-Ile-Cys-Gly) bears strong resemblance to that of the Na^+, K^+-ATPase α subunit (Leu-Glu-Gly-Ile-Arg-Gly). The putative actin binding domain of the Na^+, K^+-ATPase α subunit is conserved in three out of four α isoforms (Cantiello, 1995). This direct interaction between actin and the Na^+, K^+-ATPase suggests a novel functional role of the cytoskeleton in the regulation of Na^+, k^+-ATPase mediated Na^+ and K^+ transport.

Regulation of Na^+, K^+-ATPase Activity by Phosphorylation; Role of Protein Kinases

Protein kinases and phosphatases play a central role in signal transduction events in the cell. Interactions between cell surface receptors and their extracellular

ligands (hormones, growth factors, cytokines or drugs) activate intracellular signaling pathways that result in increased or decreased cellular activity. Very little information is available about the involvement of Na^+, K^+-ATPase in intracellular cascades. However, available evidence points to the α subunit of the Na^+, K^+-ATPase as a target of kinase-mediated phosphorylation (Bertorello and Katz, 1995). Much evidence for such interactions comes from *in vitro* (Bertorello *et al*., 1991; Fisone *et al*., 1995) and *in vivo* (Béguin *et al*., 1994) studies. The α subunits appears to be a substrate for phosphorylation by both protein kinase A (PKA) and protein kinase C (PKC) (Bertorello *et al*., 1991). Although there are 36 sites that could qualify as potential PKC motifs in rat $\alpha 1$, there are species-specific differences in the phosphorylation of Na^+, K^+-ATPase by PKC, which have been shown to have a structural and conformational basis (Feschenko and Sweadner, 1994; Feschenko and Sweadner, 1995). The protein kinases A and C are classified as serine/throenine kinases on the basis of their target amino acid residues. Attempts are currently underway to identify key amino acid residues involved in these phosphorylation events. Protein kinases may present cells with a physiological control mechanism for fine tuning Na^+, K^+-ATPase activity by phosphorylation of amino acid residues that inhibit or stimulate the Na^+, K^+-ATPase.

Under strict hormonal regulation, Na^+, K^+-ATPase plays a critical role in the reabsorption of Na^+ in the basolateral membrane domain of cells lining the nephron (Farman, 1996). The prevalence of the $\alpha 1\beta 1$ isozyme in kidney epithelial cells (renal tubule cells) have made them popular models for investigating the influence of phosphorylation on Na^+, K^+-ATPase activity and expression. Phosphorylation of Na^+, K^+-ATPase α subunits may be induced experimentally by treating renal tubule with drugs or hormones. Early studies using this *in vitro* model suggested that phosphorylation of the Na^+, K^+-ATPase inhibits the activity of the enzyme (Bertorello *et al*., 1991). Other investigators later confirmed this by demonstrating phosphorylation of Na^+, K^+-ATPase α subunit results in a decline in its transport activity (Middleton *et al*., 1993). More recent studies using renal tubule cells indicate that dopamine-induced phosphorylation of the rat α subunit (residue Ser-18) leads to clathrin-dependent Na^+, K^+-ATPase internalization and thus contributes to reduced Na^+, K^+-ATPase activity (Chibalin *et al*., 1999). In renal epithelial cell lines transfected with mutant rat α subunits (construct encoding for Na^+, K^+-ATPase in which Ser-18 had been mutated by site-directed mutagenesis) dopamine treatment showed no effects. These results suggest that Ser-18 is essential for clathrin-dependent internalization of α subunits into defined intracellular compartments following dopamine treatment. Clearly, kinase mediated phosphorylation of the α subunit does occur and phosphorylated Na^+, K^+-ATPase is internalized and degraded by cells. However, defining the precise physiological regulation of this mechanism requires further work. It is possible that specific phosphatase enzymes could reverse the internalisation and degradation induced by phosphorylation? If so, this would provide a reversible mechanism for regulating the plasma membrane abundance of Na^+, K^+-ATPase by a "membrane shuttle" mechanism (Bertorello and Katz, 1995). Earlier we discussed the possible role of the actin cytoskeleton in regulating the activity of Na^+, K^+-ATPase. The actin cytoskeleton may also participate in Na^+, K^+-ATPase regulatory mechanisms involving phosphorylation events. Actin appears to

serve as a signaling molecule, as illustrated, for example, by its role in activation of epithelial Na^+ channels by c-AMP-dependent protein kinase. Furthermore, monomeric or G actin has been shown to stimulate Na^+, K^+-ATPase activity *in vitro* (Cantiello 1995). Thus, it may be postulated that activation of protein kinases or actin-binding proteins could influence the local levels of monomeric actin, thus facilitating interactions with Na^+ dependent transport systems including Na^+, K^+-ATPase.

Role of the γ Subunit Isoform in Ion Transport and Development

The third and most recently identified subunit of the Na^+, K^+-ATPase system is the γ subunit. This subunit was initially described as a putative proteolipid component by Forbush *et al*. (1978) who suggested that purified Na^+, K^+-ATPase and microsomal fractions enriched in Na^+, K^+-ATPase contained a ~10 kD entity. Subsequent studies by Hardwicke and Freytag (1981) and Collins *et al*. (1982) provided further proof for the existence of a proteolipid component present in equimolar quantities with α-β protomers. The γ subunit was closed finally by Mercer and colleagues in 1993 providing definitive proof that this proteolipid entity is a specific component and not a contaminant or a degradation product of either the α or β subunit. This subunit is a small polypeptide of 58 amino acids with a predicted molecular mass of 6.5 kD–10 kD which co-purifies with the α and β subunits of Na^+, K^+-ATPase. The γ polypeptide is a type 1 transmembrane membrane protein (exposing its NH2 terminus to the extracellular face of the plasma membrane) lacking a signal sequence and hydropathy analysis reveals the presence of a single hydrophobic domain that is sufficient to cross the membrane. Unlike the α and β isoform subunits, the γ subunit is not glycosylated. The net charge distribution on the γ subunit is polarized; the N-terminus is negative whereas the C-terminus is positive indicating that this characteristic is important for the membrane topology, assembly and function of the γ subunit. It is present in many organs and tissues characterized and co-immunoprecipitation. Studies of the α, β and γ subunits with isoform specific antibodies have demonstrated specific association of the three subunits in catalytically competent Na^+, K^+-ATPase. Strong evidence that the γ subunit is a specific component of the Na^+, K^+-ATPase comes from studies indicating that this subunit is involved in forming the site for cardiac glycoside binding (Mercer *et al*., 1993). Forbush and co-workers (1978) who first postulated the existence of the γ subunit were also the first to show that photo-affinity-labeled ouabain derivatives bind to the γ subunit physically implicating the γ proteolipid component near the ouabain-binding site. Studies by Béguin and co-workers (1997) suggest that in the *Xenopus Laevis* oocyte expression system the γ subunit does not interact with any proteins except Na^+, K^+-ATPase α and β isoforms simultaneously co-expressed with it. Their studies also suggest that the γ subunit does not associate with individual α or β subunits but interacts with assembled, transport competent α-β promoters.

The precise physiological function of the γ subunit is unknown. In contrast to the β subunit the γ subunit is not an essential component for the structural and functional maturation of the Na^+, K^+-ATPase (Geering *et al*., 1996; Ueno *et al*., 1997). Association of the γ subunit is not needed for the formation of stable α-β complexes, the ER exit or cell surface expression and activation of functional Na^+,

K^+-ATPase (Béguin *et al*., 1997). On the contrary, the γ subunit itself requires assembly with the Na^+, K^+-ATPase in order to become stably expressed and targeted to the plasma membrane as unassembled γ subunits are found to be retained within the cell and degraded in some model systems. Thus, it is the level of cell surface α-β complexes that determine the plasma membrane expression of γ subunits.

Electrophysiological studies indicate that the γ polypeptide is involved in the activation of Na^+, K^+-ATPase by K^+. Experimental approaches involving heterologous expression of combinations of α, β and γ subunits in yeast have failed to reveal any role in enzymatic activity or modulation of ion transport (Scheiner-Bobis and Farley, 1994). Since yeast does not possess an endogenous Na^+, K^+-ATPase, such experimental approaches are fundamentally flawed. The function of the γ subunit will only be revealed if animal cell systems are used. Elegant recent studies by Holstead-Jones and colleagues suggest that the expression of the γ subunit isoform is essential during pre-implantation development. They used specific antibodies again the γ subunit to demonstrate abundant expression and localization of this subunit in the apical and basolateral membranes of the developing mouse blastocyst trophectoderm. The particularly fascinating aspect of their study was antisense disruption of γ subunit expression that resulted in delayed cavitation in the developing mouse blastocyst (see Fig. 9). These and previous studies demonstrate that the Na^+, K^+-ATPase is not only a housekeeping enzyme involved in ion homeostasis but also an agent of morphogenesis during development (Waton and Kidder, 1988; Manejwala *et al*., 1989). The sodium pumps deployed in the basolateral membranes of the epithelial trophectoderm working in conjunction with a variety of other Na^+ entry mechanisms (Na^+/H^+ exchanger, $Na^+/K^+/2Cl^-$ cotransporter) in the apical domain to generate a transepithelial flow of Na^+ and water to form the blastocoel. Any treatments that interfere with the establishment of a polarized distribution of blastocyst sodium pumps (i.e. ouabain treatment) can prevent morphogenesis (Manejwala *et al*., 1989; Watson *et al*., 1990; MacPhee *et al*., 1997).

The γ subunit shares significant homology with members of a recently recognized family of membrane proteins, characterized by single transmembrane domains that mediate ion transport. Other members of this family of transmembrane proteins include phospholemman (Palmer *et al*., 1991), channel-inducing factor (Attali *et al*., 1995) and Mat-8 (Morrison *et al*., 1994). When expressed in Xenopus oocytes, phospholemann and Mat-8 induce Cl^- selective currents. In contrast channel-inducing factor induces a K^+ current. Phospholemman can also function as a cation or anion selective channel (Kowdley *et al*., 1997). Therefore the γ subunit may behave as a cation channel independent of the α and β subunits under certain physiological conditions. Indeed recent studies in Mercer's laboratory have indicated that the γ subunit induces cation channel activity when expressed in isolation from the α and β subunits in SF-9 insect cells and *Xenopus* oocytes (Minor *et al*., 1998). The γ subunit was found to be delivered to the plasma membrane independently of other subunits. When the γ subunit was co-expressed with the α subunit, the 2 proteins co-localized in the plasmalemma. Furthermore, antibodies specific to the γ subunit also immunoprecipitated the α subunit suggesting that the γ subunit is able to physically associate with the α subunit in the plasma membrane.

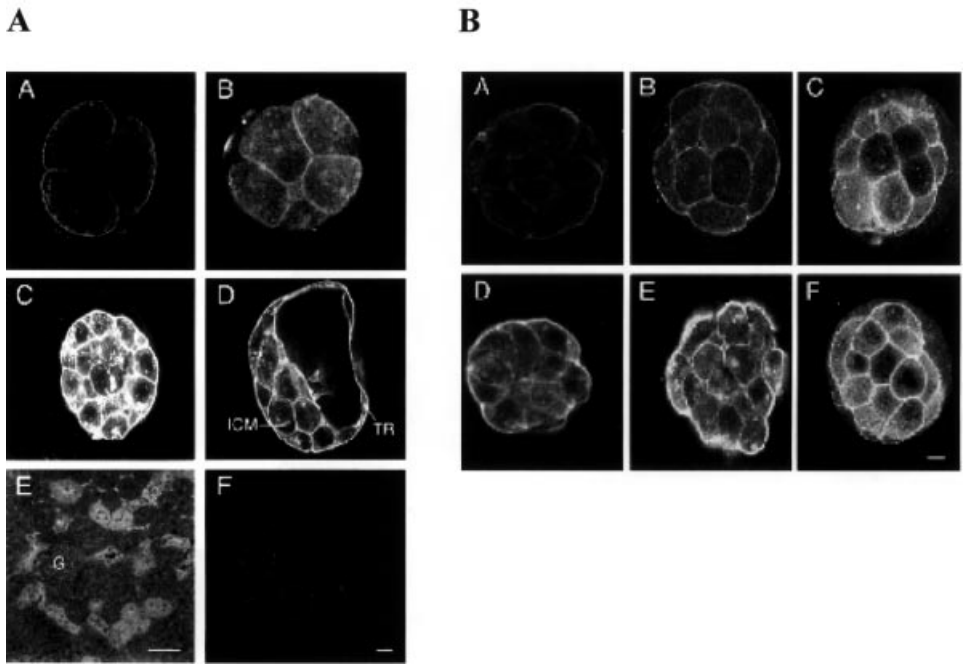

Fig. 9. *A*. Expression of the γ subunit of the Na^+, K^+-ATPase in the apical and basolateral membranes of the mouse embryo after immunostaining as demonstrated by confocal laser scanning microscopy. The γ subunit accumulates from the eight-cell stage (B). Also shown are representative four-cell (A), compacted morula (C) and blastocyst (D). A section of adult mouse kidney showing γ subunit expression in the glomerulus (E) (Bar, 25 μm). The image in F intensified for better visibility is the negative control glomerulus treated with pre-immune serum. *B*. Treatment with antisense oligonucleotides attenuated γ subunit staining in blastomere membranes (A & B) whereas nonsense oligonucleotides had no effect (D & E). Confocal images were acquired using identical magnification and laser settings to allow comparison of signal intensities between antisense and nonsense treated embryos. Embryos shown in C and F were immunostained using an α subunit antibody to show that treatment with oligonucleotides had no effect on α isoform expression (reproduced, from Holstead-Jones *et al*., 1997, The *Journal of Cell Biology*, vol., 139, pp. 1545–1552 with kind permission of G. M. Kidder and by copyright permission of The Rockefeller University Press). Copyrightcr (1997) The Rockefeller University Press.

Whatever the physiological role of the γ subunit turns out to be, it has an important influence on Na^+, K^+-ATPase activity and transepithelial ion transport. What is certain is that the γ subunit is specifically associated with α and β isoforms. However, it remains to be seen whether the γ subunit associates with other membrane proteins (P-type ATPases or even independent ion channels or transporters) or performs physiological and cellular functions not requiring the presence of α and β subunits. It is also possible that more than one isoform of the γ subunit is present in mammalian species as is the case for the α and β subunits.

A Role in Cell Adhesion and Neural Guidance

The cDNA encoding the $\beta 2$ subunit of the rat and human Na^+, K^+-ATPase was first cloned by Martin-Vasallo and co-workers in 1989 using cDNA clones containing the entire coding sequence of the rat $\beta 1$ subunit. However, the $\beta 2$ isoform was

also independently partially cloned as the Ca^{2+} dependent adhesion molecule on glia (AMOG) by Pagliusi *et al*., in the same year. Several years earlier AMOG's role in adhesion was demonstrated biochemically by Antonicek *et al*. (1987) who showed that a monoclonal antibody specific for AMOG blocked neuron-glia interactions *in vitro*. Gloor and co-workres (1990) finally obtained the complete AMOG cDNA sequence and showed that AMOG was a subunit of the Na^+, K^+-ATPase that preferentially associated with $\alpha 2$ in detergent extracts of mouse brain. Several observations have confirmed that the adhesion role of AMOG/$\beta 2$ is unique and independent of the structure and function of the Na^+, K^+-ATPase enzyme complex, at least in the mouse brain. First, purified $\beta 2$ reconstituted into liposomes have been shown to adhere to neurons *in vitro* (Antonicek and Schachner, 1988). Second, inhibition of Na^+, K^+-ATPase by ouabain does not affect the neuron-glia interactions (Gloor *et al*., 1990). Third, AMOG/$\beta 2$ is capable of promoting neurite outgrowth *in vitro* whereas $\beta 1$ has no effect (Müller-Husmann *et al*., 1993). Fourth, gene knockout studies have demonstrated that AMOG/$\beta 2$ is important in brain development since neural cells degenerate in mice deficient in the gene for AMOG/$\beta 2$ (Magyar *et al*., 1994).

The original data using AMOG specific monoclonal antibodies suggested that this protein was expressed in significant quantities in brain, but not in liver, spleen, kidney, heart or intestines (Pagliusi *et al*., 1989). It was therefore suggested that AMOG/$\beta 2$ was unique to the central nervous system. However, studies performed by Martin-Vasello *et al*. (1989) suggested that although $\beta 2$ mRNA was most abundant in rat brain, lower levels were detectable in liver, spleen, kidney and a number of other tissues including heart, adrenal glands, bladder, uterus, testis and pancreas (see Fig. 6). Therefore, the proposed adhesion function for AMOG/$\beta 2$ is probably not exclusive to the central nervous system as $\beta 2$ protein has since been found to be expressed in numerous non-excitable (non-neuronal) cell types, particularly mesenchymal cells. These include endothelial cells (Mayol *et al*., 1998) and skeletal cells including osteoblasts (Mobasheri *et al*., 1996), chondrocytes (Mobasheri *et al*., 1997a, b; Mobasheri *et al*., 1998) and tendon cells (see Fig. 10). Since glycoproteins and their branched oligosaccharides have been implicated in cell recognition events, there may be a more general role for $\beta 2$ in mediating interactions between cell and extracellular matrix macromolecules via conventional adhesion molecules bearing specific recognition epitopes for $\beta 2$. Abundant expression of AMOG/$\beta 2$ in fetal connective tissues such as cartilage (Trujillo *et al*., 1998) is indirect evidence for this hypothesis. It remains to be seen whether $\beta 2$ plays an adhesion/guidance role in cells of mesenchymal origin during development.

Novel Roles in Protein Export, Cellular Morphology and Metastatic Behaviour

Recent studies by Florkiewicz *et al*. (1998) have suggested a previously unknown role for the α subunit of the Na^+, K^+-ATPase in Basic fibroblast growth factor (FGF-2) export via an exocytic pathway independent of the endoplasmic reticulum/Golgi apparatus. The evidence provided suggests that the α subunit of the Na^+, K^+-ATPase is a molecular component of an exocytic pathway mediating translocation of newly synthesised FGF-2 to the cell surface for its subsequent export to the extracellular matrix.

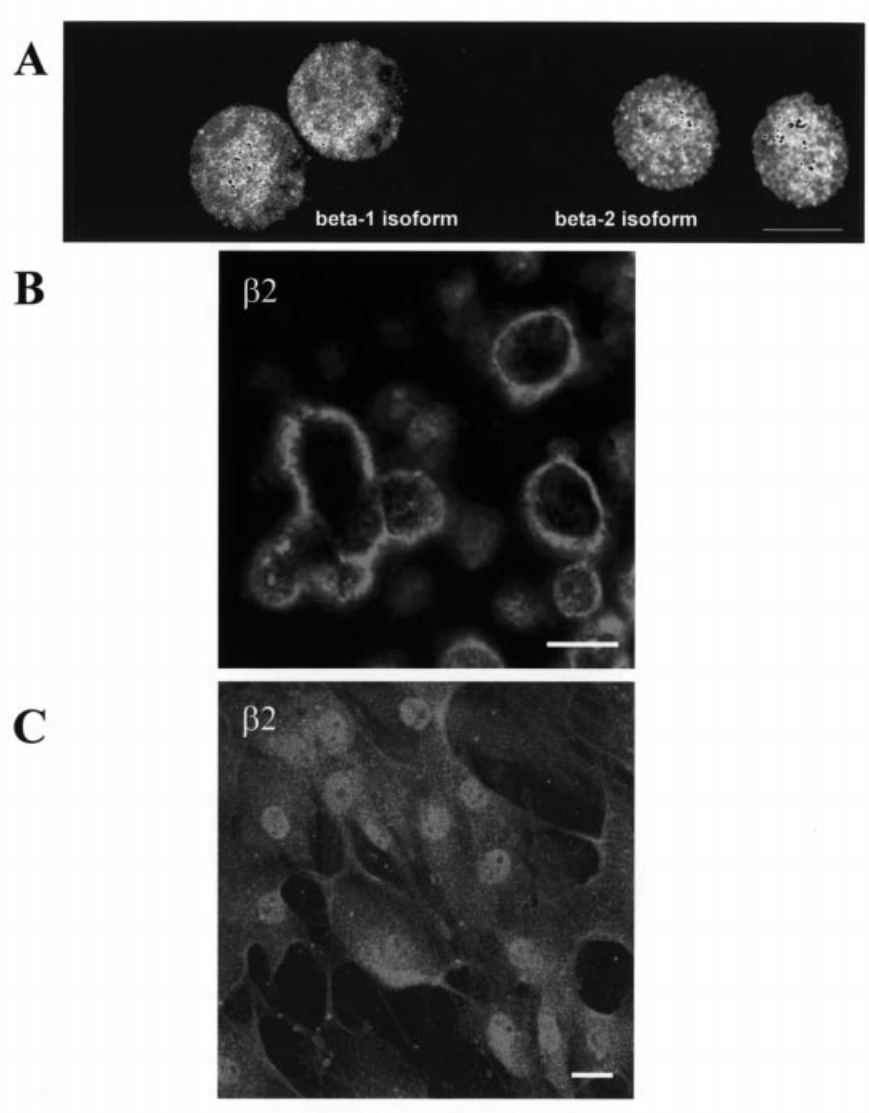

Fig. 10. Multiple Na^+, K^+-ATPase β subunits exist in cells derived from musculoskeletal tissues. Immunocytochemical preparations of cells derived from cartilage and tendon were labelled with β isoform specific antobidies and examined by immunofluorescence confocal laser microscopy. (A) Freshly isolated bovine chondrocytes immunostained with antibodies raised against the $\beta 1$ and $\beta 2$ isoforms. The $\beta 2$ isoform is abundantly expressed in human chondrocytes (B) and human tendon cells (C).

A number of reports have indicated that Na^+, K^+-ATPase activity is regulated by hormones, second messengers, protein kinase A (PKA) and protein kinase C (PKC) (Bertorello *et al.*, 1991; Aperia *et al.*, 1994; Ewart and Klip, 1995). Phosphorylation of Na^+, K^+-ATPase by protein kinases A and C has been investigated

in vitro where it has been demonstrated that the modulation of pump properties is subtle or requires additional regulatory proteins that would be present *in vivo*. Mutagenesis of the PKC site on rat $\alpha 1$ (Serine 23) has been shown to fundamentally alter physiological parameters including intracellular Na^+ and pH regulation in transfected COS cells (Belusa *et al*., 1997). In addition the above-mentioned mutation influences the morphology and adhesive properties of cultured cells transfected with the mutant enzyme. These results indicate a novel functional role for the Na^+, K^+-ATPase α subunit in cell adhesion and also in Na^+/H^+ exchange (NHE) mediated intracellular pH regulation. The NHE proteins critically depend on steep inward Na^+ gradients generated and maintained by the Na^+, K^+-ATPase; ejection of intracellular protons is accompanied by Na^+ entry via electroneutral Na^+/H^+ exchange. In view of the effects of Na^+, K^+-ATPase effectors on pump activity *per se*, it is also important to consider their impact on other Na^+-coupled transport systems. These include the Na^+/H^+ exchanger, the Na^+/Ca^+ exchanger and the Na^+–K^+–$2Cl^-$ cotransporter whose activity influence turnover of the Na^+, K^+-ATPase and in the long-term may affect the expression of the Na^+, K^+-ATPase. The observation that mutated α subunits influence the phenotype of adherent cell suggests that wild type α subunits are somehow involved in attachment to the substratum *in vitro* or to specific extracellular matrix molecules *in vivo*. Since cell attachment is mediated by focal adhesion contact sites and more specifically by integrins (Ruoslahti, 1991; Ruoslahti and Yamaguchi, 1991), plasma membrane Na^+, K^+-ATPase units may functionally interact with integrins or with additional accessory or link proteins. This phenomenon has already been observed in fibroblasts where NHE proteins are stimulated following attachment to the substratum, a process in which integrin clustering and interaction with fibronectin is of vital importance (Schwartz and Lechene, 1992). Changes in the expression of NHE proteins have been shown to alter cell shape and motility of cells (Wakabayashi *et al*., 1997). Investigating the relationship between Na^+, K^+-ATPase and cell attachment is clearly a topic for future research. However, much will be accomplished from consolidating and combining knowledge of other Na^+-dependent transport systems such as Na^+/H^+ exchange with clearly defined roles in pH regulation, ion homeostasis, cell motility and adhesion (Tominaga and Barber, 1998). It will be interesting to examine the involvement of Na^+, K^+-ATPase and related Na^+-dependent transport systems in metastatic behaviour of cancer cells. Voltage dependent Na^+ channels have been shown to play an important role in invasion of malignant cells (Laniado *et al*., 1997; Diss *et al*., 1998). Inhibition of Na^+ channels by specific toxins (such as Tetrodotoxin) in highly metastatic cancer cell lines dramatically thwarts invasive behaviour and cell motility in general (Grimes *et al*., 1995). Prolonged activity of voltage activated Na^+ channels (in the "activated" or "open ion gates" conformation) is expected to have profound effects on intracellular Na^+ and this will affect Na^+, K^+-ATPase activity which is highly dependent on intracellular Na^+ or persistent fluctuations in intracellular Na^+ may alter the density of the Na^+. Since the Na^+ affinity of the various Na^+, K^+-ATPase α subunits is variable, long-term changes in intracellular Na^+, or persistent fluctuations in intracellular Na^+ may alter the density of the Na^+, K^+-ATPase and the pattern of expression of its constituent subunit isoforms in metastatic cancer cells.

ASSOCIATED PATHOLOGIES OF NA^+, K^+-ATPASE

Thus far there are no reports of inherited mutations in the genes encoding the Na^+, K^+-ATPase. However, a growing number of heritable disorders are known to be caused by mutations in transport systems including ion channels, ion pumps and ion exchangers mutations. For example defective ion channel proteins are responsible for cystic fibrosis (Riordan *et al*., 1989; Rommens *et al*., 1989). In human patients with cystic fibrosis numerous different mutations in the gene encoding the cystic fibrosis transmembrane regulator (CFTR ion channel) have been identified (Davis *et al*., 1996). Defective ion channels are also responsible for heritable hypertension. Mutations in β and γ subunits of the amiloride sensitive sodium channel (ENaC) have been reported in Liddle's Syndrome, a form of pseudoaldosteronism (Shimkets *et al*., 1994; Tamura *et al*., 1996). Inherited alterations of calcium channels in peripheral nerves lead to bizarre neuromuscular disorders in experimental animal models (Burgess *et al*., 1997). A defect in the sodium/hydrogen exchanger gene has been associated with epilepsy in mice (Cox *et al*., 1997). A large number of other heritable mutations in ion channels have been identified recently and we refer readers to an excellent review article by Ackerman and Clapham (1997). Studies of these heritable mutations in ion transport systems have contributed to the creation of a novel and exciting branch of pathophysiology called "ion channelopathies" (Noebels, 1998). The fact that naturally occurring Na^+, K^+-ATPase mutations do not exist supports the notion that organisms that may acquire mutations in the genes encoding Na^+, K^+-ATPase cannot possibly survive due to the central role played by this enzyme system in the homeostatic function of animal cells. The critical part played by the Na^+, K^+-ATPase in embryonic development provides backing for this idea. Nevertheless, the expression, targeting and activity of Na^+, K^+-ATPase has been shown to be altered in some pathological states. Thus Na^+, K^+-ATPase plays a significant central and peripheral roles at the level of pathophysiological regulation in a number of human disorders.

Differential Expression of Na^+, K^+-ATPase in Cardiac Hypertrophy

The most striking differences between the α isoforms are in their sensitivities to cardiac glycosides (Sweadner, 1989) and in their tissue distribution patterns (Mercer, 1993). In rat, $\alpha 1$ is relatively insensitive to ouabain and to oxidants compared to $\alpha 2$ and $\alpha 3$ (Lingrel and Kuntzweiler, 1994; Xie *et al*., 1995). The expression of the α isoforms is subject to regulation by various hormones (Orlowski and Lingrel, 1990) and altered during development and experimentally induced pathological conditions (Herrera *et al*., 1988; Sweadner *et al*., 1994; Zahler *et al*., 1993). The relatively ouabain insensitive $\alpha 1$ constitutes most of the sarcolemmal Na^+, K^+-ATPase both in the adult rat heart and in the neonatal rat cardiac myocyte. The same is true for human heart (Jewell *et al*., 1992). However, while the predominant ouabain-sensitive isoform of the human and rat fetal/neonatal myocyte is $\alpha 3$ (there is little or no detectable $\alpha 2$ expression), that of adult human and rat heart is the $\alpha 2$ isoform. The switch from $\alpha 3$ to $\alpha 2$ occurs during postnatal development (Sweadner 1989; Jewell *et al*., 1992).

Numerous studies on the expression of Na^+, K^+-ATPase isoforms of fully developed cardiac muscle in different rat models of pressure-overload hypertrophy have been conducted (Book *et al*., 1994; Charlemagne *et al*., 1994; Sweadner *et al*., 1994). The general pattern that emerges from these studies is that at both transcriptional and translational levels there is no significant change in the expression of $\alpha 1$ but that $\alpha 2$ expression is repressed. Thus, it seems clear that in the rat model repression of ouabain-sensitive $\alpha 2$ isoform is a common feature of the hypertrophied phenotype. It is not known if cardiac glycosides, as drugs (Schwartz *et al*., 1988) or as the suggested humoral agent or endogenous ouabain (Blaustein, 1993) can cause hypertrophy and repression of $\alpha 2$ expression in the adult rat heart. In the primate myocardium (including human studies) the emerging picture suggests that three α subunits ($\alpha 1$, $\alpha 2$, $\alpha 3$) and one β subunit ($\beta 1$) are expressed (Shamraj *et al*., 1991). Thus the most likely Na^+, K^+-ATPase heterodimer combinations include $\alpha 1\beta 1$, $\alpha 2\beta 1$ and $\alpha 3\beta 1$ (Shamraj *et al*., 1993; Sweadner *et al*., 1994; Wang *et al*., 1996). The level of $\alpha 2$ expression is low but $\alpha 1$ and $\alpha 3$ are abundantly expressed based on direct comparisons with human brain (see Fig. 5). Studies by Zahler and co-workers (1996b) have indicated that in experimental canine hypertrophy a greater proportion of $\alpha 1$ isoform containing pumps are found. Thus, shifts in Na^+, K^+-ATPase isoforms may occur in pressure-overloaded heart. The expression level of the $\alpha 3$ isoform mRNA and protein was found to be lower in hypertrophied hearts. However, the relationship among cardiac glycoside therapy, inhibition of the three isoforms expressed in human heart, and increased cardiac contractility remains to be determined.

Na^+, K^+-ATPase and Hypertension

Little is known regarding the direct involvement of the Na^+, K^+-ATPase in other pathological states and regarding the possible underlying mechanisms that may lead to Na^+, K^+-ATPase disorders. One promising hypothesis relating the Na^+, K^+-ATPase to pathology is in essential hypertension where existence of circulating inhibitors of the Na^+, K^+-ATPase has been proposed (Blaustein, 1977). Ouabain-like substances (likely stereoisomers of ouabain) have been isolated from the hypothalamus (Tymiak *et al*., 1993) and from human plasma (Hamlyn *et al*., 1991). The presence of such compounds has been shown to be elevated in certain types of hypertension (for a review see de Waardener, 1996). In the late 1980's Ruiz-Opazo's group reported that in hypertension isoform-specific changes occur in the expression of the Na^+, K^+-ATPase (Herrera *et al*., 1988). A very recent paper by the same group suggests that the gene locus encoding the $\alpha 1$ Na^+, K^+-ATPase is a susceptibility gene for hypertension (Herrera *et al*., 1998) in the Dahl salt-sensitive rat model. Thus the data provide the basis for the study of the $\alpha 1$ Na^+, K^+-ATPase locus in human hypertension.

In the kidney the Na^+, K^+-ATPase is the driving force for Na^+ reabsorption in the basolateral membrane of cells lining the nephron and this vital function implicates the Na^+, K^+-ATPase system in the homeostatic control of extracellular volume and blood pressure (Fig. 8). Thus, any dysfunction of the renal Na^+, K^+-ATPase is expected to present severe pathophysiological consequences. Defects in renal Na^+,

K^+-ATPase activity, regulation and polarization have been suggested to be the underlying cause for hypertension and other disorders of nephrogenic origin (Laski and Kurtzman, 1996; Geering, 1997). Although no direct evidence has been provided to support this hypothesis, it is certain that elevated blood pressure, where chronic or acute, is associated with abnormal or dysregulated subapical distribution of Na^+ transporters (amiloride sensitive Na^+ channels, Na^+/H^+ exchange) and basolateral Na^+, K^+-ATPase activity in the proximal tubule. A defect in Na^+ reabsorption may arise following a dysregulation of Na^+, K^+-ATPase activity (short and long-term). Several hypotheses have been proposed to explain the possibility of endogenous ouabain-like inhibitors of the Na^+, K^+-ATPase existing in circulation (Hamlyn *et al*., 1982; Hamlyn *et al*., 1988; Hamlyn *et al*., 1991) and in the hypothalamus (Tymiak *et al*., 1993). These endogenous inhibitors which are likely to be isomers of ouabain have been shown to be elevated in certain types of hypertension (Hamlyn *et al*., 1991; de Wardener, 1996). Increased levels of endogenous ouabain-like substances would be expected to inhibit the renal Na^+, K^+-ATPase system. Thus, "over-inhibited" Na^+, K^+-ATPase in the basolateral membrane of the renal tubule would be expected to interfere with systemic Na^+ homeostasis with severe consequences for blood pressure. Furthermore, over inhibition of the Na^+, K^+-ATPase system (high affinity sites followed by low affinity sites) in vascular smooth muscle will also result in elevated blood pressure. Recent studies by Mayol *et al*. (1998) suggest that endothelial cells express at least four isozymes of the Na^+, K^+-ATPase consisting of low affinity $\alpha1\beta1/\alpha1\beta2$ sites and high affinity $\alpha3\beta1/\alpha3\beta2$ sites. Ouabain dose response curves show that the low affinity sites are three-fold more abundant than high affinity sites. Endogenous ouabain-like substances would be expected to inhibit first the high affinity Na^+, K^+-ATPase sites followed by the low affinity $\alpha1$ containing systems. The Na^+, K^+-ATPase would be critically involved in Ca^{+2} homeostasis and cell volume regulation in endothelial cells. In addition to these important functions, the Na^+, K^+-ATPase may perform specific endothelial and vascular functions (such as release of relaxing and contracting factors, modulating the levels of angiotensin converting enzyme and modifying local nitric oxide synthesis and release). Early studies of Na^+, K^+-ATPase isoform expression suggested the presence of only one class of low affinity sites consisting of $\alpha1$ isoforms (Zahler *et al*., 1996a, b). The presence of high affinity sites containing $\alpha3$ isoforms suggests that the Na^+, K^+-ATPase can play various roles in the endothelium dependent regulation of vascular tone by maintaining the equilibrium between vasconstrictive and vasodilative factors.

Involvement of Na^+, K^+-ATPase in Neurological Disorders

The Na^+, K^+-ATPase is most abundantly expressed in epithelia and in the central nervous system (CNS). Disturbances in CNS Na^+, K^+-ATPase activity have previously been proposed as being involved in the pathophysiology of bipolar mood disorder (El-Mallakh and Wyatt, 1995). Bipolar mood disorder is characterized by extreme mood swings from major depression to mania. The Na^+, K^+ATPase is responsible for generating and maintaining membrane potential and thus disturbances in its activity could have grave consequences for neuronal functioning. Evidence suggests that in bipolar patients there is a general reduction in CNS glucose

metabolism (Schwartz *et al*., 1987) and ATP production compatible with a reduction in Na^+, K^+-ATPase activity. Interestingly, patients suffering from digoxin/digitalis neurotoxicity frequently show symptoms of mania and depression (Eisendrath and Sweeney, 1987) and there is evidence for altered lymphocyte responses in patients with manic depressive psychosis (Wood *et al*., 1991). Recent evidence suggests an allelic association between bipolar disorder and the gene coding for the $\alpha 3$ isoform of Na^+, K^+-ATPase in an Irish population (Mynett-Johnson *et al*., 1998). It remains to be seen whether a similar allelic association will emerge in other populations.

Based on morphological similarities between neuropathological changes in spongiform encephalopathies and the changes frequently observed in the brains of animals injected with ouabain it has been suggested that the Na^+, K^+-ATPase enzyme itself may be one of the molecular targets of the infective prion entitles (Calendriello *et al*., 1995). In man, spongiform encephalopathies consist of Kuru, Creutzfeld–Jakob disease and Gerstmann–Straussler–Scheinker syndrome. Examples in domestic animals include scrapie (sheep) and bovine spongiform encephalopathy (mad cow disease). The plasma membrane Na^+, K^+-ATPase may be an obvious target in spongiform encephalopathies but there are also numerous other likely protein candidates that may be affected by the infective prions. Clearly, this and other more speculative hypotheses await further examination.

CONCLUSIONS

The Na^+, K^+-ATPase is a key regulator of cellular ion homeostasis. The existence of multiple isoenzymes consisting of different isoform subunits each exhibiting different kinetic properties confer distinct functional capabilities to a host cell under specific environmental conditions. This will permit highly specialized physiological fine tuning in different functional scenarios in different tissues and cell types. The complexity of Na^+, K^+-ATPase isoenzyme composition and isoform distribution underscores the subtlety of its expression, subunit assembly, plasma membrane targeting, regulation and physiological role in various tissues. The realization that NA^+, K^+-ATPase participates in a plethora of other cellular activities merely adds to its existing complexity and confirms its central role in the cell. However, despite the multitude of genetic, biochemical and immunological technologies currently available much work remains to be done. Knowledge of the precise 3-dimensional structure of the Na^+, K^+-ATPase still eludes us and until its crystal structure is elucidated, we may only guess before a detailed molecular mechanism is put forward to explain how the hydrolysis of ATP is coupled to the transport of cations.

ACKNOWLEDGEMENTS

We are grateful to Dr. Jacob I. Sznajder (University of Illinois at Chicago) for critical evaluation of the manuscript before submission. Research in the authors' laboratories is supported by grants from the British Heart Foundation (JFL), Programa Sectorial de Promocion General del Conocimiento, Ministerio de Educacion y Cultura, Spain (grant number PB97.1488) (P M-V, the Faculty of Science and

Engineering, University of Westminster (grant number VCX 1024), Teaching Company Directorate (Department of Trade & Industry, United Kingdom), and Intersep Ltd. (AM, MDB, and MT).

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