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Molecular and Functional Studies of the Gamma Subunit of the Sodium Pump

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This article reviews our studies of the γ subunit of the sodium pump. γ is a member of the FXYD family of small, single transmembrane proteins and is expressed predominantly in the kidney tubule. There are two major variants of γ which function similarly to bring about two distinct effects, one on K'_{ATP} and the other, on K_K, the affinity of the pump for K^+ acting as a competitor of cytoplasmic Na^+. In this way, γ is believed to provide a self-regulatory mechanism for maintaining the steady-state activity of the pump in the kidney. Our studies also suggest that K^+ antagonism of cytoplasmic Na^+ activation of the pump is relevant not only to the presence of γ in the kidney, but probably some hitherto undefined factor(s) in other tissues, most notably heart. The interesting possibility that not only γ but other members of the FXYD family regulate ion transport in a tissue-specific manner is discussed.

KEY WORDS: Gamma subunit; sodium pump.

INTRODUCTION

Although the basic functions of the sodium pump are the maintenance of Na^+ and K^+ homeostasis, tissue-specific modification of its behavior may be critical to specialized functions. While the nature of the α subunit isoform of the α/β Na,K-ATPase pump complex may be a primary determinant of the intrinsic kinetic properties of the enzyme, there is an increasing body of evidence suggesting the Na,K-ATPase is subject to complex short- and long-term regulation either by post-translational modifications or by other cell-specific components, which may interact with α/β pumps and modulate their kinetic properties (for review, see Therien and Blostein, 2000). Many earlier reports focused on the role of membrane lipids of which the main effects were related to membrane fluidity and thickness. A striking and mechanistically well-characterized tissue-specific modulator of the Na,K-ATPase is the L_p antigen of low-K^+ (LK) ruminant red cells, so-called because of its association with the L blood group antigens and its highly specific effects on the sodium pump (reviewed in Dunham and Blostein, 1997). However, the molecular nature of this protein remains unknown. Interactions of the Na,K-ATPase with components of the cytoskeleton have also been reported and the consequences of such interactions include correct processing and targeting of sodium pumps to the appropriate membrane compartment as well as regulation of activity. To date, however, there has been relatively little information on the nature and mechanistic basis of sodium pump modulation by specific membrane components, due mainly to the difficulty in identifying and separating such components from the enzyme complex.

Members of a family of small, single transmembrane proteins characterized by a FXYD motif, which include the γ subunit of renal Na,K-ATPase, are expressed in a tissue-specific fashion. Although the existence of gamma (γ) had been suggested almost 30 years ago (Rivas *et al*., 1972), and was subsequently referred to as the γ subunit (Reeves *et al*., 1980), only later was its specific association with the sodium pump demonstrated by Forbush and co-workers (Forbush *et al*., 1978). As described below, we

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now believe that the γ subunit is a mainly kidney-specific regulator of the Na,K-ATPase. A detailed description of seven members of this family has been obtained by analysis of gene transcripts in the EST data base (Sweadner and Rael , 2000). This revealed the FXYD motif prior to the transmembrane segment and several other conserved residues within the transmembrane and cytoplasmic segments. The better characterized other members of this family include phospholemman (PLM), expressed in mouse (Bogeav *et al*., 1998), dog (Palmer *et al*., 1991), rat and human (Chen *et al*., 1997), channel-inducing factor (CHIF) in rat (Attali *et al*., 1995) expressed in colon and kidney, and an 8-kDa mammary tumor-associated protein (Mat-8) in mouse (Morrison and Leder, 1994) and man (Morrison *et al*., 1995). While the latter members of this family have been described as ion channels or specific regulators of ion channels, there is new evidence suggesting that they may have other functions.

This article presents an overview of our studies of the molecular and mechanistic basis of tissue diversity in behavior of the ubiquitous $\alpha 1$ mammalian (rat) isoform. Several interrelated issues are discussed. The major focus is the tissue-specific expression and functional consequences of γ-subunit association with the α/β pump complex. Another aspect concerns the tissue-specific nature and mechanism underlying K^+ antagonism of cytoplasmic Na^+-activation, which is relevant not only to the presence of γ in the kidney, but, presumably, some heretofore undefined factor(s) in other tissues, most notably heart. Finally, we discuss very recent evidence that other members of the FXYD family interact with the sodium pump in a tissue-specific fashion.

RESULTS AND DISCUSSION

The Gamma Subunit (FXYD2)

Modulation of K'_{ATP} *Secondary to a Shift in the* $E_1 \leftrightarrow E_2$ *Conformational Equilibrium*

The γ subunit, first described in purified kidney Na,K-ATPase preparations (Forbush *et al*., 1978), associates in approximately equimolar amounts, with the α and β subunits (Collins *et al*., 1982; Hardwicke and Freytag, 1981; Reeves *et al*., 1980). Molecular cloning of the γ subunits of rat, mouse, cow, and sheep indicated a molecular weight of ≈6500 (Mercer *et al*., 1993). Cloning and sequencing of the human (Kim *et al*., 1997) and *Xenopus* γ subunits (Béguin *et al*., 1997) have also been reported. Sequence comparisons show ≈75% homology overall and much higher (93%) for only mammalian sequences. Further structural analysis has shown that γ comprises a single transmembrane domain and has a N-terminus out, C-terminus in topology (Béguin *et al*., 1997; Therien *et al*., 1997). As described elsewhere (Therien *et al*., 1997) and shown in Fig. 1, Western blot analysis of a variety of tissues, including rat glomerular cells, medulla, axolemma, heart, red blood cells, glomerular epithelial cells (GEC cell line), HeLa, lung, stomach, spleen and intestine (not shown), and cultured cells derived from rat kidney (NRK-52E) using anti-γ (C-terminal) antiserum shows γ protein only in kidney and not in cell lines derived from kidney. The presence in tubule-rich tissue from other species and absence in cell lines derived from them (pig and dog kidney vs. LLC-PK and MDCK cell lines) is noted as well.

On SDS–PAGE, the γ subunit of kidney runs as a doublet (apparent M_r's ≈8 and ≈9 kDa) (Mercer *et al*., 1993; Therien *et al*., 1997; Fig. 1) and a doublet is observed following *in vitro* expression in the presence, but not absence, of pancreatic microsomes (Mercer *et al*., 1993; Béguin *et al*., 1997). Recent mass spectrometry analysis of rat kidney γ indicates that the two major bands are splice variants. Gamma(a) (γa, upper band on SDS–PAGE) has a mass of 7184.0 +/− 1 Da (carbamidomethyl cysteine) (Kuster *et al*., 2000), which corresponds closely to that for the published sequence without the initiator methionine (Therien *et al*., 1999), while gamma(b) (γb, lower band) has a mass of 7337.9 +/− 1 Da. Tryptic peptide mapping and sequencing by mass spectrometry revealed that the seven N-terminal residues of γa, TELSANH, are replaced by Ac-MDRWYL in γb, but otherwise the chains

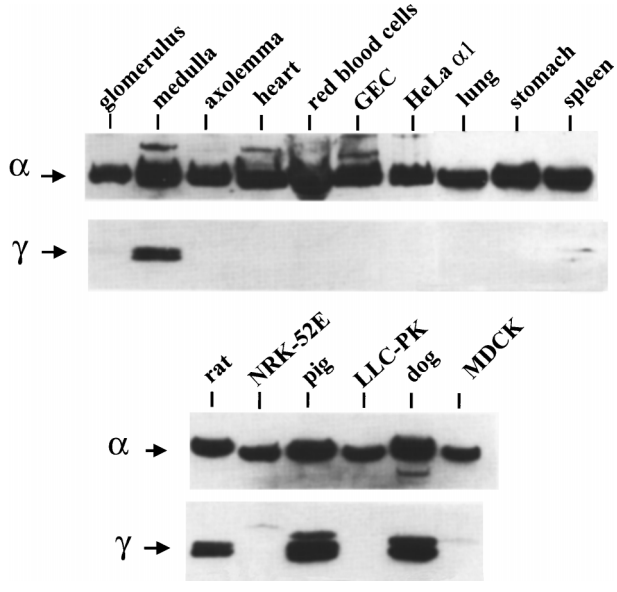

Fig. 1. Immunoblot analysis of γ expression in rat tissues.

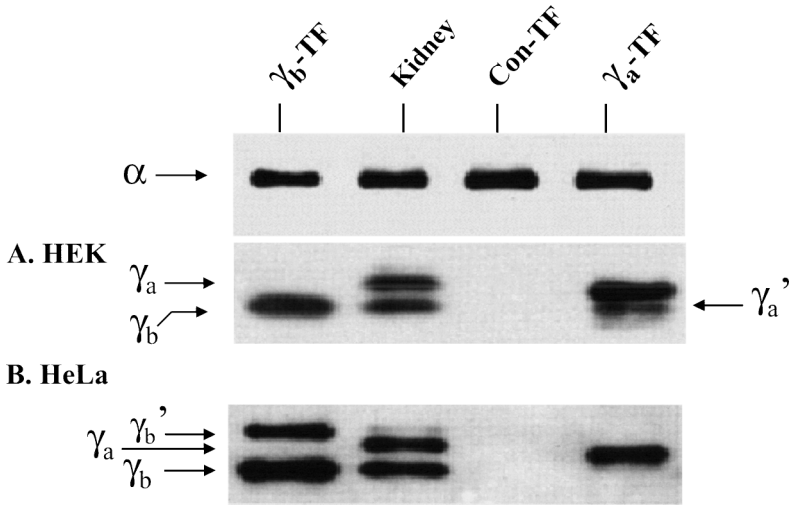

Fig. 2. Immunoblot analysis of γa and γb expression in HEK and HeLa cells. Taken from Kuster *et al*., 2000. The cloning and transfection of rat γa and γb cDNA's were carried out exactly as described for wild-type HeLa cells (Kuster *et al*., 2000), except that a stable cell line expressing rat α1 was used (α1-HeLa cells obtained as a gift from E. Jewell and J. B Lingrel). Cells were selected by growth in 400 μg/ml hygromycin and 1 μM ouabain (Kuster *et al*., 2000). Western blot analysis was carried out as described previously (Therien *et al*., 1997) using antibodies against the C-terminus (γC33; Therien *et al*., 1997) and against the N-termini of γa and γb. Quantitative phosphorimaging was carried out using the Storm Phospho-Imager (Molecular Dynamics).

are identical. These sequences are identical to those obtained by searching the EST database (Sweadner and Rael, 2000). Additional bands seen on Western blots of transfected cells has led to some confusion (Arystarkova *et al*., 1999), which is clarified by experiments in which the cDNA's for both variants were expressed in both HEK and HeLa cells (Kuster *et al*., 2000). As shown in Fig. 2, two major bands are expressed in HeLa cells. They correspond to γa (upper band) and γb (lower band) of the renal Na,K-ATPase. Additional minor bands seen after transfection, namely γa$'$ in HEK and γb$'$ in HeLa cells, imply that these are cell-specific posttranslational modifications (Kuster *et al*., 2000).

For functional analysis, two approaches were used. One series of experiments tested the effects of anti-γ (C terminus) antibodies on the renal enzyme. In the other series, the kinetic behavior of γ-transfected cells was compared to that of mock (vector alone)-transfected cells. The latter were performed initially with γa-transfected human embryonic kidney (HEK) cells and, more recently, with γa and γb expressed in both HEK and HeLa cells.

We first showed that anti-γ antiserum raised against the C-terminus of γ inhibits Na,K-ATPase activity of the kidney enzyme, but not of tissues that do not express γ (Therien *et al*., 1997). Further analysis showed that anti-γ decreases the apparent affinity for ATP probably by stabilizing the E_2 form(s) of the enzyme. There are several observations that support this conclusion (as shown in Table I): (1) anti-γ inhibits Na,K-ATPase activity such that inhibition increases at acidic pH under which condition the

Table I. Gamma Effects on V_{max}, K'_{ATP}, and the $E_1 \leftrightarrow E_2$ Conformational Equilibrium[a]

Parameter measured	Effects of anti-γ(C-terminal)	Effect of γ transfection
1. Na,K-ATPase as a function of pH	Inhibition increases at acidic pH; decreases at alkaline pH	n.d.
2. K'_{ATP}	Increases K'_{ATP}	Decreases K'_{ATP}
3. K^+ sensitivity of Na-ATPase	Increased K^+ inhibition at low (μM) ATP	Decreased K^+ inhibition at low (μM) ATP
4. Vanadate sensitivity of Na-ATPase	Decreases I_{50} for vanadate	Increased I_{50} for vanadate
5. V_{max} and EP_{max}	V_{max} decreased ($\approx$25%); no effect on EP_{max}	n.d.

[a] Membranes were prepared and enzyme assays were carried out as described previously (Therien *et al*., 1997). For studies of sensitivity to vanadate, Na-ATPase assays were carried out without and with vanadate, with low and high ouabain added for each, respectively. Data taken from Therien *et al*. (1999, 2000) and H. X. Pu *et al*. (2001). n.d., not determined.

$E_2(K) \rightarrow\rightarrow E_1$ segment of the reaction becomes more rate limiting; (2) displacement of the E_1/E_2 conformational equilibrium toward E_2 by anti-γ was evidenced by an increase in K'_{ATP}; and (3) anti-γ increases sensitivity to K^+-inhibition of Na-ATPase at micromolar ATP concentration under which condition $E_2(K) \rightarrow\rightarrow E_1$ is rate limiting. Although a small effect of anti-γ on apparent K^+ affinity (increase) was observed at suboptimal ATP concentration (Therien *et al*., 2000), a consistent opposite effect could not be detected with γ-transfected cells, possibly due to their higher background hydrolysis. In fact, in assays of ouabain-sensitive $^{86}Rb^+$ (K^+) influx, a significant difference in the apparent affinity for extracellular K^+ between either γa- or γb-transfected cells compared to control mock-transfected cells could not be detected. (4) Based on the premise that anti-γ increases E_2 forms, including E_2P, it was predicted that anti-γ-treated kidney enzyme should be more sensitive to vanadate than control membranes. That this is, indeed, the case is shown by the modest (≈2-fold), but significant, decrease in the IC_{50} for vanadate inhibition effected by anti-γ ($p < 0.01$). (5) Finally, anti-γ appears to decrease the catalytic turnover of the enzyme, as indicated by the decrease in V_{max} without an effect on maximal phosphoenzyme.

It is important to note that, in general, the behavior of anti-γ-treated, compared to nonimmune serum treated-kidney enzyme mirrors the behavior of mock-transfected compared to γ-transfected cells, respectively. This is exemplified by the results for HeLa cells summarized in Table I. Accordingly, it may be inferred that γ increases V_{max} and catalytic turnover, since anti-γ has the opposite effect on the renal enzyme. (Variability in specific activity among different membrane preparations precluded comparisons of V_{max} of γ- vs. mock-transfected cells.)

Taken together, these results provide strong evidence that an important role of the γ subunit is to shift the conformational equilibrium of the Na,K-ATPase reaction toward E_1 and that anti-γ disrupts the relevant interaction between γ and the α/β Na,K-ATPase complex. The observation that γ decreases sensitivity to vanadate and, conversely, that anti-γ treatment of the renal enzyme increases sensitivity to vanadate, strongly support the conclusion that the change in K'_{ATP} reflects primarily an effect on the E_1/E_2 conformational equilibrium. Our recent transfection studies show that the aforementioned properties of γ shown in Table I, hold true for its two major variants, γa and γb (H. X. Pu *et al*., 2001). Quantitatively, the effects of γ are within the range predicted by our estimates of the $\gamma : \alpha$ ratio, which is at least 50% of that of the kidney enzyme.

Another finding concerning the function of γ is that it has a second distinct effect on the catalytic function of the Na,K-ATPase. Thus, our earlier studies revealed a

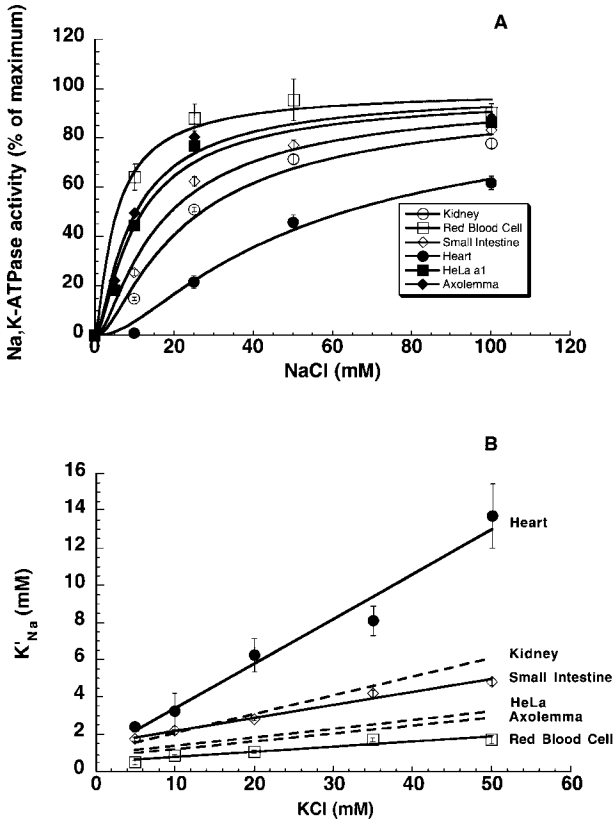

Fig. 3. Tissue-specific differences in K'_{Na} at high K^+ concentration reflect differences in K^+ antagonism of cytoplasmic Na^+ activation. (A) Na^+ activation profiles determined at 50 mM KCl (Fig. 1, Therien and Blostein, 1999). (B) Dependence of K'_{Na} on K^+ concentration (Fig. 2, Therien and Blostein, 1999). Reprinted with permission from the American Physiological Society.

notably greater effect of K^+ as an inhibitor at cytoplasmic Na^+ activation sites of kidney pumps compared to most other tissues (see Fig. 3). Originally, we dismissed the notion that this property was due to the presence of γ, since anti-γ did not abrogate the effect. However, our recent transfection studies have revealed that both γ variants increase K^+/Na^+ antagonism (H. X. Pu *et al*., 2001). When Na,K-ATPase assays are carried out at relatively high K^+ concentration, this effect is manifested as a lower apparent affinity for Na^+ of γ-compared to mock-transfected cells. A detailed analysis of K'_{Na} measured as a function of varying K^+ concentration, indicates that the effect is due to a decrease in K_K with little effect on K_{Na}. These results are summarized in Table II. This effect of γ on K^+/Na^+ antagonism is seen equally with both γ variants and, interestingly, this function of γ is not altered by antibodies, which react with the undenatured enzyme, namely either against the C-terminus reactive with both variants,

Table II. Gamma Effects on Na^+ and K^+ Interactions with Na^+ Activation Sites[a]

Cells	K_{Na}	K_K	K_{Na}/K_K
α1-HeLa-mock	0.86	19.1	0.045
α1-HeLa-γa	0.83	11.7	0.071
α1-HeLa-γb	0.87	11.8	0.074

[a] For experiments with HeLa cells expressing the ouabain-resistant rat α1 catalytic subunit ("α1-HeLa"), the cells are grown in 1 μM ouabain and assayed in medium containing a low (10 μM) ouabain concentration. Activities shown are the differences in ATP hydrolysis measured in the presence of low (10 μM) and high (5 mM) ouabain concentrations. Kinetic evaluation of K^+ antagonism of Na^+ activation at cytoplasmic sites was based on the Albers–Post model and the assumption that Na^+ and K^+ bind randomly at three equivalent cytoplasmic sites and was carried out as in previous studies using the relationship $v = V/\{1 + K_{Na}/[Na](1 + [K]/K_K)\}$ where [Na] and [K] are the cytoplasmic concentrations of Na^+ and K^+, respectively, and K_{Na} is the affinity for Na^+ binding at cytoplasmic activation sites in the *absence* of K^+; K_K is the affinity for K^+ acting as a competitive inhibitor of Na^+ binding at cytoplasmic sites. Good fits of the data to the linear relationship between the apparent affinity constant for Na^+, K'_{Na}, and $[K^+]$, i.e., $K'_{Na} = K_{Na}(1 + [K]/K_K)$, allowed the quantification of K_{Na} and K_K.

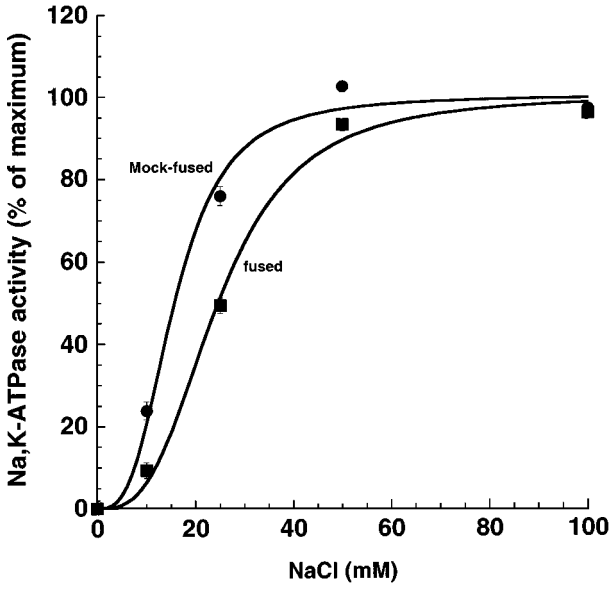

Fig. 4. Na^+ activation of kidney pumps either fused or mock-fused into dog red blood cells (Fig. 4 of Therien and Blostein, 1999). Reprinted with permission from the American Physiological Society.

or the TELSANH peptide of γa (H. X. Pu *et al.*, 2001). (Antibodies raised against the N-terminus of γb are reactive only with SDS-denatured enzyme, which precludes meaningful interpretation of its failure to alter the effects of γb on $\alpha 1\beta 1$ pumps.)

Overall, it is evident from our studies that both γ variants alter the kinetics similarly, with no evidence of a significant difference between the two on catalytic function. It may also be noted that the functional effects do not depend on tissue-specific modifications of the γ subunit, although such modifications can be observed in Hela cells (γb′) or HEK cells (γa′). Expression of γa in NRK-52E kidney cells has been reported to modulate (decrease) Na^+ and K^+ affinities (Arystarkova *et al.*, 1999). Although this functional effect appears similar to that described here for γa, there are puzzling experimental differences. First, compared to kidney enzyme, the level of γ expression was much lower (15–20%) than in experiments carried out with the HeLa transfectants ($\geq$50%; Fig. 2) despite the similar functional effect. Second, the effect was observed only in cells expressing a doublet, unlike the studies with Hela cells which, like kidney (see Kuster *et al.*, 2000), express only a single γa band. Since there does not seem to be a modified (γa′) in intact kidney membrane, the relationship of this observation in studies with NRK-52E (Arystarkova *et al.*, 1999) to an effect in intact kidney has not been clear.

The recognition that the γ subunit induces at least two functional effects, only one of which is abrogated by anti-γ (C-terminal), suggests that there must be more than one region of interaction between the γ and α subunits on which the functional sites reside. Presumably, the effect on the E_1/E_2 equilibrium and apparent ATP affinity involves the C-terminal sequence KHRQVNEDEL and the K^+/Na^+ antagonism is mediated by other sequences in the molecule. At the level of mechanism, while stabilization of the E_1 conformation by the γ subunit explains the increase of apparent ATP affinity, such an effect cannot also explain the increased K^+/Na^+ antagonism. An increased intrinsic K^+ binding affinity at one of two cytoplasmic K^+ sites could lead to an increased cytoplasmic K^+/Na^+ antagonism, without simultaneously affecting the E_1/E_2 equilibrium. The singularity of two effects is underscored by the observation (Therien and Blostein, 1999; see Fig. 4) that polyethylene glycol-mediated fusion of kidney pumps into cells devoid of pumps (dog erythrocytes) abrogates the kidney-specific increase in K^+/Na^+ antagonism, but not anti-γ-mediated inhibition of overall activity.

Is Tissue-Specific K^+ Antagonism at Cytoplasmic Na^+ Activation Sites a General Mechanism for Pump Regulation?

K^+/Na^+ antagonism was first noted in studies of $\alpha 1\beta 1$ pumps of kidney, α1-transfected HeLa, and axolemma (Therien *et al.*, 1996), and, more recently, in

Table III. Apparent Affinities for Na^+ and K^+ Binding to Na^+ Activation Sites of $\alpha 1$ Pumps

Tissue/cell	K_{Na}[a] (mM)	K_K[a] (mM)	K_{Na}/K_K[a]
Red blood cell	0.51 ± 0.16[c]	18.6 ± 9.1	0.027 ± 0.005[g]
Axolemma[b]	0.78 ± 0.05	18.7 ± 1.8	0.042 ± 0.002
HeLa[b]	0.91 ± 0.02	19.9 ± 0.7	0.046 ± 0.001
Small intestine	1.46 ± 0.16[d]	20.8 ± 3.6	0.070 ± 0.005[g]
Kidney[b]	1.02 ± 0.09	10.0 ± 1.2[e]	0.102 ± 0.003[g]
Heart	0.95 ± 0.71	3.96 ± 3.3[f]	0.239 ± 0.025[g]

[a] K_{Na} is the affinity constant for Na^+ in the absence of K^+ and K_K the affinity constant for K^+ as a competitive inhibitor. Values of K_{Na}, K_K, and K_{Na}/K_K were determined from plots in Fig. 2 and Eq. (3) of ref. 4. Reprinted with permission from the American Physiological Society.

[b] Values taken from Table II of Therien and Blostein (1999).

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

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

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

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

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

other tissues, namely heart, small intestine, and red blood cells (see Fig. 3). A detailed kinetic analysis of K^+/Na^+ competition was carried out for six rat tissues (Table III). The results indicate that the order of susceptibilities of $\alpha 1$ pumps of rat tissues to K^+/Na^+ antagonism (represented by K_{Na}/K_K, the ratio of the apparent affinity for Na^+ binding at cytoplasmic activation sites in the absence of K^+ to the affinity constant for K^+ as a competitive inhibitor) is red blood cells < axolemma ≈ rat $\alpha 1$-transfected HeLa cells < small intestine < kidney < heart. This is due primarily to a lower K_K of $\alpha 1$ of heart and kidney. K_{Na} values were generally similar except for the small intestine (higher K_{Na}). A more detailed analysis of the rates of K^+ occlusion and deocclusion (Therien and Blostein, 1999) support a minimal simple model, whereby Na^+ competes with K^+ for binding to the cytoplasmic cation binding site of E_1 to form either (1) the $E_1P(Na)$ in the forward reaction or (2) $E_2(K)$ in the backward reaction.

It is interesting to note that a role of the γ subunit in interactions of the Na,K-ATPase with K^+ may be relevant to earlier findings that the γ subunit is a component of the protein complex found in so-called "19-kDa membranes," the product of tryptic digestion following occlusion of K^+ or Rb^+ by the enzyme to form $E_2(K)$ (Or *et al*., 1996), and that partial protection by K^+ ions against tryptic digestion of the γ subunit is seen in renal microsomes (Therien *et al*., 1997).

Comparison of Kidney and Heart

As shown in Table III, K_{Na}/K_K is highest in both kidney and, to an even greater extent, heart which, in the adult rat, comprises at least 80% $\alpha 1$. However, there is a clear distinction between the underlying nature of this effect in heart compared to kidney. When the rat heart enzyme preparation is subjected to SDS treatment with concentrations above that generally used to permeabilize the membranes (0.60 mg/ml), there is a notable decrease in K^+/Na^+ antagonism, as evidenced in the Na^+-activation profiles measured at relatively high K^+ concentration. Thus, the apparent Na^+ affinity of heart (measured at 50 mM K^+) is decreased ≈ twofold when the detergent is increased to 0.9 mg/ml, whereas that of the kidney remains unaltered (R. Blostein and A. Wilczinski (2000)).

Reversible modulation of K^+/Na^+ antagonism is a potentially important mechanism of sodium pump regulation since the concentration of K^+ in most cells is roughly tenfold higher than that of Na^+. Alteration in the susceptibility of the sodium pump to inhibition by K^+ would allow for changes in activity without changes in cation concentrations. Such a regulatory mechanism would be especially important in the heart, where the pump has a role in determining the set point for Na^+/Ca^{2+} exchange and, hence, contractility of cardiac muscle (Blaustein, 1977). The nature of the putative regulatory component in cardiac tissue is currently under investigation.

Physiological Implications

The physiological significance of the γ subunit could be that it provides a self-regulatory mechanism for maintaining the steady-state activity of the pump in the kidney. This notion is underscored by the abundance of both variants in the medullary thick ascending limb (H. X. Pu *et al*., 2001), suggesting that its functional effects are tailored to meet the requirement of Na^+ and K^+ homeostasis in the prevailing environmental conditions and, in particular, by the observations of the dual effects on the kinetic properties, the one on K'_{ATP} and the other on K_K, the affinity of the pump for cytoplasmic K^+ acting as a competitor of Na^+. The effect of γ on K'_{ATP} was discussed recently in terms of its importance in maintaining pump activity under putative anoxic parts of the medulla, that is, to increase ATP utilization and maintain optimally high intracellular K^+ and low Na^+ under energy-compromised conditions (Therien *et al*., 1999, 2000). Such a regulator of K'_{ATP} should alter the pump's affinity for the nucleotide only moderately, for

an excessive increase would effect even greater decreases in ATP concentration, thus leading to compromised cell viability. The ability of γ to increase K^+/Na^+ antagonism at the cytoplasmic surface (H. X. Pu *et al*., 2001), may provide a means of acute regulation of the steady-state Na^+ concentration. A lowered effective cytoplasmic Na^+ affinity for activating the pump, due to a regulatory interaction, may be tailored to fit cells in which the steady-state Na^+ concentration is higher than in cells which lack the regulator, but which, nevertheless, must respond to changes in Na^+ entry. Thus the optimal affinity for cytoplasmic Na^+ ions should be one at which there is plenty of reserve capacity for responding to changes in cell Na^+ at the prevailing set-point of Na^+ concentration. The recent report of a putative dominant-negative mutation (G41R) in the γ subunit of the Na,K-ATPase (Meij *et al*., 2000) may be relevant to a role of γ in maintaining intracellular Mg^{2+} secondary to elevating intracellular Na^+. Such a relationship between intracellular Na^+ and Mg^{2+} was seen not only in sublingual mucous acini (Zhang and Melvin, 1996), but also in the kidney (G. Quamme, personal communication 2001).

The lack of a notable difference between γa and γb with respect to their effects on pump kinetics may not be surprising. The two variants differ with respect to only the six or seven residues at the extracellular amino terminus. Accordingly, the two variants may influence differentially such properties as membrane targeting, pump turnover, or basolateral signaling and affect the rate of active Na^+ and K^+ transport by altering the density of pumps in the basolateral membrane. It is also plausible that each of these variants may influence differentially, in a cell-specific manner, some interactions of the pump with the extracellular matrix. Our experiments cannot rule out the possibility of a heretofore undetected more subtle distinction between the two variants.

In contrast to their functional similarities, γa and γb show differences in their localization along the kidney tubule (H. X. Pu *et al*., 2001). Using anti-γ (C-terminus) and antibodies to the rat α subunit as well as antibodies to identify cell types, double immunofluorescence experiments carried out in the laboratory of N. Farman showed highest expression is in the medullary portion of the thick ascending limb (TAL), which contains both γa and γb, with no other positive tubular segments in the medulla. In the cortex, most tubules express γ, but at lower levels. Antibodies specific for γa and γb showed differences in their cortical location: γa is specific for cells in the macula densa and principal cells of the cortical collecting duct, but not cortical TAL. In contrast, γb, but not γa, is present in the cortical TAL. Thus, the distinction between γa and γb may be related to cell-specific location of the pumps rather than to different modulatory effects on function.

Interaction of Other Members of the FXYD Family with the Na,K-ATPase

CHIF or FXYD4 was identified by differential screening for aldosterone-induced transcripts (Attali *et al*., 1995). CHIF cDNA codes for an 87 residue polypeptide with a hydrophobic signal peptide preceding the FXYD motif. Recent evidence using an *in vitro* expression system suggest that the signal peptide may not be cleaved, implying the existence of two transmembrane domains (Shi *et al*., 2001). CHIF mRNA and protein is specifically expressed in kidney collecting duct (CD) (cortical CD < outer medulla CD < inner medulla CD) and in distal colon surface cells (Capurro *et al*., 1996; Wald et al., 1996), but not in a variety of other tissues. In the colon, CHIF mRNA is upregulated by aldosterone, dexamethasone, or low Na^+ diet (via changes in plasma aldosterone) or by a high K^+ diet. In the kidney, only regulation by K^+ intake is apparent (Wald *et al*., 1996, 1997). These observations form the basis for the hypothesis that CHIF is involved primarily in regulation of K^+ homeostasis.

Although CHIF was found originally to induce K^+ channel activity in *Xenopus* oocytes, later work has shown this to be an erratic phenomenon. Immunocytochemical localization shows the presence of CHIF in the basolateral membrane of renal cortical collecting duct principal cells and at increased levels in outer and inner medullary collecting duct, but not in other nephron segments (Capurro *et al*., 1996; Shi *et al*., 2001; Garty *et al*., 2001). Since this expression pattern exactly complements that of the γ subunit, and given the homology between the proteins, it is conceivable that CHIF could replace γ as a regulator of Na,K-ATPase in the specific cell types. In support of this hypothesis, coimmunoprecipitation of CHIF with α subunit of Na,K-ATPase in colon membranes has been recently demonstrated (Garty *et al*., 2001). Dependence of the coimmunoprecipitation on Na,K-pump ligands indicates that the interaction is quite specific.

A phospholemman-like protein (PLMS) has been described in purified shark rectal gland Na,K-ATPase preparations (Mahmmoud *et al*., 2000). Like cardiac PLM, PLMS can undergo phosphorylation by protein kinase A and C and activation of the Na,K-ATPase occurs as a result. Similar effects are achieved with low concentrations of a non-ionic detergent and it is suggested that either phosphorylation or the detergent cause dissociation of the PLMS from the γ subunit. Mahmmoud *et al.* (2000)

propose that phosphorylation of cardiac PLM may regulate cardiac Na,K-ATPase by a similar mechanism.

The two examples discussed above raise the tantalizing prospect that other FXYD proteins act as tissue-specific regulators of the Na,K-ATPase, in addition to any other roles they may have. It now remains to be established whether or not this concept has validity.

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