Modified Kidney Sodium Potassium Adenosine-Triphosphatase Kinetics in Protein Energy Malnutrition

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Summary Erythrocyte membrane and kidney microsomal Na, K-ATPases have been shown to be elevated in children suffering from kwashi-orkor and in protein energy malnourished (PEM) rats. Kinetic properties of kidney microsomal Na, K-ATPase were studied in PEM rats to understand further the mechanism of this elevated activity. From the Arrhenius plots no significant differences were seen in the critical temperature and energy of activation of Na, K-ATPase between the control (C), energy-restricted (ER), and protein-restricted (PR) rats. Changes in the apparent $K_{0.5}$ values of Na^+ , K^+ , and ATP were of much smaller magnitude, and increased $V_{\max}$ was found to be mainly responsible for the observed increase in Na, K-ATPase activity in PEM rats. The Hill coefficient with ATP as substrate was found to be 1.62 in all three groups. $V_{\max}/K_m$: a broad index of physiological efficiency, remained unaltered with K^+ as a substrate in the ER rats, but was higher by 60% in PR rats as compared with the control value. In the Na^+ -activated component, the physiological efficiency in protein restriction was higher by 60% in relation to energy restriction. These results lead to the conclusion that the increased Na, K-ATPase activity in kidney microsomal preparations, from PR rats is attributable to modifications in the enzyme site and that energy restriction, in fact, results in a lowering of the physiological efficiency of Na, K-ATPase by affecting the Na^+ -site. Altered lipid microenvironment if any, does not appear to contribute to changes in Na, K-ATPase activity in PEM since neither Arrhenius plots nor Hill coefficients (ATP) showed any modification.

Key Words: Na, K-ATPase, protein restriction, energy restriction

Sodium-potassium-activated adenosine triphosphatase (ATP-phosphohydro-

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lase, EC 3.6.1.3: Na, K-ATPase) a biochemical expression of the "Na⁺-pump," is involved in the uphill transport of Na⁺ and K⁺ across the plasma membrane [1, 2]. The enzyme system has a ubiquitous distribution in all mammalian cell membranes and is crucial to the maintenance of potential across the membrane via maintaining an ionic equilibrium. In performing this task in different cells, it contributes to the active Na⁺-reabsorption in the kidney and transmission of nerve impulses in the neuron [3]. Due to these vital functions this enzyme system is highly responsive to any perturbation in the cell, particularly the ones concerned with alterations in ion permeability. In addition, being an integral membrane protein, it also responds to an altered microenvironment, which is reflected mostly by modifications in Arrhenius and Hill plots [4, 5].

Our own interest in this transport system stems from an earlier study on protein energy malnutrition (PEM) amongst the pre-school children in many developing countries including India. Accumulation of fluid (edema) is one of the most obvious clinical signs in these patients. Ouabain-sensitive Na,K-ATPase activity in the leucocytes and erythrocytes of these patients seems to be elevated [6, 7]. In subsequent studies, this elevation in erythrocyte membrane Na,K-ATPase was shown to be an adaptation for preventing the continued cellular accumulation of Na⁺ in these patients. A failure of such an adaptive mechanism causes intracellular accumulation of electrolytes and fluid causing edema [8–10].

In simultaneous studies using an experimental model of this disorder in which rats receiving a protein-deficient diet simulate some of the clinical signs of human kwashiorkor [11], we found similar elevation of kidney microsomal Na,K-ATPase in PEM rats [12].

Na,K-ATPase, an integral membrane protein with vectorial localisation across the membrane, is activated by Na⁺ from the inner surface of the membrane. Na⁺ is considered to activate phosphorylation step and K⁺ the dephosphorylation step from the outer surface of the membrane [9]. The aim of the present experiment was to determine a possible mechanism for the increased kidney microsomal Na,K-ATPase activity in PEM rats from a study of its kinetic characteristics.

MATERIALS AND METHODS

Details of the diet compositions and rats used in these studies have been reported earlier [13, 14]. Briefly, weaning Wistar strain male rats were fed a 22% casein diet till they attained an average body weight of 90 g. They were then divided into 3 groups. Group I, the control (C), received 22% casein diet *ad lib*; group II rats, the same diet in restricted amounts so as to maintain their average body weight similar to that of group III which received a 1% casein diet and served as a protein-restricted group (PR). Group II thus served as the energy-restricted group (ER). The rats were maintained on their respective diets for 7–9 weeks, after which time the PR rats show major symptoms of kwashiorkor [11].

All the reagents used were of analytical grade. Ouabain and ATP were ob-

tained from Sigma Chemical Co., (St. Louis, MO). Sodium ATP was converted to Tris-ATP by the method of Epstein and Whittam [15] for all kinetic studies. Sodium ortho vanadate (pure form) was a gift from Dr. Ramanadham (NIH, Bethesda).

Enzyme preparation. Rats were fasted overnight and sacrificed by cervical dislocation. The kidneys were removed and immersed immediately in ice-cold homogenising medium containing 0.25 M sucrose, 0.03 M histidine-NaOH buffer (pH 7.4), and 0.001 M EDTA. They were then decapsulated, freed from adhering blood, weighed, and homogenised in 9 vols of homogenising medium in a motor-driven Teflon pestle for 1 min. The homogenate was centrifuged at $600 \times g$ for 10 min. The supernatant was removed, and the pellet was washed with 5 vols of homogenising medium and again centrifuged at $600 \times g$ for 15 min. The pellet was discarded and the supernatant further centrifuged at $105,000 \times g$ for 1 h. The membrane-rich pellet was resuspended in homogenising medium to give approximately 10 mg protein/ml, frozen and stored overnight at -20°C , and then analyzed for enzyme activity.

Assay of Na,K-ATPase activity. The enzyme activity was assayed with 50–60 μg of kidney microsomes in a reaction medium containing 20 mM Tris-HCl (pH 7.4), 140 mM NaCl, 14 mM KCl, 3 mM MgCl_2 , 3 mM Tris-ATP, and 0.2 mM EDTA in a final volume of 0.5 ml. Ouabain, when used, was at a concentration of 2 mM. Reactions were allowed to proceed for 10 min at 37°C before they were terminated by addition of 0.5 ml of 10% TCA. Tubes were then centrifuged to sediment the precipitate and the P_i released by the enzyme action was measured in the supernatant by the method of Fiske-Subbarow [16]. Proteins were estimated by Lowry's method [17]. Na,K-ATPase activity was defined as the difference between activities in the absence and presence of ouabain. One unit of enzyme was defined as that amount releasing 1 μmol of P_i per hour per mg protein of microsomes.

Arrhenius plots, transition temperature, and activation energies. Arrhenius plots were prepared by assaying the enzyme activity at different temperatures ranging between 2 and 30°C at $4\text{--}5^{\circ}\text{C}$ intervals. At lower temperatures (between $2\text{--}10^{\circ}\text{C}$) the reactions were continued for up to 30 min, and at higher temperatures, for 10 min. For the calculation of the transition temperature (T_e) the logarithm of the specific activity value at each temperature was plotted against the reciprocal of the absolute temperature. The values of T_e were directly read from the graph and the activation energies above and below the T_e were calculated by the Arrhenius equation.

Enzyme kinetics. Kinetic characteristics of the enzyme from the three groups of rats were studied with, Na^+ , K^+ , and ATP as substrates. Enzyme activity was measured at various concentrations of Na^+ (2–100 mM), K^+ (0.5–20 mM), and ATP (0.1–4 mM) while keeping the other two constant. The activity at any concentrations of Na^+ , K^+ , or ATP was taken as the difference between the activity at that concentration and that in the absence of the ion.

Effect of 1 μM vanadate [Sodium ortho vanadate (V)] on Na,K-ATPase from the three groups of rats was studied at different concentrations of Na^+ . Microsomes were incubated with 1 μM vanadate for 15 min at 37°C before the start of the reaction.

RESULTS

The Arrhenius plots of Na,K-ATPase from the 3 groups of rats gave biphasic curves with a deflection point at a certain temperature called the transition temperature (T_c). This temperature depends upon and reflects the composition of the membrane.

From the results presented in Table 1 it can be seen that the T_c of Na,K-ATPase was not altered due to food or protein restriction. The activation energies (E_a) calculated from the slopes of the curves using the Arrhenius equation were not different above the T_c but below the T_c the activation energy in PR rats was higher compared with the value in the control. In ER also the activation energy was higher, but not statistically so.

Table 1. Critical temperature (T_c) and activation energies (E_a) of kidney microsomal Na, K-ATPase from PEM rats.

Group	<i>N</i>	T_c (°C)	E_a (> T_c)	E_a (< T_c)
			(Kcal/mol)	
C	6	17.7±0.50	4.2±0.34	22.7±1.19
ER	5	17.0±0.80	4.6±0.50	25.2±1.37
PR	5	17.2±0.40	4.4±0.50	26.4±1.40*

Values are means $\pm$ SEM. T_c and E_a are calculated from the Arrhenius plots drawn from the mean values of activities. *N*, Number of rats in each group; * $p < 0.05$ vs. C.

Table 2. Kinetic constants of kidney microsomal Na,K-ATPase in PEM rats.

Group	Na^+			K^+			ATP	
	V_{max}^a	K_m (mM)	V_{max}/K_m	V_{max}^a	K_m (mM)	V_{max}/K_m	K_m (mM)	Hill coefficient (ΔH)
C	15.3±1.00 (9)	8.1±1.64	1.897	25.6±0.83 (6)	3.6±0.83	7.114	2.1±0.04 (6)	1.6±0.06
ER	18.8±2.00 (10)	12.2±1.30	1.528	31.6±3.40 (6)	3.8±0.65	8.412	1.6±0.03‡ (6)	1.6±0.02
PR	20.8±1.56 (10)†	8.6±0.30	2.434	29.0±0.80 (5)	2.4±0.29	11.930	1.6±0.05‡ (6)	1.6±0.06

Values are means $\pm$ SEM. Numbers in parentheses indicate the number of rats in each group.

^a $\mu\text{mol Pi/h/mg}$ protein. Apparent K_m 's for Na^+ and K^+ were derived from Lineweaver-Burk plots. K_m for ATP was calculated from Hill plots. V_{max}/K_m were calculated from mean values of V_{max} and K_m . † $p < 0.01$ vs. C; ‡ $p < 0.001$ vs. C.

Table 3. Effect of vanadate on the kinetic constants of kidney microsomal Na,K-ATPase from PEM rats using Na⁺ as substrate.

Group	N	No vanadate			Vanadate (1 μ M)		
		K_m Na ⁺ (mM)	V_{max}^a	V_{max}/K_m	K_m Na ⁺ (mM)	V_{max}^a	V_{max}/K_m
C	9	8.1 $\pm$ 1.20	15.3 $\pm$ 1.00	2.0 $\pm$ 0.23	7.2 $\pm$ 1.13	6.1 $\pm$ 0.09 ^b	0.9 $\pm$ 0.14 ^b
ER	10	12.3 $\pm$ 1.34	18.8 $\pm$ 2.01	1.7 $\pm$ 0.28	17.9 $\pm$ 3.80*	8.2 $\pm$ 1.14 ^b	0.5 $\pm$ 0.09 ^{*,b}
PR	10	8.6 $\pm$ 0.31	20.3 $\pm$ 15.7 $\dagger$	2.8 $\pm$ 0.45	12.9 $\pm$ 1.00 $\ddagger$	8.3 $\pm$ 0.36 ^b	0.7 $\pm$ 0.09 ^b

Values are means $\pm$ SEM. ^a μ mol Pi/h/mg protein; ^b $p < 0.002$ vs. no vanadate; * $p < 0.02$ vs. C; $\dagger$ $p < 0.01$ vs. C; $\ddagger$ $p < 0.002$ vs. C.

With Na⁺ and K⁺ the enzyme followed Michaelis Menten kinetics and could be fitted in the Lineweaver Burk plots. With ATP, the enzyme gave a sigmoidal curve and was linearised by Hill plots, and Hill coefficients were determined from the slopes of these plots.

As shown in Table 2, the apparent K_m 's of Na,K-ATPase for ATP as calculated from the Hill plots were significantly lower in both ER and PR rats compared with that of the control, indicating an increased affinity of the enzyme for ATP in these two groups. However, the Hill coefficients were not altered. On the other hand the K_m values for K⁺ and Na⁺ did not change due to PR but the affinity for Na⁺ was found to be lower in ER rats. The V_{max} with both Na⁺ and K⁺ were higher in ER and PR rats though a statistically significant difference was observed only with Na⁺.

Vanadate showed a concentration-dependent inhibition of the enzyme activity from all the three groups. However, when tested at different concentrations of Na⁺, vanadium behaved as a noncompetitive inhibitor of Na,K-ATPase from control animals, whereas toward the enzymes from the experimental rats, it behaved as a mixed type of inhibitor; i.e., K_m was increased, but V_{max} was decreased (Table 3). The extent of inhibition (percentage wise) of V_{max} by vanadium was not much different among the three groups. However V_{max}/K_m , which broadly reflects physiological capacity of the system, was more affected by vanadium in ER (71%) and PR rats (76%) than in the controls on a normal diet (55%).

DISCUSSION

Na,K-ATPase activity has been shown to be altered in many pathological conditions [18–21], and in most of these cases the alteration is reversible and returns to normal after treatment. Such alterations may result from changes in the membrane microenvironment or from modifications in enzyme kinetics itself, though enhanced synthesis of enzymes cannot be ruled out.

It has been well established that the activity of Na,K-ATPase is influenced greatly by the physical state of the membrane lipids, which in turn affect the fluidity of the membrane [4, 22]. A very important tool for studying membrane

fluidity is the Arrhenius plot of Na,K-ATPase [23]. Most membrane-bound enzymes show a transition temperature (T_e) in their Arrhenius plots. This has been shown to be not due to any effect of temperature on the protein moiety of the enzyme, but rather to changes in the physical properties of membrane lipids surrounding and interacting with the enzyme. An inherent change in the physical state of the lipids shifts the T_e away from the normal value. The present experiment did not show any such change in the T_e among the groups indicating that the fluidity of the membrane is not much affected and thus may not be responsible for the observed increase in enzyme activity due to food restriction. Though a slight increase in the activation energies below the T_e was observed in ER and PR groups, this may not reflect any significant changes in the lipid microenvironment of the enzyme.

Kinetic studies of the enzyme with Na^+ , K^+ , and ATP as substrates showed a significant increase in the affinity for ATP with enzyme from both ER and PR groups. In erythrocytes of kwashiorkor children, on the other hand, we had observed an increase in the affinity for Na^+ [9]. There is evidence for more than one ATP binding site per molecule of the enzyme. Hill coefficients (ΔH) calculated from Hill plots with ATP as substrate further strengthen this. However, there were no differences in the ΔH values among the three groups. The increase in affinity for ATP in PEM group suggests that there may be an enhancement in the phosphorylation step of the two-step mechanism of enzyme reaction as proposed by Albers and Post *et al.* [24, 25].

Vanadate potently inhibits Na,K-ATPase from the cytoplasmic side of the membrane. The extent of inhibition thus depends on the membrane permeability to vanadate [26]. It stabilizes the pump in the E_2K configuration and slows the conformational change of $\text{E}_2\text{K} \rightarrow \text{E}_1\text{K}$ [27].

More importantly, Patrick has implicated vanadate to play an important role in edema formation in kwashiorkor [28]. In the light of observations made by Day and colleagues [29] that vanadate inhibits the extrusion of Na^+ into the basolateral spaces of the kidney tubule, it was suggested that vanadate might be the factor that may finally end the controversy surrounding the pathogenesis of edema [30].

Bond *et al.* on the other hand, have shown that at low Na^+ concentrations Na,K-ATPase is extremely sensitive to vanadate. At higher concentrations Na^+ occupies the low-affinity site and reverses the inhibition of Na,K-ATPase by vanadate competitively [31]. In the present experiment also, vanadate had a greater inhibitory effect on the enzyme at low than at high Na^+ concentrations (data not presented). Moreover, the different effect of vanadate on the enzyme from the three groups points to membrane modification on the inner side, i.e., the vanadium binding site.

The above experiments thus point out that the observed changes in enzyme activity in PEM rats may not be due to changes in membrane fluidity as was anticipated, but rather due to slight modification on the inner side of the membrane

leading to changes in enzyme kinetics with ATP. This probably enhances the phosphorylation step of Na,K-ATPase which is supposed to be rate-limiting. Observations on the vanadate effect further strengthen this assumption.

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