Sensitive Assay and Kinetic Property of Urinary Inhibitor of Na^+ , K^+ -ATPase in Sodium-loaded Patients with Essential Hypertension

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A sensitive assay method to evaluate the inhibitor of Na^+ , K^+ -ATPase in human urine was developed by measuring the inorganic phosphate liberated from ATP in vitro using Na^+ , K^+ -ATPase from porcine cerebral cortex. Ouabain inhibited the Na^+ , K^+ -ATPase by competing with the potassium ion (an apparent $K_i = 2.6 \pm 0.89 \times 10^{-8} \text{ M}$, $n = 8$) under the condition of 100 mM NaCl, 4.5 mM MgSO_4 and 0.56 mM ATP. The apparent K_m value of KCl was 0.4 mM. Factors inhibiting Na^+ , K^+ -ATPase were detected in the post-salt fraction on Sephadex G-15 chromatography following the ethanol extraction of lyophilized fresh urine of sodium loaded human subjects (300 meq Na^+ /day, for 4 days) with essential hypertension. Two active fractions around the 400 daltons following salt were eluted on Sephadex G-15 chromatography. The slower eluted factor competed kinetically with potassium ion, but the inhibitory activity was lost within two days during storage at 4°C . The faster-eluted inhibitor lost its activity within a day. These results indicate that the unstable inhibiting factors of Na^+ , K^+ -ATPase exist in human urine and one of these factors inhibits ouabain sensitive Na^+ , K^+ -ATPase by binding to the potassium binding site (or very close to it), which exists at the outer surface of the cell membrane of this enzyme.

IT has been postulated by Haddy and Overbeck¹ that the participation of the circulating natriuretic factor, which inhibits Na^+ , K^+ -ATPase, in the pathogenesis of essential hypertension is enhanced with sodium-loading. From this point of view, the circulating natriuretic factor or the endogenous digitalis-like factor has been studied in many laboratories.^{2,3} This factor is thought to be of low molecular weight (< 500), stable and very polar.²

Gruber et al. reported that this factor in the plasma of volume-expanded dogs inhibited the Na^+ , K^+ -ATPase and cross-reacted with the goat

antidigoxin antibody,⁴ thus designating this endogenous digitalis-like substance to endoxin. On the other hand, Hamlyn et al.⁵ observed that the circulating inhibitor of Na^+ , K^+ -ATPase increased in plasma of patients with essential hypertension when compared with normotensive subjects; but the digitalis-like substance reacting with the anti-digoxin antibody did not increase in essential hypertension. Although the existence of the factor binding to the anti-digoxin antibody is controversial, these results suggest that at least the Na^+ , K^+ -ATPase inhibitor may exist in urine of the volume-expanded dog or in the plasma of essential hypertensive patients.

In this paper, we studied the inhibitor of Na^+ , K^+ -ATPase in the urine of sodium-loaded patients with essential hypertension by a sensitive assay method. In addition, the kinetic property of

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Fig. 1. Double reciprocal plots of Na^+ , K^+ -ATPase activity and the KCl concentration in the presence ($5 \times 10^{-7} \text{ M}$, open circle) and absence (closed circle) of ouabain.

Fig. 2. Sephadex G-15 fractionation of urine extracts.

this inhibitor was also studied to clarify the inhibiting mechanism in Na^+ , K^+ -ATPase system.

MATERIALS AND METHODS

Chemicals: Na^+ , K^+ -ATPase from porcine cerebral cortex and vanadium-free ATP disodium salt were obtained from Sigma Co. Ammonium molybdate was purchased from Merck Co. Other reagents were obtained from Wako Pure Chemical Co., Osaka, Japan.

Preparation of urinary inhibitor of Na^+ , K^+ -ATPase: Fresh urine from sodium-loaded patient with essential hypertension (300 meq Na^+ /day, for 4 days) was lyophilized and the resulting powder was extracted with ethanol (10 ml ethanol/100 ml of original urine) to avoid the concomitant extraction of large amount of NaCl

Fig. 3. An apparent molecular weight and an elution volume on Sephadex G-15.

and other salts. The extracted ethanol solution was evaporated to dryness under vacuum at below 40°C . The brown amorphous solid was dissolved in one ml H_2O and applied on Sephadex G-15 chromatography and eluted with H_2O .

Assay of Na^+ , K^+ -ATPase: The reaction mixture (2.7 ml) contains 40 mM TES- NaOH buffer pH 7.4, 100 mM NaCl , 4.5 mM MgSO_4 , 5.0 mM EGTA, 0.02% n-octyl- β -D-glucoside, 0.56 mM ATP, 0.025 unit of Na^+ , K^+ -ATPase and various concentrations of KCl . For the screening assay of Na^+ , K^+ -ATPase inhibitor, 0.1 mM KCl was used. Porcine Na^+ , K^+ -ATPase (5 units) was dissolved in 5 ml of TES buffer (pH 7.4). This solution was ultrasonified by Sonifier B-12 (Branson sonic power company, Danbury, Connecticut, USA) at 100 watts for 15 seconds under ice-bath cooling to prepare a fine micelle solution.

The enzyme reaction was started by the addition of ATP at 25°C . After standing 10 min, 0.03 ml of 1% Triton X-100 and 0.3 ml of 2.5% ammonium molybdate solution were added. The absorbance at 660 nm was measured after 10 min at 25°C . Under this assay condition, the substrate ATP did not disturb the estimation of inorganic phosphate. But by changing the above condition, i.e. increasing the ATP concentration, developing time and developing temperature, ATP itself disturbed the assay of inorganic phosphate as false positive.

RESULTS

The activation of Na^+ , K^+ -ATPase by K^+ ion

Fig. 4. Double reciprocal plots of Na^+ , K^+ -ATPase activity and the KCl concentration in the presence (open circle) and the absence (closed circle) of the slower eluted fraction on Sephadex G-15. The sample volume used for each assay was 0.135 ml.

Fig. 5. Change of inhibitory activity of the slower eluted fraction on Sephadex G-15 during storage at 4°C . Curve A is a theoretical inhibition curve of slower-eluted fraction assayed on the same day as a function of a potassium ion. Curve B is that of the same fraction assayed after 24 hours.

followed the Michaelis-Menten form at fixed concentrations of NaCl (100 mM), MgSO_4 (4.5 mM) and ATP (0.56 mM). The concentration of KCl required for a half maximal rate ATP hydrolysis (an apparent K_m value) was $4 \times 10^{-4} \text{ M}$ by the Lineweaver-Burk plot (Fig. 1). Ouabain showed the competitive inhibition with the potassium ion and the apparent K_i value of ouabain was $2.6 \pm 0.89 \times 10^{-8} \text{ M}$ (Fig. 1).

A crude ethanol extract was obtained from 1 liter of fresh urine as described in the methods and the evaporated residues were dissolved in 10 ml water. A typical fractionation pattern on Sephadex G-15 of this solution is shown in Fig. 2. The two fractions inhibiting Na^+ ,

K^+ -ATPase inhibitor were observed following salt fraction. These fractions were eluted between the fractions of FAD and picric acid used as standard compounds (Fig. 3).

The kinetics of the slower-eluted inhibitor of Na^+ , K^+ -ATPase was studied with respect to the concentration of K^+ . At fixed concentrations of Na^+ (100 mM) and ATP (0.56 mM), a higher K^+ concentration was required for the half maximal rate of action after an addition of post-salt fraction. The inhibition mechanism of Na^+ , K^+ -ATPase by the urinary inhibitor of Na^+ , K^+ -ATPase was a competitive type (Fig. 4). This inhibitory factor was unstable and 56% of the inhibitory activity was lost within a day by measuring the inhibitory activity at 0.1 mM KCl (Fig. 5) and was lost completely after 48 hours during storage at 4°C . On the other hand, the activity of the faster-eluted inhibitor of Na^+ , K^+ -ATPase inhibitor had completely disappeared within a day by storage at 4°C .

DISCUSSION

An endogenous inhibitor of Na^+ , K^+ -ATPase was detected by measuring the inhibition of Na^+ , K^+ -ATPase activity. The assay method of Na^+ , K^+ -ATPase used in this study was very sensitive and satisfied the conditions necessary to study the kinetics of the Na^+ , K^+ -ATPase inhibitor. The apparent K_m value of KCl was $4 \times 10^{-4} \text{ M}$ which is the same value reported by Post and Sen⁶. The apparent K_i value of ouabain was $2.6 \times 10^{-8} \text{ M}$.

The two Na^+ , K^+ -ATPase inhibitory factors were detected following salt fraction on Sephadex G-15 chromatography. The inhibitory activity was very labile even with the storage at -70°C .

The factor inhibited the Na^+ , K^+ -ATPase by the competition with potassium ion. This is very important, because the potassium binding site of the Na^+ , K^+ -ATPase is present at the outer surface of the cell membrane and this may facilitate the binding of the endogenously circulating Na^+ , K^+ -ATPase inhibitor to the Na^+ , K^+ -ATPase molecule. Vanadium ion is also a potent inhibitor of Na^+ , K^+ -ATPase, but the vandadium ion is a competitive inhibitor of Na^+ ion. This may indicate that the vanadium ion must enter into the cell to inhibit the Na^+ , K^+ -ATPase in vivo.

For the in vitro assay of Na^+ , K^+ -ATPase

inhibitor, the results indicate that as the concentrations of potassium ion become lower, the inhibiting rates of the Na^+ , K^+ -ATPase by the inhibitor become greater (Fig. 4 and 5). The concentration of KCl for the detection of Na^+ , K^+ -ATPase inhibitor using Na^+ , K^+ -ATPase is usually used at 10 or 20 mM by investigators. This concentration is too high when compared with the K_m level (0.4 mM) of KCl as in our experiment. These results indicate that the concentration of potassium ion for the screening assay of urinary inhibitor of Na^+ , K^+ -ATPase should be below K_m level to magnify the inhibiting rate and to avoid the false negative data by high concentrations of KCl.

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