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SODIUM TRANSPORT DEFECT OF OUABAIN-RESISTANT RENAL Na,K-ATPase

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The murine renal Na,K-ATPase is resistant to cardiac glycosides. It is not yet known however whether altered active transport is associated with the drug-resistance. To investigate this problem Na,K-ATPases were purified from the outer medulla of both rat and rabbit kidneys and reconstituted identically into liposomes. The Na-stimulation of the Na,K-ATPase activity before reconstitution and of the Na-transport after reconstitution was measured. A Na-defect inherent in the ouabain-resistant rat Na,K-ATPase was discovered indicating a link between the cardiac glycoside sensitivity and the Na-transport.

The development of resistance to drugs and toxins is a fundamental principle in nature. Na,K-ATPase (EC 3.6.1.37), the membrane transport system responsible for creating and stabilizing the vital transmembrane Na/K-gradient, carries a receptor (1,2) for cardiac glycosides with widely variable sensitivity to these highly toxic drugs (3-6). The physiological significance of this variability, however, is not yet understood. To answer this question, it is essential to know whether the variable receptor sensitivity is associated with a change in the Na,K-exchange process.

In the present study we wished to check whether a change in active transport is associated with the ouabain-resistance of the alpha-subunit. Purified renal Na,K-ATPase was used to rule out any influence of intracellular proteins. The active Na-transport of the ouabain-resistant rat enzyme was compared to the Na-transport of the ouabain-sensitive rabbit enzyme after identical reconstitution into liposomes. We discovered a Na-transport defect typ-

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ical for the ouabain-resistant rat Na,K-ATPase. Our results reveal a link between the cardiac glycoside affinity and the active Na-transport.

MATERIALS AND METHODS

Na,K-ATPase purification

The enzyme was purified by a slightly modified version of the $DodSO_4$ extraction (7) procedure: 12.6 mg of microsomal protein was incubated in 7 ml of 3 mM Na_3ATP, 25 mM imidazole, 1 mM Na_2EDTA, 2.5 mM lithium-$DodSO_4$ (Serva) for 20 min at 25°C. The solution was then put on 4 ml 15% sucrose, 16 ml 25% sucrose, 25 mM imidazole, 1 mM Tris-EDTA (Merck, Titriplex II neutralized with Tris), pH 7.5, 0°C, and centrifuged for 110 min at 48 000 x g. The pellet was suspended in 0.5 ml 1% sucrose, 25 mM imidazole, 1 mM Tris-EDTA, pH 7.2 and put at -70°C. Protein was determined by the Pierce BCA assay (Rockford, USA).

Na,K-ATPase activity

The Na-dependent ADP production was measured by the linked enzyme-assay at 25 or 37°C as follows: 0.5 to 2 µg Na,K-ATPase protein was added to 1 ml of 0.3 mM NADH (grade I, Boehringer), 2.5 mM phosphoenolpyruvate(cyclohexamine)-Tris (Sigma), 8 µl pyruvate-kinase/lactate-dehydrogenase (Boehringer), 30 mM imidazole (Merck), 1 mM Tris-EDTA, 2.5 mM Tris-ATP (prepared according to ref.8 from Na_3ATP grade I, Boehringer), 5 mM $MgCl_2$, 10 or 50 mM KCl (suprapure, Merck), pH 7.2. Increased NaCl (suprapure, Merck) concentrations were added. The oxidation rate of NADH was recorded at 340 nm simultaneously in 4-6 cuvettes in a automated enzyme kinetic accessory using both a Philips Unicam SP180 and a Varian Cary 210 spectrophotometer. One oxidized NADH molecule reflects the production of 1 ADP molecule, i.e., the rate of ATP hydrolysis by Na,K-ATPase.

Reconstitution of Na,K-ATPase into liposomes

Na,K-ATPase (180 µg protein) was suspended in 60 µl of 30 mM histidine (Fluka), 1 mM Tris-EDTA, 5 mM $MgCl_2$, 50 mM RbCl (suprapure, Merck), 23 mM cholic acid (Merck), pH 7.2, 0°C. The supernatant resulting from a 10 min spin at 100 000 x g (Beckman, Airfuge) was added to 50 µl of 5 mM $MgCl_2$, 30 mM histidine, 1 mM Tris-EDTA, 23 mM cholic acid, 0.8 mg phosphatidylcholine (grade I, Lipid Products, Nutfield, U.K.), 0.2 mg phosphatidylserine (Lipid Products), pH 7.2, 0°C. Liposomes were formed by 15 h dialysis at 0°C (10) in 250 ml of 50 mM KCl, 5 mM $MgCl_2$, 1 mM Tris-EDTA, 30 mM histidine, pH 7.2. Two different rat and two different rabbit enzyme preparations of similar purity and specific acitivity were reconstituted identically.

Transport measurement

Three µl aliquots of liposomes were incubated at 25°C with 3 µl of 50 mM KCl, 5 mM $MgCl_2$, 30 mM histidine, 1 mM Tris-EDTA, 9 kBq ^{22}Na (Amersham), 1-100 mM NaCl, pH 7.2, with or without 10 mM Tris-ATP. The ^{22}Na uptake was stopped by adding 120 µl of 50 mM KCl, 30 mM imidazole, 1 mM Tris-EDTA, pH 7.2, 0°C (stop-solution) to the liposomes. The external isotope was removed in a Sephadex G-50 medium (Sigma) column (1 x 20 cm) at 0°C in stop-solution; the washed liposomes were collected within the first 10 min at a flow rate of 0.8 ml/min. The ^{22}Na content of the liposomes was determined by beta scintillation counting in Aquasure (Kontron).

RESULTS

The primary function of renal Na,K-ATPase is the transepithelial transport of Na ions; hence, the Na-transport is the first parameter to examine in looking for putative functional changes caused by ouabain-resistance. The Na-dependence both of the active Na-transport and of the Na,K-ATPase activity was measured.

A Na-defect of the ouabain-resistant rat Na,K-ATPase activity was discoverd in comparison to the ouabain-sensitive rabbit kidney enzyme (Table I). The low Na-affinity characteristic for the rat enzyme was manifest only below 50 mM NaCl; there, the activity of the rat enzyme decreased more sharply and was only 55% of the rabbit enzyme at 1 mM NaCl (Table I). This Na-defect is not dependent on the absolute values of the specific Na,K-ATPase activity. The values shown in Table I were obtained with a rat enzyme of higher activity than rabbit enzyme. In other experiments a rat enzyme of lower specific activity (1780 μmol/mg per h) was compared to a

Table I. Na-defect of ouabain-resistant renal Na,K-ATPase before reconstitution

[Na] mM	Na,K-ATPase activity (% of maximum)		
	Rabbit	Rat	Rat/rabbit
1	5.1 ± 0.1	2.8 ± 0.1	0.55
5	36.2 ± 0.6	22.4 ± 0.7	0.62
10	60.5 ± 1.1	50.6 ± 0.8	0.84
50	94.6 ± 1.4	94.8 ± 3.0	1.0
100	100	100	1.0

The enzyme activities of purified rabbit and rat Na,K-ATPase were compared at 37°C in 10 mM KCl, 5 mM $MgCl_2$ at increased NaCl concentrations by the linked enzyme assay described in Methods. The maximal Na,K-ATPase activity (at 100 mM NaCl) of the rabbit and of the rat enzyme was set at 100%. The Na,K-ATPase activity of both enzymes was determined simultaneously (n = 8 at 1 mM Na and n = 6 at 5-100 mM Na). The level of significance of the difference between rat and rabbit was <0.001 at 1-10 mM Na. The specific Na,K-ATPase activity at 100 mM NaCl was 1393 ± 24 μmol/h per mg for the rabbit and 1594 ± 56 μmol/h per mg for the rat enzyme (SE; n=6). The ouabain-insensitive component was below 2% for both rabbit and rat at 50 μM or 1 mM (rat) ouabain.

rabbit enzyme of higher activity (2200 μmol/mg per h). The resulting rat/rabbit ratios (not shown) were very close to the results of Table I. The Na,K-ATPase activity of rat and rabbit enzymes was determined also in the conditions used for active transport experiments in liposomes, i.e., 25°C instead of 37°C, 50 mM RbCl instead of 10 mM KCl; again, values very similar to Table I were found (not shown).

To determine whether the Na-defect of the Na,K-ATPase activity plays a role in active transport, rat and rabbit Na,K-ATPases were incorporated identically into liposomes and the ATP-induced ^{22}Na-uptake at 1 mM NaCl was measured. Fig. 1 shows that the ATP-induced Na-uptake by the rat enzyme was only 30% of rabbit. The ^{22}Na-uptake in the absence of ATP was close to background in both preparations (not shown). Thus, the Na,K-ATPase defect seen before reconstitution is expressed as a Na-transport defect of similar size after reconstitution of the enzymes into liposomes. The close agreement of the values before and after reconstitution indicates that the Na-defect is solidly anchored in the ouabain-resistant

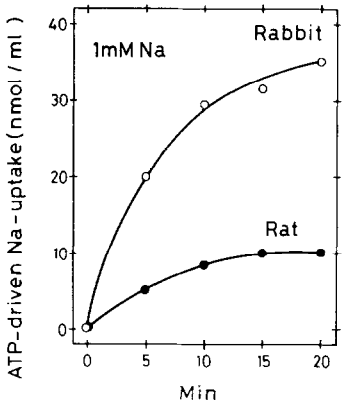

Figure 1. **Na-transport defect of ouabain-resistant Na,K-ATPase after reconstitution.** Na,K-ATPase purified from rabbit (○) and rat (●) kidney with specific activities of 1600 and 2000 μmol/mg per h respectively were reconstituted identically into liposomes. The active ^{22}Na-uptake (○,●) was started by the addition of 1 mM external ^{22}NaCl and 10 mM Tris-ATP at 25°C. No ^{22}Na entered in the absence of ATP within 30 min. The ^{22}Na-uptake was measured by the gel filtration procedure at the times shown. Experimental details are described in Methods.

Table II. *Na-transport defect of ouabain-resistant renal Na,K-ATPase after reconstitution*

[Na] mM	^{22}Na-uptake (% of maximum) Rabbit	Rat	Rat/rabbit
1	1.0 ± 0.1	0.5 ± 0.1	0.50
5	7.8 ± 1.1	4.5 ± 0.3	0.58
10	13.9 ± 1.1	12.0 ± 1.6	0.86
100	100	100	1.0

Liposomes were formed identically with rabbit (1400 μmol/mg per h) and rat enzyme (1780 μmol/mg per h) in a buffered solution containing 50 mM RbCl and 5 mM $MgCl_2$. The active ^{22}Na-upake rates were determined after 2 min incubation in the presence of 10 mM external Tris-ATP at 25°C. No ^{22}Na entered in the absence of ATP. Rat and rabbit liposomes were processed in parallel. The results are from two rat and two rabbit liposomes preparations; n = 5 at 100 mM Na; n = 3 at 1-10 mM Na. The technology for reconstitution of Na,K-ATPase and for transport assays is described in Methods.

renal Na,K-ATPase; if not, it would be altered by the reconstitution procedure which involves intense detergent and lipid treatment of the enzyme.

To check whether the Na-transport defect is also attenuated at Na concentrations above 50 mM in analogy to the Na,K-ATPase defect shown in Table I, we determined initial ^{22}Na-uptake veloci-

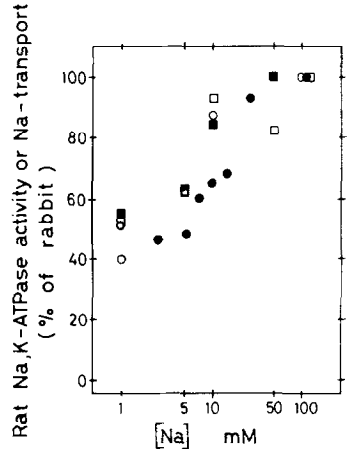

Figure 2. **Na-defect of the ouabain-resistant rat kidney Na,K-ATPase before and after reconstitution.** Na,K-ATPase activity before reconstitution (●,■) and Na-transport after reconstitution (○,□) were measured at increased Na concentrations as described under Tables I and II. The Na,K-ATPase activity and Na-transport of rat enzymes of 2000 (●,○) and 1780 μmol/mg per h activity (■,□) are expressed in % of the rabbit enzymes processed in parallel (1600 and 1400 activity respectively) after normalization of the maximal transport and enzyme activites (at 100 mM NaCl) to 100%.

ties at increasing Na concentrations. The results in Table II show that the rat/rabbit transport ratio rose from 0.5 (at 1 mM Na) to 1.0 (at 100 mM Na) in perfect agreement with the effect of increasing Na concentrations on the rat/rabbit ratio of the enzyme activity (Table I).

Fig. 2 illustrates that the defective Na-transport after reconstitution of the rat kidney enzyme corresponds to the impaired Na,K-ATPase activity before reconstitution. The defect is around 50 % at 1 mM NaCl and then the rat enzyme gradually approaches the rabbit enzyme by a sudden rise in the Na-transport and in the Na,K-ATPase activity around 10 mM NaCl (Fig. 2). Thus, the same Na-defect appears at different specific Na,K-ATPase activities, at 25 or at 37°C, at 10 mM, 50 mM KCl or 50 mM RbCl, and before and after reconstitution of the enzyme into liposomes. The stability of the defect infers that it is inherent in the rat enzyme.

Purified rat and dog kidney Na,K-ATPases have been compared before with regard to their affinities for Na, K and ATP but no difference was found because the authors did not look specifically for a difference in the Na-affinity below 10 mM Na (10). Indeed, an ATP-regenerating incubation medium and a high resolution spectrophotometer for continuous recording of the linear enzymatic activity rate is an absolute requirement for a precise and reproducible comparison of the minimal enzyme activity (20 to 40 times reduced at 1 mM Na) with the maximal activity seen at 100 mM Na. In contrast, the active ^{22}Na-uptake into liposomes is better measured in low turnover conditions, namely, at 25°C with 1 mM $^{22}NaCl$ of high specific radioactivity; long-lasting linear transport rates are then observable.

DISCUSSION

The ouabain-resistance of the rat kidney enzyme is located in the alpha subunit of the Na,K-ATPase molecule as demonstrated

by hybridization studies of alpha and beta subunits from resistant and sensitive species (11,12). The responsible region has been localized (by site-directed mutagenesis) in the primary sequence of the alpha subunit (13). Substitution of glutamine-111 by arginine and of asparginine-122 by aspartic acid at the two lipid boundaries of the extracellular H1-H2 loop is sufficient to confer ouabain-resistance to the ouabain-sensitive sheep kidney enzyme. Price and Lingrel (13) propose that ouabain can still bind to the unchanged extracellular H3-H4 region but that its release is facilitated by the additional charges on the H1-H2 loop of the alpha subunit. Thus, the sensitivity of Na,K-ATPase seems to be controlled by the release-rate of cardiac glycosides. The release-rate, in turn, is supposed to depend on a critical conformational change in the H1-H2 region (13). The same altered conformational change may be responsible for the specific Na-transport defect we are describing in the present work. In fact, several distinct antigenic sites have been detected by immunochemical comparison of the ouabain-sensitive lamb kidney with the ouabain-resistant rat kidney enzyme before denaturation by dodecyl sulfate (14). As the antigenic sites are located on the intracellular protein protrusion, the three-dimensional structure of this part of the ouabain-resistant alpha-subunit must be altered too. Thus, the presence of charged amino acids at the lipid-protein boundary seems to modify not only the membrane insertion of the rat kidney alpha subunit but also the intracellular protein folding. A minor spatial displacement of the Na-binding region may be sufficient to account for the Na-defect described herein.

In the present work the ouabain-resistant renal Na,K-ATPase was compared to the ouabain-sensitive enzyme; both are coded by the alpha-1 gene (13). Na,K-ATPase alpha-2 and alpha-3 isoforms with higher ouabain-sensitivities have been detected in non-renal

tissues (6). Recently, however, Na,K-ATPases with various ouabain-affinities have been found in the collecting tubule of isolated rabbit nephrons suggesting that the kidney may contain isoforms too (15). Indeed, the renal alpha-subunit can also be resolved into a doublet if tetradecyl sulfate is added to the solutions used for the gel electrophoresis (16); such alpha-heterogeneity is an empirical test for the presence of Na,K-ATPase isoforms (6). On the other hand, Na,K-ATPase isoforms with low ouabain-affinity have also lowered Na-affinity (6,17). We show herein that two renal Na,K-ATPases both of alpha-1 origin but with distinct ouabain-affinities transport Na-ions differently. The results demonstrate a link between the cardiac glycoside sensitivity and the active Na-transport of renal Na,K-ATPase.

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