ISSN 0006-2979, Biochemistry (Moscow), 2008, Vol. 73, No. 9, pp. 990-994. © Pleiades Publishing, Ltd., 2008.
Published in Russian in Biokhimiya, 2008, Vol. 73, No. 9, pp. 1230-1235.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM07-383, July 6, 2008.

Effect of Colchicine on Sensitivity of Duck Salt Gland Na,K-ATPase to Na^+

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Received November 26, 2007
Revision received February 27, 2008

Abstract—Low molecular mass proteins of the FXYD family that affect the sensitivity of Na,K-ATPase to Na^+ and K^+ are known to be present in Na,K-ATPases in various tissues. In particular, in Na,K-ATPase from kidney a γ-subunit (with electrophoretic mobility corresponding to molecular mass of about 10 kD) is present, and Na,K-ATPase preparations from heart contain phospholemman (electrophoretic mobility of this protein corresponds to molecular mass of 13-14 kD), which provides for the interaction of heart Na,K-ATPase with cytoskeletal microtubules. Disruption of microtubules by colchicine removes phospholemman from heart Na,K-ATPase preparations. The goal of the present study was to reveal a low molecular mass protein (probably a member of FXYD family) in preparation of Na,K-ATPase from duck salt glands. Immunoprecipitation of solubilized duck salt gland Na,K-ATPase using antibodies against α1-subunit results in the coprecipitation of a 13 kD protein with the Na,K-ATPase complex. Treatment of homogenate from duck salt glands with colchicine removes this protein from the purified preparation of Na,K-ATPase. Simultaneously, we observed a decrease in the sensitivity of Na,K-ATPase to Na^+ at pH 6.5. However, colchicine treatment of homogenate from rabbit kidney does not affect either the sensitivity of Na,K-ATPase obtained from this homogenate to Na^+ or the content of 10 kD protein (presumably γ-subunit). The data suggest that phospholemman (or a similar member of the FXYD family) tightly interacts with Na,K-ATPase from duck salt glands and binds it to microtubules, simultaneously participating in the regulation of the sensitivity of Na,K-ATPase to Na^+.

DOI: 10.1134/S0006297908090058

Key words: Na,K-ATPase, colchicine, FXYD family proteins, duck salt glands

Na,K-ATPase (the Na-pump) is an enzyme of plasma membrane responsible for Na^+ and K^+ transport across the membrane against their electrochemical gradients. The enzyme consists of catalytic α-subunit (molecular mass of about 100 kD) that provides for ATP hydrolysis and cation transport [1], and glycosylated β-subunit with molecular mass of about 55-65 kD (molecular mass of protein part is about 35 kD) that is necessary for the delivery and correct embedding of α-subunit into the plasma membrane, and also for protecting of α-subunit from the influence of external factors [2]. Beside this, β-subunit was identified as a factor of adhesion of glial cells [3]. Both subunits are integral membrane proteins and are found in molar ration 1 : 1 in purified preparations of Na,K-ATPase [4]. α- and β-subunits are present in several isoforms that are characterized by different distribution in different tissues and can combine with each other in different variants [2, 5].

γ-Subunit with molecular mass of about 7 kD (its electrophoretic mobility corresponds to that for a protein with molecular mass of about 10 kD) was found in preparations of Na,K-ATPase from kidney, where almost solely the α1β1-isoenzyme is represented [6]. The γ-subunit is one of the FXYD family proteins that have in their amino acid sequence this signature peptide. Proteins of the FXYD family have a transmembrane domain and an N-terminal domain exposed to the extracellular medium. Seven proteins from this family are known today, and most of them are represented in preparations of Na,K-ATPase from different sources: specific interaction with Na,K-ATPase was shown for six members of this family [7].

Protein FXYD1 (phospholemman) is a part of Na,K-ATPase occurring in skeletal muscles, heart, and kidney juxtaglomerular apparatus [8]. Phospholemman contains 72 amino acid residues and has electrophoretic mobility corresponding to that for protein with molecular mass of 13-14 kD [9]. It was also found that phospholemman can interact with cytoskeleton microtubules:

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colchicine treatment of heart homogenate results in the release of phospholemman connected to cytoskeleton from the membrane [10].

Na,K-ATPase from kidney contains γ-subunit (FXYD2) existing as two isoforms, γ_a and γ_b, which are created as a result of alternative splicing of one gene and are different in N-terminal amino acid sequence and electrophoretic mobility [11]. Protein FXYD4 (corticosteroid hormone induced factor, CHIF) was found in kidney and colon [12]. Although the FXYD proteins have a self-dependent function (they can form channels after polymerization [13]), after binding with Na,K-ATPase these proteins can modulate the ATPase activity. Mostly it leads to a change in the affinity of Na,K-ATPase to Na^+ and K^+ [14].

The subject of the present study is the Na,K-ATPase from duck salt glands, which like Na,K-ATPase from mammalian kidney is represented by the α1β1-isoenzyme [15]. It is still unknown whether or not FXYD protein occurs in preparations of Na,K-ATPase from duck salt glands. According to data of Martin and Sachs, who analyzed duck salt gland Na,K-ATPase preparation with very high activity (about 2800 μmol P_i/h per mg protein) by Laemmli PAGE, this preparation contains only α- and β-subunits. However, it should be noted that these authors did not pay special attention to low molecular mass proteins of the preparation [16].

The purpose of this study is to reveal: i) whether or not duck salt gland Na,K-ATPase contains a low molecular mass protein with structure similar to that of FXYD proteins and which is removed from the preparation by colchicine treatment, and ii) whether or not such a protein affects the sensitivity of Na,K-ATPase to Na^+.

MATERIALS AND METHODS

ATP (disodium salt) and colchicine were from Fluka (Germany); protease inhibitor cocktail was from Amresco (USA); sucrose, EDTA, SDS, glycine, Tris-HCl, and protein A-agarose were from Sigma-Aldrich (USA); Triton X-100 and imidazole were from Merck (Germany); acrylamide, methylene-bis-acrylamide, and other reagents for PAGE were from Bio-Rad (USA); protein markers for PAGE (10-170 kD) were from Fermentas (Lithuania). Other reagents of analytical grade purity were produced in Russia.

The disodium salt of ATP was converted to the imidazole salt as follows: 1.1 g ATP (disodium salt) was dissolved in 5 ml of distilled water and the solution was applied to a column (V = 6 ml) with Dowex 50WX×8 that was previously converted into the H^+-form. Eluate with pH in the range 2.0-3.5 was collected into 6 ml of cooled 2.5 M imidazole solution. The ATP concentration in the solution was calculated by measurement of its optical density at 257 nm using molar extinction coefficient 14.3 $M^{-1}\cdot cm^{-1}$.

Preparations of Na,K-ATPase from duck salt glands and rabbit kidney were purified as described earlier [17, 18]. The purification procedures of enzymes from both sources include the following steps: i) separation of microsomal fraction from homogenate using differential centrifugation; ii) extraction of admixtures of proteins from microsomal fraction by the treatment of this fraction with a low concentration of SDS at 26°C; iii) separation of extracted proteins by centrifugation of the suspension in a discontinuous sucrose gradient that sediments membrane fraction containing purified Na,K-ATPase. Enzyme preparations were suspended in 20 mM Tris-EDTA buffer including 250 mM sucrose and stored before use at −70°C.

To destroy cytoskeletal microtubules, colchicine was added to homogenate of duck salt glands or rabbit kidney medullar layer (final concentration 1.6 μM) in the presence of a cocktail of proteinase inhibitors, and the mixture was kept for 30 min at 4°C. The enzyme was further purified in the same way as without colchicine treatment. Protein concentration was determined according to method of Lowry and coauthors [19] using BSA as a standard.

Polyacrylamide gel electrophoresis in the presence of SDS was carried out according to the Laemmli method [20] using 3.5% stacking and 10% running gel. Before applying the samples to the gel, they were incubated at 37°C for 5 min in standard Laemmli buffer. After electrophoresis, gels were washed for 1 h with 10% solution of acetic acid with 25% isopropanol and then stained with 0.5% solution of Coomassie Brilliant Blue R-250 prepared using 10% acetic acid and 25% isopropanol. Gels were destained by washing.

To reveal low molecular mass proteins, we used Tricine cathode buffer with 9.6% stacking and spacer gels and 18% running gel [21]. Protein samples were treated as described above. Proteins were visualized using Coomassie Brilliant Blue R-250 or by silver staining.

All steps of silver staining were carried out under constant agitation. Gels were treated for 1 h with solution containing 10% acetic acid and 40% ethanol, washed two times for 1 min with 10% ethanol, and then 5 times for 5 min with distilled water. After that the gels were washed for 2 min with solution containing 0.15% $KFe(CN)_6$, 0.3% $Na_2S_2O_3$, and 0.5% Na_2CO_3, then wash 4 times for 5 min with distilled water and treated for 30 min with 0.1% solution of silver nitrate. Then the gels were washed for 5 min with 2.5% Na_2CO_3 and stained for 5-10 min with 2.5% solution of Na_2CO_3 containing 0.02% formaldehyde. After staining, gels were placed for 5 min in 1% acetic acid solution.

Na,K-ATPase activity was determined by measuring P_i concentration that was increased as the result of ATP hydrolysis. The P_i concentration was measured in accordance with the method of Rathbun and Bethlach [22]. The incubation medium contained 130 mM NaCl,

20 mM KCl, 3 mM ATP (imidazole salt), 3 mM $MgCl_2$, and 30 mM imidazole (pH 7.5). To determine the sensitivity of Na,K-ATPase to Na^+, 3 mM KCl was used, and the NaCl concentration was changed from 0.3 to 25 mM, while the pH was 7.5 or 6.5.

Na,K-ATPase immunoprecipitation was carried out as described earlier [23]. Purified Na,K-ATPase preparation was treated with 1% Triton X-100 dissolved in buffer for the immunoprecipitation that has the following composition: 20 mM Tris-HCl (pH 7.4), 250 mM sucrose, 1 mM dithiothreitol, 2 mM EDTA, and cocktail of protease inhibitors. Then it was centrifuged at 100,000*g* for 1 h, and the pellet was discarded. Protein concentration was measured in the supernatant, and 5 μl of protein A-agarose suspension was added to 10-20 μg of solubilized Na,K-ATPase (protein concentration 5-10 mg/ml) obtained from homogenate of tissues both treated and untreated by colchicine. The mixture was kept with agitation for 1 h at room temperature. After that samples were centrifuged (1500*g*, 5 min), supernatant was separated from pellet, and 20 μl of protein A-agarose and 50 μl of antibodies against Na,K-ATPase α1-subunit were added to the supernatant. Samples were incubated with agitation for 1.5 h at room temperature, and after that they were centrifuged under the same conditions. The pellet was washed 3-5 times with immunoprecipitation buffer and 2 times with 20 mM imidazole solution (pH 7.4). Laemmli buffer (40 μl) was added to the pellet, and the suspension was incubated for 10 min at 80°C. The immunoprecipitate was analyzed by PAGE with Tricine buffer, and the gels were silver stained.

Statistics. Each point on the plots represents a mean value from five measurements. The parameter spread shows the standard deviation.

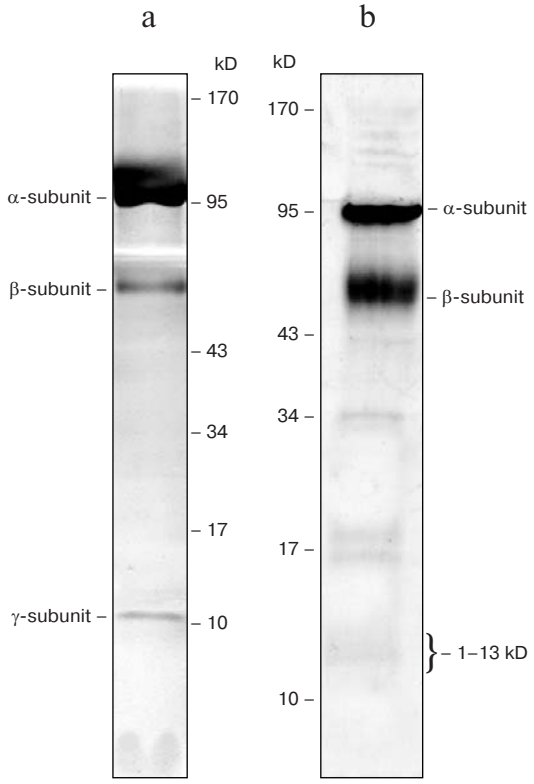

Fig. 1. Electrophoretic analysis of protein composition of preparations of Na,K-ATPase from rabbit kidney (a) and duck salt glands (b). SDS-PAGE was preformed according to the method of Laemmli, and gels were stained with Coomassie Brilliant Blue R-250. Each lane was loaded with 12 μg of protein.

RESULTS AND DISCUSSION

Results of SDS-PAGE analysis of protein compositions of Na,K-ATPase preparations obtained from rabbit kidney and duck salt glands are presented in Fig. 1 (a and b, respectively). It can be seen that besides Na,K-ATPase α- and β-subunits (they are seen on gels as bands corresponding to proteins with molecular mass 100 and 55 kD, respectively) there is a 10 kD protein in the Na,K-ATPase preparation obtained from rabbit kidney that seems to be a γ-subunit of the enzyme (Fig. 1a). Besides α- and β-subunits, a number of other proteins (a 34 kD protein and also several proteins with lower molecular masses, in particular, two proteins with molecular masses of about 17, 13, and 11 kD) are present in the preparation of duck salt gland Na,K-ATPase (Fig. 1b). It should be noted that the Na,K-ATPase preparations from duck salt glands and rabbit kidney whose protein compositions are shown in Fig. 1, had rather high activity (about 1600 and 1800 μmol P_i/h per mg protein, respectively).

Because Na,K-ATPases from duck salt glands and rabbit kidney are of the same isoenzyme (α1β1) [15], we expected the properties of Na,K-ATPases obtained from these sources would be similar. In fact, we observed similar dependence of Na,K-ATPases obtained from duck salt glands and rabbit kidney on the NaCl concentration at pH 7.5 (data not shown). However at pH 6.5 we found differences between the sensitivity of these enzymes to Na^+ (Fig. 2): the values of I_{50} for NaCl were 1.7 mM for duck salt glands Na,K-ATPase (curve *1*) and 5.8 mM for Na,K-ATPase from rabbit kidney (curve *2*). Perhaps the different sensitivity of Na,K-ATPase obtained from these two sources to Na^+ is due to the presence of other regulatory proteins in the duck salt gland Na,K-ATPase. To elucidate what proteins present in duck salt gland Na,K-ATPase tightly interact with complex α1β1, we conducted experiments with immunoprecipitation of solubilized Na,K-ATPase using antibodies against Na,K-ATPase α1-subunit and analysis of protein composition of the immunoprecipitate.

As can be seen from Fig. 3a, which shows the result of electrophoretic analysis of the immunoprecipitate, only four protein bands are seen on the gel. Two of them

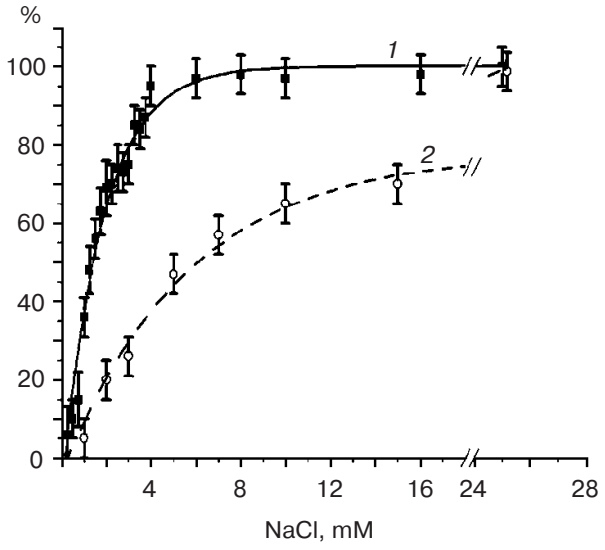

Fig. 2. Dependence of the activity of Na,K-ATPase from duck salt glands (*1*) and rabbit kidney (*2*) on the NaCl concentration. Enzyme activity in the medium with 25 mM NaCl and 10 mM KCl (pH 6.5) was taken as 100% because it did not increase when the NaCl concentration was further increased.

(with molecular mass 50-55 and 25 kD) correspond to heavy and light chains of antibodies that were used for immunoprecipitation. A band corresponding to heavy chains of antibodies overlaps with the band corresponding to the β-subunit of the enzyme. Beside this, there are bands on the gel that correspond to Na,K-ATPase α-subunit (100 kD) and to a protein with molecular mass of about 13 kD. Thus, solubilization of Na,K-ATPase by Triton X-100 results in additional purification of the enzyme, but a protein with molecular mass of about 13 kD that appears to be tightly bound to αβ-complex is retained in the preparation and co-immunoprecipitated with it. The electrophoretic mobility of this protein is similar to that of phospholemman. To reveal whether this protein is connected with the microtubules of cytoskeleton, we conducted immunoprecipitation of Triton X-100-solubilized Na,K-ATPase obtained from homogenate of duck salt glands after its treatment with colchicine. The electrophoretic analysis of the immunoprecipitate is shown in Fig. 3b. It can be seen that immunoprecipitate obtained using antibodies against α1-subunit from solubilized enzyme purified from duck salt glands homogenate after its treatment by colchicine contains almost no protein with molecular mass of about 13 kD (Fig. 3b). Thus, the 13 kD protein is tightly bound with duck salt gland Na,K-ATPase and is removed from purified preparation of this enzyme if the homogenate used as the source for purification of Na,K-ATPase was treated by colchicine.

Colchicine treatment of homogenate led to a decrease in specific activity of Na,K-ATPase in preparations obtained from both sources (duck salt glands and rabbit kidney) by about 20% compared with the control. Colchicine treatment also resulted in a change in sensitivity of duck salt gland Na,K-ATPase to Na^+: the I_{50} value for NaCl increases from 1.7 to 5 mM (Fig. 4); in other words, it becomes close to the value that is characteristic for rabbit kidney Na,K-ATPase. Colchicine treatment of rabbit kidney homogenate did not lead to the removal of a protein with molecular mass of about 10 kD (γ-subunit) from preparation of purified Na,K-ATPase obtained from this source. Also, the treatment of rabbit kidney homogenate by colchicine does not affect the sensitivity of Na,K-ATPase obtained from the homogenate to Na^+, at both pH 7.5 and 6.5 (data not shown).

As above, colchicine treatment of heart homogenate that results in the destruction of microtubules disturbs the

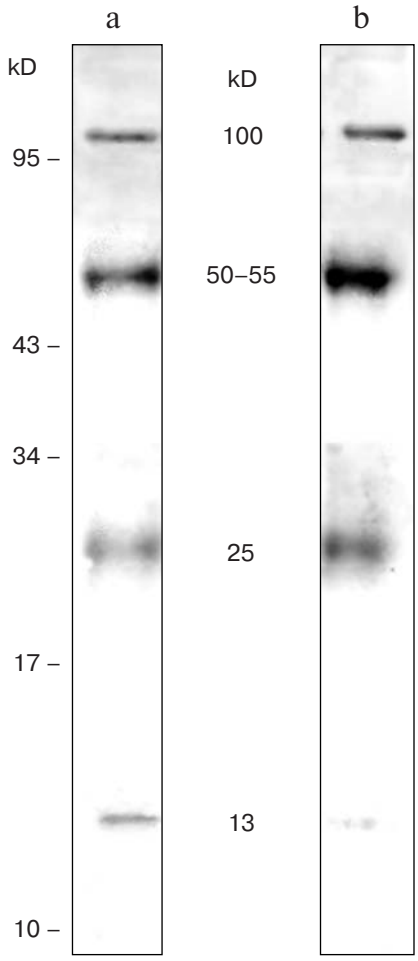

Fig. 3. Electrophoretic analysis of protein composition of immunoprecipitate that was obtained from solubilized Na,K-ATPase from duck salt glands using antibodies against Na,K-ATPase α1-subunit. The gel was loaded with immunoprecipitate (15 μg protein) of preparations obtained without (a) and after (b) incubation of the homogenate with 1.6 μM colchicine. SDS-PAGE was preformed using Tricine cathode buffer, and the proteins were stained by silver.

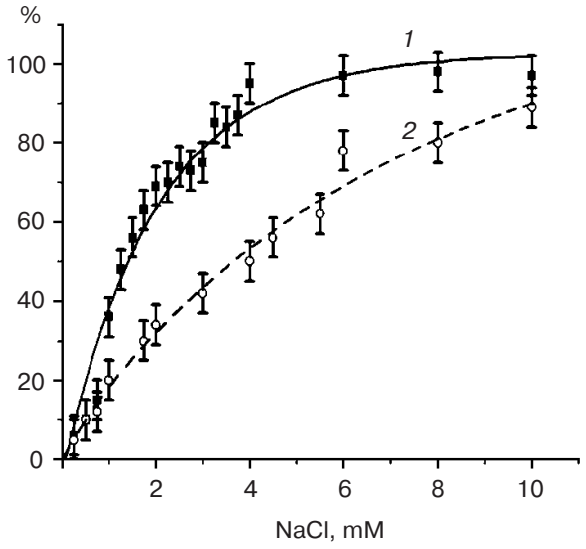

Fig. 4. Dependence of the activity of Na,K-ATPase from duck salt glands obtained without (*1*) and after (*2*) incubation of homogenate in the presence of 1.6 μM colchicine on the NaCl concentration. Enzyme activity in the medium with 25 mM NaCl and 10 mM KCl (pH 6.5) was taken as 100%.

interaction of Na,K-ATPase with phospholemman, which is a link between the Na,K-ATPase and microtubules [12]. It appears that a similar process takes place in the case of colchicine treatment of duck salt gland homogenate. Our data suggest that the 13 kD protein that tightly interacts with Na,K-ATPase from duck salt glands and effects on the sensitivity of this enzyme to sodium ions seems to be a phospholemman or another protein that is similar to it. However, further studies should be carried out to test this suggestion.

This work was supported by the Russian Foundation for Basic Research (grant No. 05-04-48239).

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