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A Protein Whose Binding to Na,K-ATPase Is Regulated by Ouabain

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Abstract—Immunoprecipitation of Na,K-ATPase from kidney homogenate by antibodies against α1-subunit results in the precipitation of several proteins together with the Na,K-ATPase. A protein with molecular mass of about 67 kD interacting with antibodies against melittin (melittin-like protein, MLP) was found in the precipitate when immunoprecipitation was done in the presence of ouabain. If immunoprecipitation was done using antibodies against melittin, MLP and Na,K-ATPase α1-subunit were detected in the precipitate, and the amount of α1-subunit in the precipitate was increased after the addition of ouabain to the immunoprecipitation medium. MLP was purified from mouse kidney homogenate using immunoaffinity chromatography with antibodies against melittin. The addition of MLP to purified FITC-labeled Na,K-ATPase decreases fluorescence in medium with K^+ and increases it in medium with Na^+. The enhancement of fluorescence depends upon the MLP concentration. The N-terminal sequence of MLP determined by the Edman method is the following: HPPKRVRSRLNG. No proteins with such N-terminal sequence were found in the protein sequence databases. However, we revealed five amino acid sequences that contain this peptide in the middle part of the chain at distance 553 amino acids from the C-terminus (that corresponds to protein with molecular mass of about 67 kD). Analysis of amino acid sequence located between C-terminus and HPPKRVRSRLNG in all found sequences has shown that they were highly conservative and include WD40 repeats. It is suggested that the 67-kD MLP either belongs to the found protein family or was a product of proteolysis of one of them.

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Key words: Na,K-ATPase, immunoprecipitation, melittin, ouabain, WD-repeats

Na,K-ATPase provides for active transport of Na^+ and K^+ across the plasma membrane of animal cells. The enzyme consists of at least two subunits: catalytic α-subunit with molecular mass of about 110 kD and β-subunit that is a glycoprotein with molecular mass of about 55 kD (protein part of about 35 kD). Both subunits are represented by several isoforms that are encoded by different genes and are differently expressed during ontogenesis in various tissues [1, 2]. Cardiotonic steroids such as ouabain or digoxin are known to bind to Na,K-ATPase specifically and to inhibit its activity [3].

Data obtained recently suggest that the effect of ouabain at low nontoxic concentrations results not only in the inhibition of Na,K-ATPase and in the corresponding change in Na^+ and K^+ concentrations inside the cell, but also can provide for the binding of some intracellular proteins to Na,K-ATPase that, in turn, can trigger the development of various effects including cardiac hypertrophy [4], endocytosis of Na,K-ATPase [5], or death of some types of cells [6]. For example, in cardiomyocytes the binding of ouabain to Na,K-ATPase leads to the activation of Src-kinase, which results in the phosphorylation and transactivation of epidermal growth factor receptor (EGFR) [4]. It was shown that there is multifocal interac-

Abbreviations: EGFR) epidermal growth factor receptor; ERP78/BiP) 78 kD glucose-regulated protein; FITC) fluorescein isothiocyanate; MLP) melittin-like protein; PBS) phosphate buffered saline; PBST) phosphate buffered saline with Tween-20; PMSF) phenylmethylsulfonyl fluoride; PVDF) polyvinylidene difluoride.

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tion between the α1-subunit of Na,K-ATPase and Src-kinase that is regulated by ouabain [7]. The data imply that Na,K-ATPase acts as an intracellular signal transducer that is induced by the binding of ouabain to the enzyme.

This idea is supported by the finding of ouabain and related cardiotonic steroids (that for a long time were believed to have only plant origin) in animal tissues, particularly in adrenal glands and hypothalamus [8, 9]. It is now accepted that endogenous cardiotonic steroids are a new class of steroid hormones whose receptor is Na,K-ATPase.

Besides Src-kinase, many other proteins have been shown to interact with Na,K-ATPase, including GRP78/BiP (78 kD glucose-regulated protein) [5], IP-3-kinase (phosphatidylinositol-3-phosphate kinase) [10, 11], catalytic subunit of protein phosphatase 2 [12], ankyrin [13], and adducin [14]. The binding of Src-kinase [7] and GRP78/BiP [5] to Na,K-ATPase is regulated by the binding of ouabain to the pump. We have shown earlier that method of immunoprecipitation is suitable for revealing the set of proteins interacting with Na,K-ATPase [15]. Using this approach and method of two dimensional electrophoresis for the separation of proteins of the immunoprecipitate, we have found that the addition of ouabain to a line of colon epithelial cells (Caco-2) results in the binding to the Na,K-ATPase of more than 20 proteins [16]. Some of these proteins can be considered as candidates for the initiators of signal cascades produced by ouabain.

It was shown earlier that melittin, an amphiphilic peptide from bee venom consisting of 26 amino acid residues, interacts with certain proteins. Among them are calmodulin [17], myosin light chain kinase [18], some other protein kinases [19, 20], as well as P-type ATPases (Ca-ATPase, H,K-ATPase, Na,K-ATPase) that are inhibited by melittin [21-23]. Melittin is known also to activate phospholipase A2 [24]. It was suggested that melittin imitates a protein module participating in protein–protein interactions. Two phospholipase A2-activating proteins with molecular mass of about 28 and 70 kD have been revealed that have structural similarity with melittin [25, 26]. These proteins are members of the β-transducin (G_β) superfamily and contain WD-repeats [27, 28]. Using anti-melittin antibodies J. Cupolletti et al. detected a protein with molecular mass of about 67 kD in rabbit gastric mucosa [29]. Activation of acid secretion in parietal cells of gastric mucosa induced by histamine was shown to result in the reversible association of this cytoplasmic 67-kD melittin-like protein (MLP) with the membrane fraction containing H,K-ATPase. It is known that activation of acid secretion leads to the morphological rebuilding of apical membrane of parietal cells and to the inserting of H,K-ATPase into this membrane [30]. Thus 67-kD MLP having structural similarity with melittin can participate in endo/exocytosis of gastric H,K-ATPase, which is a close relative of the Na-pump.

Taking into account the evidence concerning the interaction of melittin with Na,K-ATPase and the existence of MLPs, we suggested that some of these proteins can interact with Na,K-ATPase in the site of melittin binding. The goal of present work is to reveal among the proteins that bind to Na,K-ATPase a protein or proteins that have structural similarity with melittin, and to identify these proteins.

MATERIALS AND METHODS

Materials. Monoclonal antibodies against α1-subunit of Na,K-ATPase (α6F) were obtained from DSHB (USA). EDTA, sodium deoxycholate, SDS, glycine, Tris-HCl, sucrose, ouabain, pepstatin A, leupeptin, phenylmethylsulfonyl fluoride (PMSF), hydrogen peroxide, and protein A-agarose were from Sigma-Aldrich (USA); Affi-Gel 10, nitrocellulose membranes, Silver Stain Kit, acrylamide, methylene-bis-acrylamide, and other reagents for SDS-PAGE were from Bio-Rad (USA); polyvinylidene difluoride (PVDF) membranes were from ICN Biomedical (USA). Triton X-100, glutaraldehyde, and imidazole were from Merck (Germany); Coomassie Brilliant Blue was from Serva (Germany). Bee venom melittin (free of phospholipase A_2) obtained by HPLC chromatography was purchased from Aura (Moscow, Russia). Other reagents of the highest purity available were produced in Russia.

Purification of Na,K-ATPase. Purified Na,K-ATPase was obtained from pig and rabbit kidney using the method described by Jorgensen [31]. Enzyme preparations were stored in sucrose EDTA-Tris buffer at –70°C. Protein concentration was determined by the method of Lowry et al. [32] using BSA as a standard.

Purification of Na,K-ATPase α-subunit. Purified Na,K-ATPase from kidney was subjected to SDS-PAGE in accordance with the Laemmli procedure [33] using 3.5% stacking and 3.5-20% gradient running gel. Proteins were separated simultaneously on two gels with thickness of 2 mm. After electrophoresis, two strips were cut off both sides of the gel, fixed, and stained with Coomassie Brilliant Blue R-250 for the identification of α-subunit. The corresponding region of unstained gel was separated and cut into small pieces, which were put in a chamber for electro-elution (Model 422 Electro-Eluter; Bio-Rad). Electro-elution was performed during 3 h with a current 10 mA per tube.

Polyclonal antibodies against Na,K-ATPase α1-subunit from pig kidney were generated using as antigen α1-subunit purified by electro-elution. To obtain antibodies against melittin, we used as antigen melittin that was cross-linked by 6% glutaraldehyde [23] before the administration. The immunization protocol was the following: the first injection was made to a rabbit subcutaneously using 0.4-0.5 mg of α1-subunit or 0.1 mg of melittin with

complete Freund adjuvant in ratio 1 : 1 (v/v). The booster injections (totally four) were made with incomplete Freund adjuvant within time intervals 3-4 weeks. Cross-linked melittin was injected into rabbits together with lidocaine.

To test the raising of antibodies, blood was taken from the ear vein and titer of antiserum was determined using ELISA (for antibodies against melittin) or dot-ELISA (for antibodies against Na,K-ATPase α1-subunit). Then the antibodies were purified using immunoaffinity chromatography with corresponding antigen immobilized on Affi-Gel 10.

The 67-kD MLP was purified using affinity chromatography. We used a method that was developed in the laboratory of Professor J. Cuppoletti (Cincinnati, USA) but was not published earlier (the method was used with the permission of its author). The following modifications were introduced compared with the original method: instead of anti-melittin serum, we used purified antibodies against melittin for the immobilization on the Affi-Gel 10, a mixture of protease inhibitors was added to the homogenate, and NaN_3 was added to some buffers.

Affi-Gel 10 (3 ml) was washed by 10 volumes of cold distilled water and then by five volumes of 100 mM MOPS, pH 7.4. Purified antibodies against melittin were incubated with Affi-Gel 10 overnight with stirring at 4°C. Then 100 μl of 1 M ethanolamine solution was added, and the mixture was incubated for 1 h with stirring at 4°C. A column (1.5 × 1 cm) was packed with Affi-Gel 10 with attached antibodies and washed by 10 volumes of phosphate buffered saline with Tween-20 (PBST) containing 0.2% NaN_3. Homogenate was prepared from the whole mouse kidney or from rat outer medulla. Kidneys of five mice (total weight about 2 g) or the same amount of rat outer medulla were cut by scissors into small pieces and homogenized using a Potter homogenizer (glass-Teflon) in 10 volumes of the following buffer: 30 mM imidazole, pH 7.4, 5 mM EDTA, 130 mM NaCl, 1.1% Triton X-100, 0.25% CHAPS, 2 mM PMSF, 0.1 mM leupeptin, 1 mM pepstatin A. The homogenate was filtered through four layers of gauze, and protein concentration was measured by the Bradford method [34]; it usually varied between 10-15 mg/ml. Solubilized homogenate was applied to the column. After that, the column was washed with 10 volumes of PBST containing 0.2% NaN_3, 2 mM PMSF, 0.1 mM leupeptin, and 1 μM pepstatin A. The 67-kD MLP protein was eluted with five volumes of glycine-HCl, pH 2.5. When protein started to elute from the column, 800-μl aliquots were collected into plastic tubes containing 200 μl of 1 M Tris-HCl, pH 8.0, and then the samples were analyzed using SDS-PAGE. Fractions containing protein with molecular mass of about 67 kD were pooled and subjected to dialysis for 18 h at 4°C against 5 mM imidazole, pH 7.4. Then the protein was frozen, freeze-dried, and dissolved in 100 μl of 20 mM imidazole, pH 7.4.

Immunoprecipitation was done as described earlier [15]. Sample buffer for SDS-PAGE (10-20 μl) was added to the immunoprecipitate and samples were incubated for 10 min at 80°C. Protein composition of the immunoprecipitate was determined by SDS-PAGE as described above and by Western-blotting. Protein bands were stained with Coomassie Brilliant Blue R-250 or by the silver staining procedure.

Western blot analysis. Proteins separated by SDS-PAGE were transferred to nitrocellulose membrane by trans-blotting for 1 h at 100 V in a Mini Trans-Blot Electrophoretic Transfer cell (Bio-Rad). After that the membrane was washed 2 times with PBST, blocked for 1 h with 5% fat-free milk solution in PBST, and incubated for 1 h at room temperature with monoclonal antibodies against Na,K-ATPase α1-subunit or with polyclonal antibodies against melittin diluted by PBST solution 250 and 1000 times, respectively. Then the membrane was washed and incubated with secondary anti-rabbit or anti-mouse antibodies conjugated with horseradish peroxidase (dilution 1 : 1000). After washing the membrane with PBST (5 times) antigen–antibody complexes were visualized by staining with 3,3′-diaminobenzidine and H_2O_2. To measure the amount of protein in stained bands, they were scanned using a HP Scanjet 4400c and the optical density of bands was determined using the OneDScan program.

Fluorescence experiments. Purified Na,K-ATPase from rabbit kidney was subjected to the dialysis overnight against 50 mM imidazole, pH 7.5, 0.2 mM EDTA. Na,K-ATPase was labeled with FITC as described earlier [35]. Na,K-ATPase (1 mg/ml) was incubated in the dark at room temperature with stirring for 1 h in medium containing 50 mM Tris-HCl, pH 9.0, 2 mM EDTA, 5 μM FITC. The reaction was stopped by addition of 10 volumes of ice-cold solution of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA. FITC-labeled Na,K-ATPase was precipitated by centrifugation at 105,000*g* for 1 h. The pellet was gently washed with 6 ml of distilled water, suspended in a small volume of 0.25 M sucrose, 50 mM Tris-HCl, pH 7.5, and assayed for the protein. The amount of incorporated label was determined from absorbance measurements at 495 nm. The concentration of bound FITC was calculated using extinction coefficient 84,800 $M^{-1} \cdot cm^{-1}$. Fluorescence was measured on a Hitachi F-3000 spectrofluorimeter in a cuvette with volume 1.5 ml containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA. Protein concentration was 10-15 μg/ml. Fluorescence was excited at 495 nm, and fluorescence was measured at 520 nm (slit widths were 5 and 1.5 nm, respectively). All fluorescence changes were expressed as percentage of total fluorescence that was measured without addition of ligands (in the presence of Tris-HCl and EDTA).

Determination of N-terminal amino acid sequence of MLP. MLP purified from mouse kidney homogenate was

subjected to separation by SDS-PAGE. PVDF membranes were incubated for 20-30 sec in 100% methanol and then 20-60 min in the buffer used for trans-blotting (25 mM $NaHCO_3$, 0.1% SDS, 20% methanol). Proteins were transferred from gel to PVDF membrane by the electro-trans-blotting procedure for 1.5 h (100 V). To visualize 67-kD MLP position on the membrane, two strips were cut off both sides of the membrane. One strip was stained by Amido Black, and the second strip was stained with antibodies against melittin. A protein band corresponding to 67-kD protein and stained by anti-melittin antibodies was cut off. N-Terminal sequence of this protein was read using the Edman degradation procedure in the Laboratory of Protein Chemistry, Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Moscow.

Sequence analysis. Search and analysis of homological amino acid sequences was performed using the program BLASTP 2.2.9 [36]. Sequence alignment was carried out using CLUSTAL W (1.83) multiple sequence alignment (http://www.ebi.ac.uk/clustalw/) [37]. Domain structure of proteins was analyzed with program RPS-BLAST 2.2.9 [38].

RESULTS AND DISCUSSION

Immunoprecipitation. Using polyclonal antibodies against Na,K-ATPase, we have shown earlier [15] that more than 10 proteins were co-immunoprecipitated with Na,K-ATPase from solubilized homogenate of rat outer medulla. These proteins can be considered as proteins that tightly interact with Na,K-ATPase. Using antibodies against Na,K-ATPase α1-subunit, we obtained immunoprecipitate of Na,K-ATPase with interacting proteins from solubilized homogenate of rat and mouse kidney. Western-blot analysis with staining by antibodies against α1-subunit confirmed that Na,K-ATPase α1-subunit is present in the immunoprecipitate (Fig. 1a, top panel).

We have found that if ouabain were added to the immunoprecipitation buffer, the immunoprecipitate contained 67-kD protein that was stained by anti-melittin antibodies (Fig. 1a, bottom panel, lane *2*). It is interesting that in the absence of ouabain the amount of this 67-kD protein was rather small (Fig. 1a, bottom panel, lane *1*, and diagram).

When immunoprecipitation was done using anti-melittin antibodies, we found in the immunoprecipitate a protein with molecular mass of about 100 kD that was stained by monoclonal antibodies against Na,K-ATPase α1-subunit (Fig. 1b, lane *1*). The amount of α1-subunit in the immunoprecipitate was higher by about 2-fold if ouabain (0.5 mM) was added to the immunoprecipitation medium (Fig. 1b, lane *2*). The data described above suggest that Na,K-ATPase interacts with the 67-kD protein stained with anti-melittin antibodies (MLP), and this

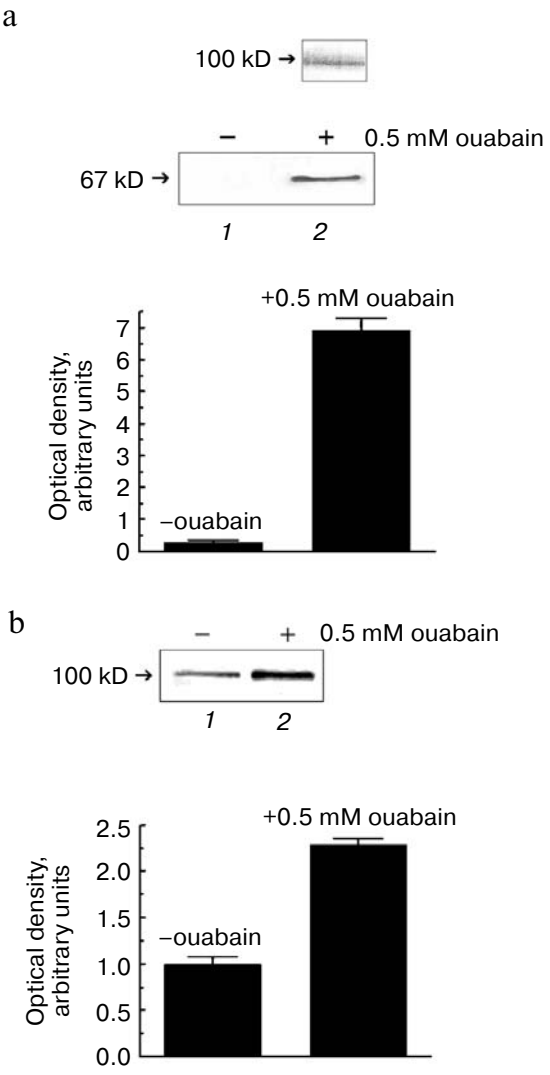

Fig. 1. Influence of ouabain on the binding of melittin-like protein to rat kidney Na,K-ATPase studied by immunoprecipitation. a) A representative result of Western blot analysis obtained from three independent experiments showing the products of immunoprecipitation carried out using polyclonal antibodies against the Na,K-ATPase α1-subunit: *1*) in medium without ouabain; *2*) in medium with ouabain (0.5 mM). Proteins were identified using monoclonal (α6F) antibody against the α1-subunit of Na,K-ATPase (top) and polyclonal antibodies against melittin (bottom). The diagram represents the optical density of bands corresponding to the MLP stained by anti-melittin antibodies. Optical density in the medium without ouabain was takes as 1.0. The data represent mean value from three independent experiments. b) A representative result of Western blot analysis obtained from three independent experiments showing the products of immunoprecipitation carried out using anti-melittin antibodies: *1*) in medium without ouabain; *2*) in medium with ouabain (0.5 mM). The proteins were identified using monoclonal antibodies to Na,K-ATPase α1-subunit (α6F). The diagram represents the optical density of bands corresponding to the Na,K-ATPase α1-subunit stained by monoclonal antibodies. Optical density in the medium without ouabain was taken as 1.0. The data represent mean value from three independent experiments.

interaction significantly increases when ouabain is bound to Na,K-ATPase.

Purification of 67-kD MLP. To reveal whether or not Na,K-ATPase directly interacts with 67-kD MLP, we studied the interaction of these two purified proteins. With this aim, 67-kD MLP was purified using immunoaffinity chromatography. Solubilized homo-genate from mouse kidney obtained as described in "Materials and Methods" was applied to the column with immobilized anti-melittin antibodies. After removing proteins that were nonspecifically bound to immobilized antibodies by the addition of PBST containing 300 mM NaCl (Fig. 2a, peak *3*), specifically bound proteins were eluted with 0.1 M glycine-HCl, pH 2.5 (Fig. 2a, peak *4*). Aliquots of eluted solution were collected and subjected to SDS-PAGE. Peak *4* contained mainly 67-kD protein with small amounts of two additional proteins having molecular mass of about 45 and 28 kD. The purest fraction including only 67-kD protein (Fig. 2b) was used in further fluorescence experiments and for the determination of N-terminal sequence.

Studies of the interaction between Na,K-ATPase and MLP by measurement of fluorescence. To study the direct interaction of Na,K-ATPase with 67-kD MLP, we used purified Na,K-ATPase from rabbit kidney. According to SDS-PAGE data, this preparation of Na,K-ATPase contained only two proteins with molecular masses of about 100 and 55 kD that correspond to α- and β-subunit, respectively (data not shown). Purified Na,K-ATPase was labeled with FITC as described above, and the stoichiometry of labeling consisted of 0.35 mol FITC/mol Na,K-ATPase.

The addition of NaCl (1 mM) to FITC-labeled Na,K-ATPase results in increase in the fluorescence by 4.5 ± 1.5% (Fig. 3a). When KCl (10 mM) was added to FITC-labeled Na,K-ATPase its fluorescence decreased by 20 ± 2.5% (Fig. 3b). The data are in agreement with results obtained earlier [35] demonstrating that NaCl increased the fluorescence of FITC-labeled Na,K-ATPase, and KCl decreased it. If MLP was added to FITC-labeled Na,K-ATPase in the absence of cations we did not observe any change in fluorescence. If then NaCl was added to the mixture FITC-labeled Na,K-ATPase–MLP, it resulted in an increase in the fluorescence that was bigger than in the presence of only NaCl (Fig. 3b). When KCl was added to the mixture FITC-labeled Na,K-ATPase–MLP, we observed the quenching of fluorescence that was less than was induced by KCl alone (Fig. 3b).

If MLP was added to FITC-labeled Na,K-ATPase after the addition of ouabain (0.5 mM), no change in the fluorescence was observed (Fig. 3c). However when 1 mM of NaCl was added to the complex of FITC-labeled Na,K-ATPase–ouabain the subsequent addition of MLP significantly increased the fluorescence (Fig. 3c).

The addition of MLP to FITC-labeled Na,K-ATPase in the presence of NaCl enhanced the fluorescence in concentration-dependent manner (Fig. 4a, curve *1*), and saturation was observed at MLP/Na,K-ATPase molar ratio of about 1 : 1. If NaCl was not added to the solution we did not observe any change in the fluorescence even at the ratios MLP/Na,K-ATPase that were close to the saturating values. Also, no fluorescent

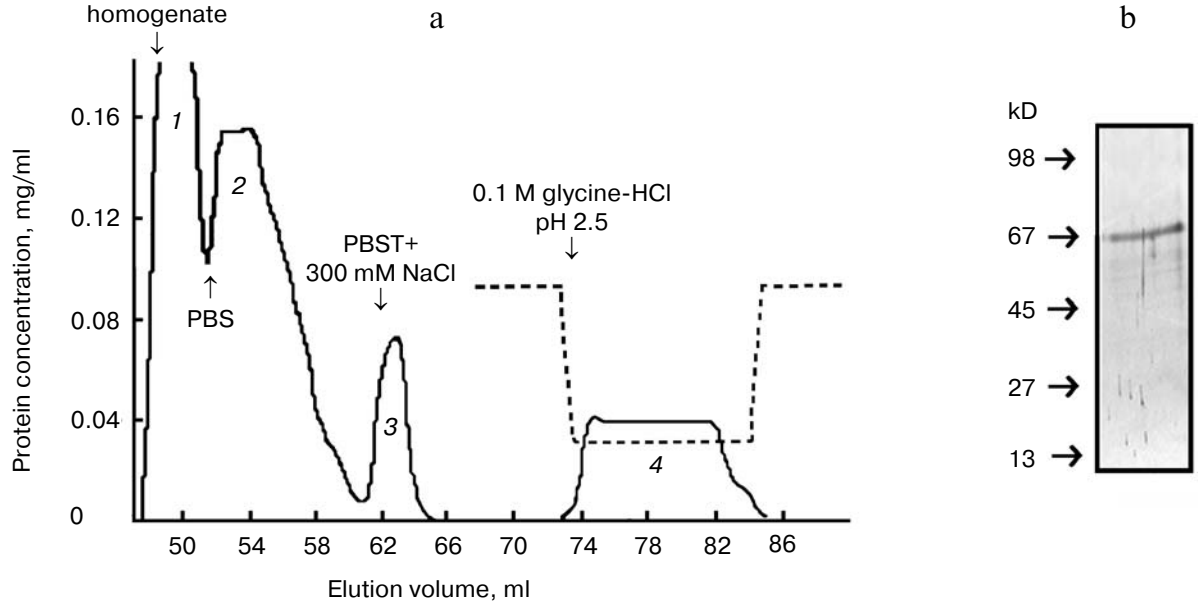

Fig. 2. Purification of 67-kD MLP using immunoaffinity chromatography with immobilized anti-melittin antibodies. a) Profile of protein elution from the column containing Affi-gel 10 beads with immobilized anti-melittin antibodies. b) Results of SDS-PAGE analysis of peak *4* obtained by elution of proteins from the column using 0.1 M glycine-HCl, pH 2.5. Silver staining.

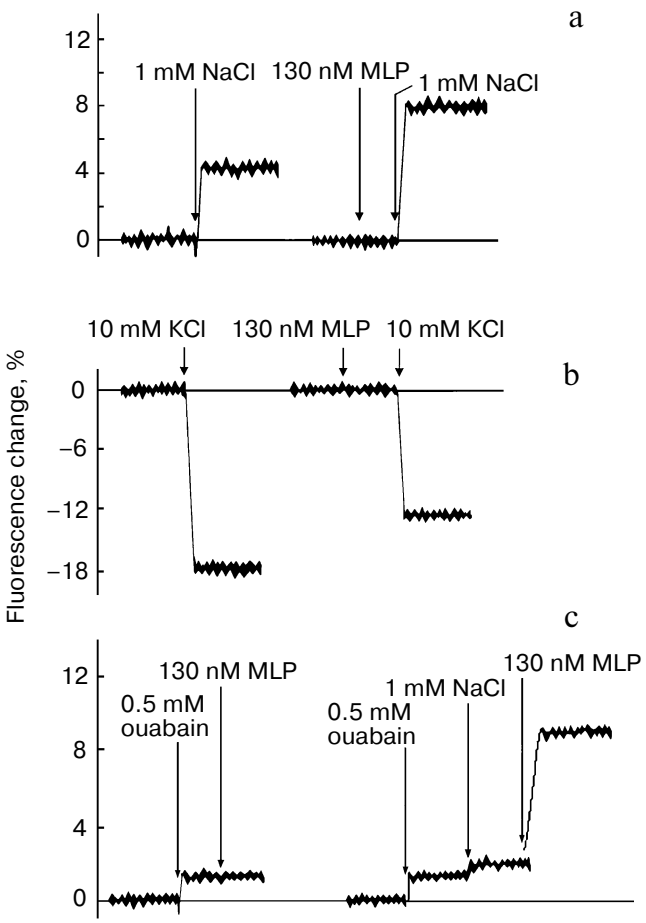

Fig. 3. Effects of 67-kD MLP on the fluorescence of FITC-labeled Na,K-ATPase from rabbit kidney in the presence of different ligands. a) Changes in the fluorescence of FITC-labeled ATPase in response to the addition of NaCl in the absence (left) and in the presence (right) of 67-kD MLP. b) Changes in fluorescence of FITC-labeled Na,K-ATPase in response to the addition of KCl in the absence (left) and in the presence (right) of 67-kD MLP. c) Effects of purified 67-kD MLP on the fluorescence of FITC-labeled Na,K-ATPase in the presence of ouabain (left) and ouabain + NaCl (right).

response was detected when BSA was added to FITC-labeled Na,K-ATPase in the presence of NaCl (Fig. 4a, curve *2*).

It is known that melittin also affects the fluorescence of FITC-labeled Na,K-ATPase [23]. In order to reveal the relationship between melittin and 67-kD MLP, we studied the effect of melittin on fluorescence of FITC-labeled Na,K-ATPase of in the presence of MLP. As can be seen from Fig. 4b, the increase in melittin/Na,K-ATPase molar ratio in the presence of BSA (curve *2*) results in the two-phases enhance of the fluorescence: the increase of the ratio melittin/Na,K-ATPase from 1 to 5 enhanced the fluorescence that did not further increase up to melittin/Na,K-ATPase ratio equal to 10. However, when melittin/Na,K-ATPase molar ratio was further increased from 10 to 30 additional enhancement in the fluorescence was observed (Fig. 4b, curve *2*). If 67-kD MLP was present in the incubation medium instead of BSA, we did not see the additional increase in the fluorescence in the range of high melittin/Na,K-ATPase ratios (Fig. 4b, curve *1*). This suggests that MLP can prevent the effect of melittin on the conformational change of FITC-labeled Na,K-ATPase that is provided at high melittin/Na,K-ATPase molar ratios.

Identification of purified 67-kD MLP. Because the genome of rat is not yet known, for the identification we used the protein from the mouse kidney homogenate. MLP purified by immunoaffinity chromatography was subjected to separation by SDS-PAGE and then was trans-blotted to PVDF membrane. To identify 67-kD MLP, its N-terminal sequence was determined by Edman degradation. The N-terminal sequence of this protein was the following: HPPKRVRSRLNG.

We did not find any protein with that N-terminal sequence in protein sequences databases. However in NCBI five amino acid sequences are present that contain the peptide HPPKRVRSRLNG in the middle part of the chain (Fig. 5). Only one from these five sequences is known as a protein: it is the protein Alfy from *Homo sapiens* containing 3526 amino acids [39]. Four other sequences containing peptide HPPKRVRSRLNG are known only from the sequences of corresponding cDNA. The distance separating the sequence HPPKRVRSRLNG from the C-terminus in all five sequences found includes 553 amino acids, which approximately corresponds to a protein with molecular mass of 67-kD.

The alignment of C-terminal parts of five found sequences including 553 amino acids showed that C-terminal sequences of two proteins (protein 3 containing WD-repeat and FYVE-domain, isoform 1 from *Mus musculus*, and mKIAA0993 from *Mus musculus*) are completely identical. Three other sequences have very high homology similarity (more than 95%).

Analysis of the C-terminal part of five sequences (shown in Fig. 5) using RPS-BLAST 2.2.9 that is suitable for conserved domain search revealed that their C-terminal parts include WD40-repeats and FYVE-domain. Thus if 67-kD MLP containing N-terminal sequence HPPKRVRSRLNG is a member of protein family presented in Fig. 5, it also should contain WD-40 repeats.

During its life cycle, Na,K-ATPase like other membrane proteins, interacts with many intracellular and membrane proteins that provide for its embedding into the membrane, interaction with the cytoskeleton, removal from the membrane, etc. Recent studies have shown that this enzyme can also participate in the transduction of signal receiving in the form of ouabain or a related molecule that binds to a specific site of Na,K-ATPase that leads to the activation of some signal cascades [4, 5]. Ouabain binding is known to change the conformation of Na,K-ATPase [40] that, in turn, can

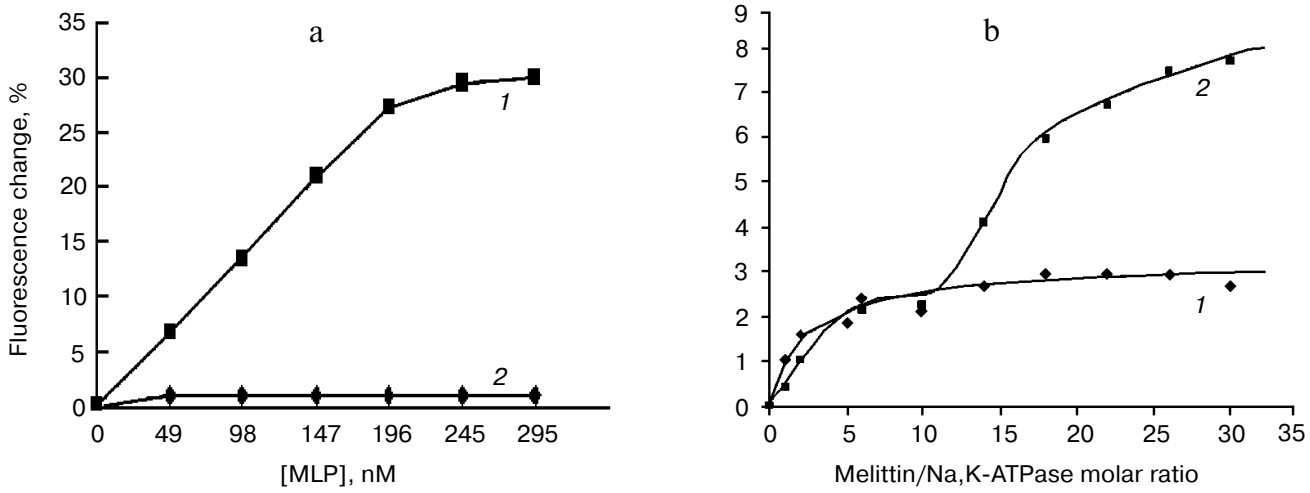

Fig. 4. Effect of purified 67-kD MLP on the fluorescence of FITC-labeled Na,K-ATPase from rabbit kidney. a) Dependence of fluorescence of FITC-labeled Na,K-ATPase on the concentration of 67-kD MLP (curve *1*) and BSA (curve *2*) in the presence of NaCl. b) Dependence of fluorescence of FITC-labeled Na,K-ATPase on the molar ratio melittin/Na,K-ATPase in the presence of 67-kD MLP (curve *1*) and in the presence of BSA (curve *2*).

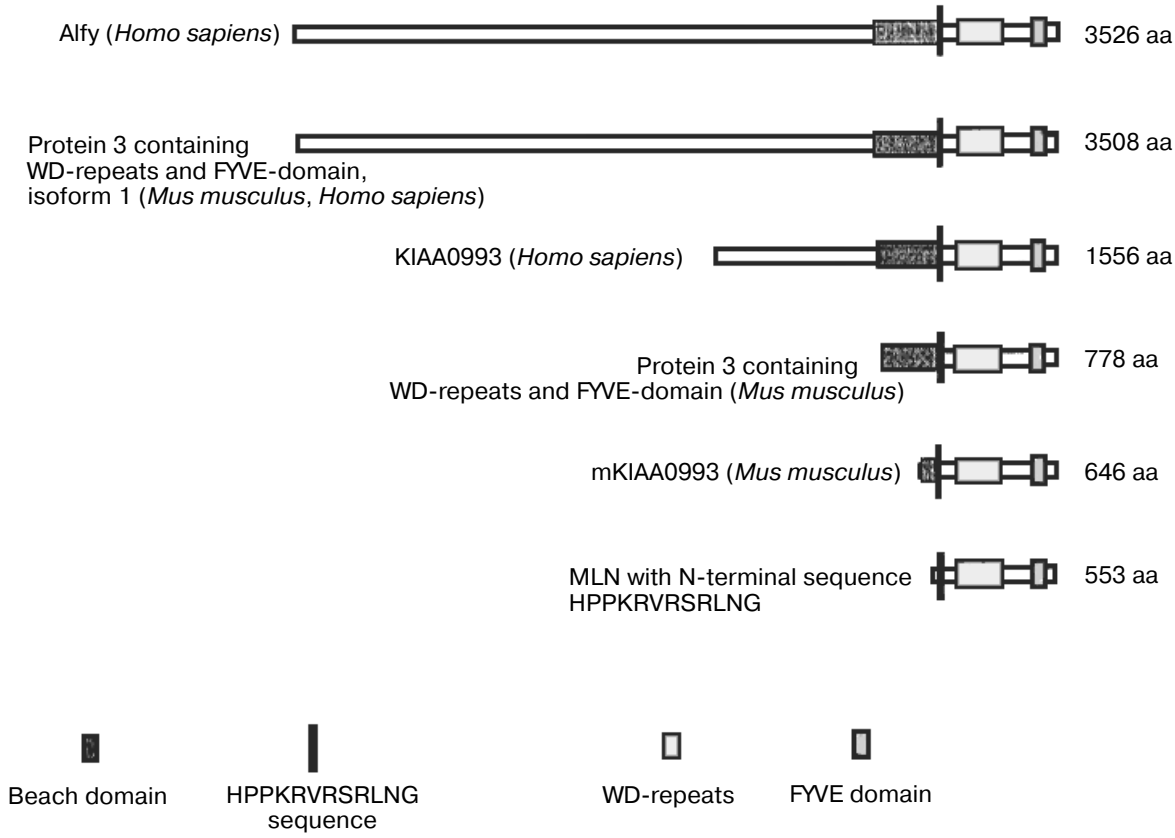

Fig. 5. Family of related proteins with sequence HPPKRVRSRLNG containing WD-repeats and FYVE-domain in C-terminal part.

induce the binding of some proteins to the Na,K-ATPase and their activation or deactivation. We found that in the cells of colon epithelium (line Caco-2) ouabain, that induced the death of these cells, provided the binding to Na,K-ATPase of about 18 proteins. Some of these proteins were identified by mass-spectrometry [16]. Among the identified proteins are the following: phosphorylated phosphatase 2C, receptor for glucocorticoids, protein

CAMTA containing ankyrin repeats, protein Rac that is activator of GTPase, and phosphorylated protein from Src-family. Other authors have identified two other proteins that interact with Na,K-ATPase after its binding to ouabain; these are Src-kinase that induces a signal resulting in the development of hypertrophy of cardiomyocytes through the phosphorylation of ERFR [4, 7], and protein ERP78/BiP that belongs to the HSP70 protein family and appears to be involved in the ouabain-induced endocytosis of Na,K-ATPase α1-subunit [5].

Our data show that among the proteins of kidney homogenate that interact with Na,K-ATPase in response to ouabain binding is a 67-kD protein (MLP) that is able to bind also antibodies against melittin, which demonstrated its structural similarity with this peptide. The fact that 67-kD MLP was found in the immunoprecipitate obtained using antibodies against Na,K-ATPase α1-subunit in the presence of ouabain as well as the Na,K-ATPase α1-subunit being revealed in the immunoprecipitate obtained using anti-melittin antibodies can be considered as evidence that Na,K-ATPase and MLP can tightly interact. Also, ouabain increased the amount of Na,K-ATPase in the immunoprecipitate obtained using anti-melittin antibodies (Fig. 1). These data together indicate that the studied proteins can be not only in one complex but also that ouabain induces or at least increases the amount of Na,K-ATPase interacting with MLP.

Results that were obtained in experiments with FITC-labeled Na,K-ATPase and purified 67-kD protein show that these two proteins interact directly but not through a protein-adaptor: the addition of purified 67-kD MLP to purified FITC-labeled Na,K-ATPase increased fluorescence in concentration-dependent manner (Fig. 4a). However, the increase in fluorescence was observed only if Na^+ was added to the incubation medium. In contrast, in the presence of K^+ MLP decreased the fluorescence of FITC-labeled Na,K-ATPase. Thus the binding of 67-kD MLP shifts the conformation of Na,K-ATPase to the E1-form.

The data also imply that either 67-kD MLP does not bind to Na,K-ATPase in the absence of Na^+ or K^+ or it can bind in medium without these cations but cannot produce a changes of Na,K-ATPase conformation that result in the change of fluorescence of FITC bound with it. However, it is clear that MLP can bind to Na,K-ATPase both in the absence and in the presence of ouabain (Figs. 3a and 3c), but for its binding or for the change in Na,K-ATPase conformation the presence of Na^+ (or K^+) is necessary (Fig. 3c).

Our data demonstrate also that purified 67-kD MLP can abolish the effect of melittin on the fluorescence of FITC-labeled Na,K-ATPase at high melittin/Na,K-ATPase ratios (more than 10). Recently we have shown that melittin induced both the inhibition of Na,K-ATPase activity and aggregation of Na,K-ATPase molecules at high melittin/Na,K-ATPase molar ratios [41]. All these data together might be an indication that melittin and 67-kD MLP can compete for a binding site located on Na,K-ATPase and saturated at high melittin/Na,K-ATPase ratios.

To identify MLP, its N-terminal sequence consisting of 12 amino acids was determined. Surprisingly, we did not find proteins with such N-terminal sequence in available databases. But we revealed that exactly the same sequence is located in the middle part of several amino acid sequences. Therefore, 67-kD MLP having N-terminal sequence HPPKRVRSRLNG might be: i) a product of posttranslational modification (proteolysis) of a protein with higher molecular mass, or ii) a product of the translation of a gene that encoded a family of proteins and is produced as result of alternative splicing. We cannot yet make a choice between these two suggestions, but we can conclude that in both cases MLP will contain WD40-repeats and FYVE-domain.

Comparison of sequences including WD-40 repeats in all sequences found in databases and in melittin shows that about 37% of amino acids in melittin and in WD40-repeats are identical, and about 40% amino acids are homologous. On one hand, it appears that namely WD40-repeat is responsible for the interaction of MLP with polyclonal anti-melittin antibodies. On the other hand, we cannot exclude that namely WD40-repeat provides the binding of MLP with the MLP-binding site of Na,K-ATPase. Further studies should be preformed for deciding between these possibilities.

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