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Identification of a Region of the Polypeptide Chain of Na,K-ATPase α-Subunit Interacting with 67-kDa Melittin-Like Protein

Yu. V. Kamanina[1,2], E. A. Klimanova[1,2], E. A. Dergousova[1,2], I. Yu. Petrushanko[2], and O. D. Lopina[1]*

[1]*Lomonosov Moscow State University, Department of Biochemistry, School of Biology, 119991 Moscow, Russia; fax: +7 (495) 939-3955; E-mail: od_lopina@mail.ru*
[2]*Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 119991 Moscow, Russia*

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Abstract—It was shown earlier that a 67-kDa protein purified from mouse kidney using polyclonal antibodies against melittin (a peptide from bee venom) interacted with Na,K-ATPase from rabbit kidney. In this study, a 43-kDa proteolytic fragment of Na,K-ATPase α-subunit interacting with the 67-kDa melittin-like protein was found. The α-subunit was hydrolyzed by trypsin in the presence of 0.5 mM ouabain (E2-conformation of Na,K-ATPase). A proteolytic fragment interacting with the 67-kDa melittin-like protein that was identified by mass-spectrometry is a region of the cytoplasmic domain of Na,K-ATPase α-subunit located between amino acid residues 591 and 775. The fragment includes a conservative DPPRA motif that occurs in many P-type ATPases. It was shown earlier that this motif of H,K-ATPase from gastric mucosa binds to melittin. We suggest that namely this motif of P-type ATPases is able to interact with proteins containing melittin-like modules.

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Na,K-ATPase is an integral enzyme of the plasmatic membrane of animal cells providing active transport of sodium and potassium ions through the membrane. The enzyme consists of at least two subunits: catalytic α- and regulatory β-subunits that are tightly bound to each other in 1 : 1 ratio.

The α-subunit has molecular mass of about 110 kDa and includes 10 transmembrane segments. They create a membrane domain inside of which is located a channel with a gate through which Na and K ions are transported. About 50% of the amino acid residues of the polypeptide chain of α-subunits are exposed to the cytoplasm, where they formed three domains: nucleotide-binding, phosphorylated, and actuator, the first two catalyzing ATP hydrolysis, all three together participating in conformational changes that are required for ion transport [1].

The β-subunit is a glycoprotein whose protein part has molecular mass of about 35 kDa, and after glycosylation the mass is increased up to 55-66 kDa. This subunit has one transmembrane domain. This subunit has regulatory function, and it delivers the α-subunit to the plasma membrane after synthesis and correct insertion of the αβ-complex into the membrane [2].

In a complex of Na,K-ATPase obtained from some sources (kidney and heart of mammals, rectal glands of shark), a third protein, the so-called γ-subunit with molecular mass of about 7.4 kDa containing one transmembrane domain, was also found. This subunit is tissue specific; it belongs to the family of FXYD proteins. These proteins also have another function (for example, they can form a channel for ions). In the content of Na,K-ATPase, the γ-subunit also has a regulatory function affecting the sensitivity of the enzyme to Na and K ions [3].

Na,K-ATPase is the only known receptor for cardiotonic steroids (in particular for ouabain), which specifically suppress the activity of this enzyme. It is also known that ouabain binding significantly changes Na,K-ATPase conformation [4].

Many different proteins can specifically interact with Na,K-ATPase, among them ankyrin [5], phosphoinositide 3-kinase [6], adducin [7], adaptor protein 2 [8], cave-

Abbreviations: CHAPS, 3-[(3-cholamidoprolyl)dimethylammonium]-1-propanesulfonate; ELISA, enzyme-linked immunosorbent assay; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline.

* To whom correspondence should be addressed.

olin 1 [9], cofilin [10], and some others. One of the proteins that was shown to interact with Na,K-ATPase in the presence of ouabain is the so-called melittin-like protein [11].

Melittin is a bee venom peptide consisting of 26 a.a. It forms amphipathic α-coil that, being included in membranes, forms pores that are permeable for water and substances soluble in water. Beside this, melittin appears to imitate some motif of intracellular proteins that participates in protein–protein interaction, in particular a site of interaction of calmodulin with its protein targets [12].

The catalytic subunit of P-type ATPases specifically binds melittin [13]. The peptide interacts with the sequence MI/LDPPR that is located in α-subunit of H,K-ATPase (M(603)DPPRAT) [14]. A similar sequence is present in all isoforms of Na,K-ATPase (MI(591)DPPRAA) and Ca-ATPase of endoplasmic reticulum (M(599)LDPPRKE). A protein interacting with antibodies against melittin and having molecular mass of about 67 kDa was found in cells of gastric mucosa and in homogenate of rabbit skeletal muscle. This protein is able to bind with Na,K-ATPase and Ca-ATPase of sarcoplasmic reticulum. Also, a protein interacting with antibodies against melittin with molecular mass of 67 kDa was purified from rabbit kidney. It can directly interact with Na,K-ATPase [11], but the site of its binding with the enzyme is unknown.

The goal of the present work was to identify the site of the Na,K-ATPase α-subunit that interacts with the melittin-like protein.

MATERIALS AND METHODS

Na,K-ATPase was purified from rabbit kidney outer medulla according to a method described by Jorgensen [15]. All buffer solutions used for the purification procedure contained a commercial cocktail of protease inhibitors and additionally 5 mM EDTA and 5 μM thiorphan, the latter being an inhibitor of the membrane protease neprilysin.

Na,K-ATPase was proteolyzed using trypsin in a solution containing 25 mM imidazole and 2 mM EDTA (pH 7.5) at 37°C in the presence or absence of 0.5 mM ouabain (time of incubation and ratio of Na,K-ATPase and trypsin as well as concentrations of Na and K ions were changed in different experiments). The proteolysis reaction was stopped by the addition of soybean trypsin inhibitor at 1 : 2 trypsin/inhibitor weight ratio.

The resulting peptide fragments were analyzed by electrophoresis using the method of Schagger and Von Jagow [16] (concentrating gel 3.5%, spacer gel 10%, and running gel 16%) with subsequent staining by Coomassie brilliant blue or by silver.

Polyclonal antibodies against melittin were obtained using a series of four subcutaneous injections of melittin into a rabbit with time interval of 30 days. Samples of melittin solution containing 100 μg of melittin were used for each injection. Melittin was cross-linked before the injection using 3% glutaric aldehyde in PBS buffer for 20 min at room temperature. Then Freund's adjuvant was added with ratio 1 : 1 (v/v). After four injections of melittin, blood was collected and diluted with PBS at ratio 3 : 1. The mixture was kept for 1.5 h at room temperature. The formed thrombus was removed by centrifugation. Proteins of the serum were salted out three times using saturated solution of ammonium sulfate to final concentration 34% for 2 h at room temperature with constant mixing. Then the sample was centrifuged at 1425*g* for 15 min. The pellet was redissolved in water. After the third salting out and centrifugation, the pellet was dissolved in borate buffer with volume equal to 1/3 of the starting volume, and then NaN_3 was added to 0.5% concentration. The titer of the antibodies estimated by an ELISA test was usually 1 : 100 or 1 : 200.

Melittin-like protein with molecular mass of 67 kDa was purified from homogenate of mouse kidney using affinity chromatography with polyclonal antibodies against melittin [11]. Fractions were analyzed using PAGE according to Laemmli [17] with staining of proteins by silver.

The sample was immunoprecipitated using protein A-agarose. Agarose was placed in PBS buffer for 1 h at room temperature and then washed three times with immunoprecipitation buffer (30 mM imidazole, 5 mM EDTA, 130 mM NaCl (pH 7.4), 1.1% Triton X-100, 0.25% CHAPS, 2 mM PMSF, 1 μM pepstatin A, 0.1 mM leupeptin). Then the protein A-agarose was precipitated by centrifugation for 2-3 min at 1500*g*, the supernatant being removed each time. The solution obtained after termination of trypsinolysis was supplemented with the buffer for immunoprecipitation and protein A-agarose in a ratio 1 : 11 : 10 (v/v), and the mixture was incubated for 1 h at room temperature with mixing. A pellet containing nonspecifically bound proteins was removed by centrifugation at 1500*g* for 5 min. The supernatant (170 μl) was supplemented with 15 μl of protein A-agarose, 50 μl of melittin-like protein, and 70 μl of polyclonal antibodies against melittin. Ouabain at concentration 0.5 mM was added in some samples. The mixture was incubated for 1 h at room temperature with mixing and then centrifuged at 1500*g* for 5 min. The pellet was washed seven times with the immunoprecipitation buffer, and then 18 μl of sample buffer for electrophoresis was added (250 mM Tris-HCl, pH 6.8, 8% SDS, 40% sucrose, 2% β-mercaptoethanol). The mixture was incubated for 10 min at 80°C. Protein composition was analyzed by PAGE.

Peptides were analyzed by tandem mass-spectrometry coupled with HPLC (LC-MS/MS) in the Laboratory of Peptide Chemistry at the Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry. The results were ana-

lyzed using the Data Analyses 2.1 program (Bruker Daltonics, USA). The NCBI database of translated sequences of nucleic acids was searched using the Mascot program (Matrixscience, USA).

RESULTS

On the basis of the data in the literature, we suggest that a site of binding of the 67-kDa melittin-like protein is located in the cytoplasmic domain of Na,K-ATPase α-subunit. Therefore, for its identification we decided to use limited proteolysis of Na,K-ATPase by trypsin in accordance with the method described by Jorgensen [18], because this procedure gives large fragments of the α-subunit. The β-subunit is not hydrolyzed by trypsin.

Tryptic cleavage was first conducted in the presence of 150 mM KCl or 150 mM NaCl, which convert Na,K-ATPase into conformations E2 and E1, respectively. The trypsin/Na,K-ATPase ratio in these experiments was changed from 1 : 5 to 1 : 30. Trypsin rather actively cleaves the enzyme α-subunit within 30 min of incubation at 37°C in the presence of KCl (Fig. 1, lanes *2-5*), whereas in the presence of NaCl trypsin causes practically no hydrolysis of this subunit (Fig. 1, lanes *6-9*). We noted the appearance of a rather large fragment with molecular mass of about 43 kDa using the trypsin/Na,K-ATPase weight ratio of 1 : 5. Appearance of a stained protein band corresponding to the fragment with molecular mass of about 43 kDa during incubation in the presence of 150 mM KCl was noted after 45 min of incubation under these conditions (Fig. 2, lanes *4* and *5*). In the presence of ouabain, which similarly to K ions converts Na,K-ATPase into conformation E2, we observed the appearance of this protein band already after 15 min incubation with trypsin (Fig. 3, lane *5*). In medium with 150 mM NaCl and 0.5 mM ouabain, we noted no hydrolysis of the Na,K-ATPase α-subunit (Fig. 3, lane *3*).

To reveal fragments of Na,K-ATPase α-subunit that interact with 67-kDa melittin-like proteins, the proteolytic fragments were immunoprecipitated using antibodies against melittin. In the medium for immunoprecipitation, Na,K-ATPase from rabbit kidney treated by trypsin for 15 min at trypsin/Na,K-ATPase ratio 1 : 5, purified melittin-like protein from mouse kidney, and antibodies against melittin were present. Ouabain at concentration 0.5 mM was also added in some samples. Polyacrylamide gel electrophoresis analysis of immunoprecipitates obtained in the presence and in the absence of ouabain showed that the protein composition of the two immunoprecipitates is

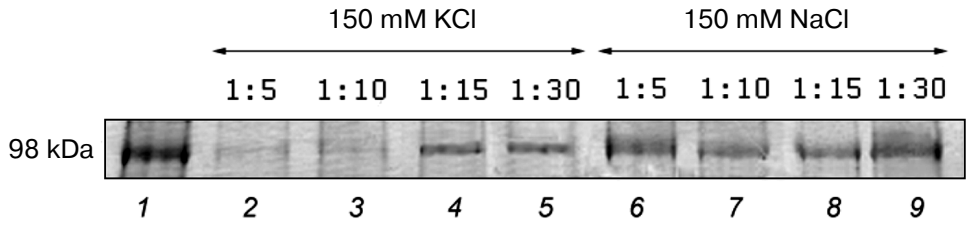

Fig. 1. Decrease in the content of Na,K-ATPase α-subunit as a result of 30-min trypsinolysis of the enzyme in medium containing 150 mM KCl (*2-5*) and 150 mM NaCl (*6-9*) at different trypsin/Na,K-ATPase ratios (noted above the lanes). Lane *1*: Na,K-ATPase α-subunit before proteolysis. SDS-PAGE was done according to the method of Schagger and Von Jagow [16]; protein was stained using Coomassie brilliant blue.

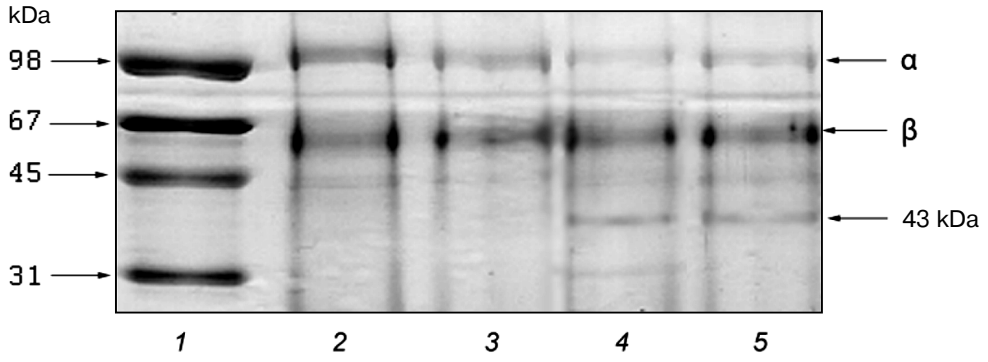

Fig. 2. Separation of proteolytic fragments of Na,K-ATPase α-subunit obtained in the presence of 150 mM KCl at trypsin/Na,K-ATPase ratio 1 : 5 and various incubation times (0 min (*2*), 30 min (*3*), 45 min (*4*), 60 min (*5*)) by SDS-PAGE according to the method of Schagger and Von Jagow [16]. Protein was stained using Coomassie brilliant blue. Lane *1*: molecular mass standards (Fermentas, USA).

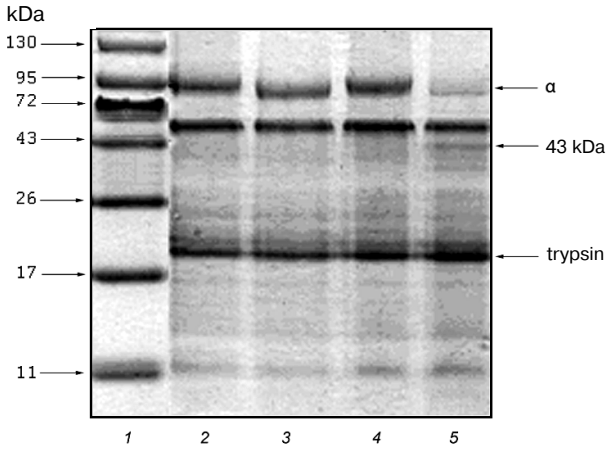

Fig. 3. Separation of proteolytic fragments of Na,K-ATPase α-subunit obtained after 15-min trypsinolysis at trypsin/Na,K-ATPase ratio 1 : 5 in medium with 0.5 mM ouabain (*2*, *3*) and 0.5 mM ouabain and 150 mM NaCl (*4*, *5*) using SDS-PAGE. Lanes: *1*) molecular mass standards; *2*, *4*) control (trypsin inhibitor was added before incubation). Protein was stained using Coomassie brilliant blue.

practically identical. Protein fragments with molecular masses 20, 25, and 55 kDa (Fig. 4) that appear to be the fragments of antibodies and a fragment with molecular mass of about 43 kDa were revealed in the immunoprecipitates. A broad band corresponding to proteins with molecular masses between 60 and 98 kDa, which most probably are 67-kDa melittin-like protein and α- and β-subunits of Na,K-ATPase, was also revealed on gels (after electrophoresis according to the method of Schagger and Von Jagow [16], proteins with low molecular masses are separated to best advantage, but proteins with high molecular masses are poorly separated). The presence in immunoprecipitates of both antibodies and Na,K-ATPase fragments demonstrates that immunoprecipitation took place. This means that the 67-kDa melittin-like protein is present in the immunoprecipitates.

A protein band with molecular mass corresponding to a proteolytic fragment of about 43 kDa that immunoprecipitates after proteolysis was cut out and analyzed by mass-spectrometry in the Laboratory of Peptide Chemistry of the Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry for identification.

The results of the analysis demonstrated that the proteolytic fragment that binds with 67-kDa melittin-like protein is the region of the cytoplasmic domain of the polypeptide chain of Na,K-ATPase α-subunit including amino acid residues 591-775. This region includes a DPPRA motif, which as shown earlier interacts with melittin in H,K-ATPase of gastric mucosa [14]. Figure 5 presents the amino acid sequence of the α-subunit of Na,K-ATPase; the fragment that according to computer analysis is probably present in the analyzed samples is underlined.

DISCUSSION

Na,K-ATPase is an integral membrane protein that changes conformation after ligand binding. Conformation E2 is induced by the binding of K ions, and the addition of Na ions turns the enzyme into E1 conformation that is characterized by lower intrinsic fluorescence as well as by lower fluorescence of fluorescent dyes bound with the enzyme, for example, fluorescein isothiocyanate [19]. Similar changes in protein conformation are observed as a result of ouabain binding. Conformational changes are coupled with the change of the structure of the site for the binding of different ligands including proteins as well as with the change of an accessibility of different peptide bonds for proteases. According to Jorgensen's data, trypsin cleaves the α-subunit of Na,K-ATPase from pig kidney close to the midpoint of the chain in the presence of KCl (between Arg438 and Ala439) with formation of two large fragments with molecular masses of about 48 and 58 kDa [18]. In accordance with the data of this author, trypsinolysis of α-subunit in medium with NaCl gives a large fragment with molecular mass of about 78 kDa and a smaller one with molecular mass of about 30 kDa (cleavage takes place between Arg262 and Ile263), and in addition a very small peptide containing 30 a.a. is released from N-terminus of the polypeptide chain. In our experience, 150 mM NaCl even in the presence of ouabain almost fully prevented cleavage of Na,K-ATPase α-subunit, probably because of its transformation into E1 form when the corresponding peptide bonds become inaccessible for trypsin.

Our experiments show that in the presence of 150 mM KCl or 0.5 mM ouabain (that, like K ions, transforms the enzyme into conformation E2), peptide bonds

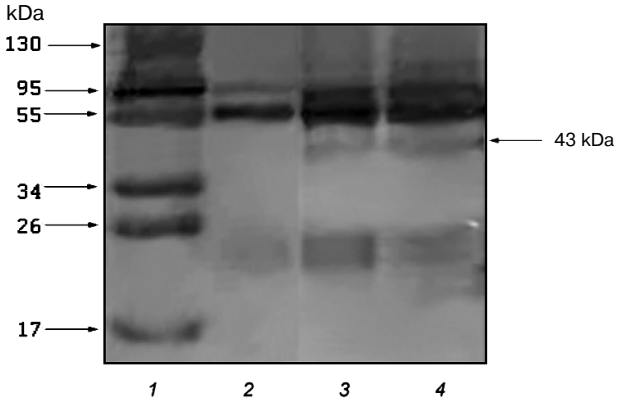

Fig. 4. Separation of proteolytic fragments of Na,K-ATPase α-subunit after immunoprecipitation with 67-kDa melittin-like protein and antibodies against melittin conducted without ouabain (*3*) and in the presence of 0.5 mM ouabain (*4*). Lanes: *1*) molecular mass standards; *2*) antibodies against melittin. The Laemmli method of electrophoresis was used in this experiment. Protein was stained using silver.

1 MGKGVGRDKY EPAAVSEHGD KKGKKAKKER DMDELKKEVS MDDHKLSLDE
51 LHRKYGTDLS RGLTTARAAE ILARDGPNAL TPPPTTPE***WV KFCRQLFGGF***
101 ***SMLLWIGA***IL CFLAYGILAA TEEDFDNDNL Y***LGVVLAAVV IITGCFSYYQ***
151 ***EA***KSSKIMES FKNMVPQQAL VIRNGEKMSI NAEDVVVGDL VEVKGGDRIP
201 ADLRIISANG CKVDNSSLTG ESEPQTRSPD FTNENPLETR NIAFFSTNCV
251 EGTARGIVIY TGDRTVMGRI ATLASGLEGG QTPIAAEI***EH FIHIITGVAV***
301 ***FLGVSFFI***LS LILEYTWLEA ***VIFLIGIIVA NVPEGLLA***TV TVCLTLTAKR
351 MARKNCLVKN LEAVETLGST STICSDKTGT LTQNRMTVAH MWFDNQIHEA
401 DTTENQSGVS FDKTSATWLA LSRIAGLCNR AVFQANQENL PILK**RA**VAGD
451 ASESALLKCI ELCCGSVKEM RERYTKIVEI PFNSTNKYQL SIHKNLNANE
501 PRHLLVMKGA PERILDRCSS ILLHGKEQPL DEELKDAFQN AYLELGGLGE
551 RVLGFCHLLL PDEQFPEGFQ FDTDEVNFPV DNLCFIGLIS **MI*DPPRA*AVP**
601 **DAVGKCRSAG IKVIMVTGDH PITAKAIAKG VGIISEGNET VEDIAARLNI**
651 **PVSQVNPRDA KACVVHGSDL KDMTSEQLDD ILKYHTEIVF ARTSPQQKLI**
701 **IVEGCQRQGA IVAVTGDGVN DSPALKKADI GVAMGIAGSD VSKQAADMIL**
751 **LDDNFASIVT GVEEGRLIFD NL*KKSIAYTL TSNIPEITPF LI***FIIANIPL
801 ***PLGTVTILCI DLGTDMVPAI SLA***YEQAESD IMKRQPRNPK TDK***LVNERLI***
851 ***SMAYGQIGMI QALGGF***FTYF VILAENGFLP FHLLGIRVDW DDRWINDVED
901 SYGQQWTYEQ RKIVEFTC***HT AFFVSIVVVQ WADLVICK***TR RNSVFQQGMK
951 N***KILIFGLFE ETALAAFLSY*** CPGMGVALRM YPLKP***TWWFC AFPYSLLIFV***
1001 ***YDEIRK***LIIR RRPGGWVEKE TYY

Fig. 5. Amino acid sequence of Na,K-ATPase α-subunit from rabbit kidney. The underlined region obtained the maximal score in the Mascot program during analysis of the peptide with molecular mass of about 43 kDa obtained after immunoprecipitation of proteolytic fragments of Na,K-ATPase in medium with 0.5 mM ouabain and 67-kDa melittin-like protein. The DPPRA motif within the underlined region is shown in slightly larger characters; transmembrane domains are shown in bold italic.

of Na,K-ATPase α-subunit become accessible for trypsin cleavage, which yielded a large fragment of the α-subunit with molecular mass of about 43 kDa. Because for peptide identification and for determination of molecular masses, we used PAGE according to Schagger and Von Jagow [16] (it allows separation of small peptides) but not the classical method of Laemmli, one cannot exclude that real molecular mass of this fragment may be higher (according to Jorgensen's data its molecular mass should be about 48 kDa). This proteolytic fragment obtained as result of trypsinolysis of α-subunit in medium with ouabain or KCl co-precipitates with 67-kDa melittin-like protein. Mass-spectrometric analysis revealed that fragment includes the amino acid sequence of the α-subunit between amino acid residues 591 and 775.

Motif DPPRA, which was shown earlier to bind melittin in the α-subunit of H,K-ATPase from gastric mucosa (M(603)DPPRAT) is located in all isoforms of Na,K-ATPase and Ca-ATPase. It is located in the large cytoplasmic loop (MI(591)DPPRAA). Therefore, a fragment that can bind 67-kDa melittin-like protein potentially can bind melittin-like structures of different proteins. We conclude that a fragment of polypeptide chain of α-subunit including amino acid residues 591-775 interacts with 67-kDa melittin-like protein. The presence or absence of 0.5 mM ouabain in the medium for immunoprecipitation does not affect the interaction of these two proteins, although increase in binding of 67-kDa melittin-like protein in the presence of ouabain was observed for native Na,K-ATPase. In other words, a site for the binding of 67-kDa melittin-like protein in the proteolytic fragment of Na,K-ATPase is accessible both in the medium with ouabain or without ouabain, in contrast to the whole α-subunits, which was shown earlier coprecipitated with 67-kDa melittin-like protein only in the presence of 0.5 mM ouabain [11]. Apparently, the absence of an effect of ouabain on the binding of melittin-like protein is due to the removal of the proteolytic fragment from the whole protein, where the ouabain-binding site located between transmembrane fragments M4, M5, and M6 is present, as well as to the removal of transmembrane fragment M5 that participates in the transfer of a signal from the ouabain-binding site to cytoplasmic part of the α-subunit.

Thus, we have shown that a proteolytic fragment of Na,K-ATPase α-subunit with molecular mass of about

43 kDa that is the C-terminal part of a large cytoplasmic domain of the subunit (amino acid residues 591-775) including motif DPPRA that as shown earlier interacts with melittin in α-subunit of H,K-ATPase from gastric mucosa (M(603)DPPRAT) binds 67-kDa melittin-like protein. The binding of this protein with the proteolytic fragment of α-subunit does not depend upon the presence of ouabain.

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