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# Melittin induces both time-dependent aggregation and inhibition of Na,K-ATPase from duck salt glands however these two processes appear to occur independently

Ekaterina A. Shorina[a], Nataliya V. Dolgova[a], Alexander M. Rubtsov[a], Kenneth B. Storey[b], Olga D. Lopina[a,*]

[a] *Department of Biochemistry, School of Biology, M.V. Lomonosov Moscow State University, Moscow 119992, Russian Federation*
[b] *Institute of Biochemistry, Carleton University, Ottawa, Ontario, Canada KIS 5B6*

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**Abstract**

Using cupric phenanthroline as a cross-linking agent, we have shown that melittin induced time-dependent aggregations of Na,K-ATPase in microsomal fractions and in preparations of purified Na,K-ATPase from duck salt glands. Incubation of melittin with these preparations also led to the progressive loss of Na,K-ATPase activity. At melittin/protein molar ratio of 5:1, we did not observe inhibition of Na,K-ATPase in the microsomal fraction but the process of enzyme aggregation occurred. At higher melittin/protein molar ratios (10:1 and 30:1), the inhibition of the enzyme and its aggregation proceeded simultaneously but the rates of these processes and maximal values achieved were different. At a melittin/protein ratio of 30:1, Na,K-ATPase inhibition may be described as a biexponential curve with the values for pseudo-first order rate constants being 2.7 and 0.15 $min^{-1}$. However, the aggregation may be presented by a monoexponential curve with a pseudo-first order rate constant of 0.15 $min^{-1}$. In purified preparations of Na,K-ATPase, the maximal aggregation (about 90%) was achieved at a melittin/protein molar ratio of 2:1, and a further increase in the melittin/protein ratio increased the rate of aggregation but did not affect the value of maximal aggregation. The results show that melittin induced both aggregation and inhibition of Na,K-ATPase but these two processes proceeded independently.
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*Keywords:* Melittin; Protein aggregation; Na,K-ATPase

## 1. Introduction

Melittin is an amphiphilic peptide from bee venom consisting of 26 amino acid residues. The peptide has a positive charge (+6 at neutral pH values) concentrated mainly at the C-terminus. Melittin is able to interact with lipid bilayers of artificial and biological membranes. Because of its positive charge, the peptide binds preferentially to negatively charged lipids (for review, see Refs. [1,2]). Melittin was shown to interact also with various proteins including calmodulin [3–5], calsequestrin [6], G-proteins [7], myosin light chains [8], and myosin light chain kinase [9]. It also affects the activity of different enzymes: melittin activates protein kinase C [10], phospholipase $A_2$ [2], and inhibits ion-motive P-type ATPases [11–15].

It has also been shown that melittin induces the aggregation of intrinsic membrane proteins. In particular, it aggregates band 3 protein in red cell membranes [16], bacteriorodopsin reconstituted into the vesicles from dimiristoylphosphatidylcholine [17], and Ca-ATPase in sarcoplasmic reticulum (SR) membranes [18]. The aggregation appeared to be due to the direct interaction of melittin with proteins (not with the lipid bilayer) because it depended on the melittin/protein but not melittin/lipid molar ratio [17]. The suggestion that melittin provided this effect by cross-linking proteins into the large aggregates was confirmed by the finding that melittin induced the aggregation of both intrinsic membrane proteins and cytoplasmic soluble proteins, in particular, myosin light chain kinase [9].

Melittin is a potent inhibitor of different P-type ion-motive ATPases including gastric H,K-ATPase [11,12], Na,K-ATPase [13] and Ca-ATPase from plasma [14] and SR membranes [15]. Therefore, melittin induces both the

* Corresponding author. Tel.: +7-95-9394434; fax: +7-95-9393955.
*E-mail address:* od_lopina@mail.ru (O.D. Lopina).

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inhibition of SR Ca-ATPase and its aggregation resulting in the restriction of the rotation mobility of enzyme molecule [15,18]. Because a correlation was observed under certain conditions between the restriction of SR Ca-ATPase rotation mobility and the loss of its activity, it was suggested that the aggregation of Ca-ATPase molecules causes the enzyme inhibition [19]. We have found that both processes (Ca-ATPase inhibition and its aggregation) are time-dependent [20,21]. Comparison of time courses of melittin-induced aggregation and inhibition of SR Ca-ATPase has shown that these two processes occurred independently [21].

We have also shown that time courses of Na,K-ATPase and SR Ca-ATPase inhibition by melittin at high melittin/protein ratios are very similar: both may be described by biexponential curves suggesting the existence of two types of melittin-binding sites [20]. The purpose of this work was to determine whether or not melittin induces Na,K-ATPase aggregation and the relationships between the aggregation and inhibition of the enzyme. The data obtained clearly show that melittin induces not only inhibition but also time-dependent aggregation of Na,K-ATPase. However, there is no correlation between these two processes.

## 2. Materials and methods

ATP, EDTA, Tris, sucrose, SDS, ouabain, alamethicin, NADH, phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase were from Sigma-Aldrich (USA). Bee venom melittin, free of phospholipase $A_2$ activity, obtained by HPLC chromatography as described by Wille [22] and Voss et al. [15], was purchased from Aura (Moscow, Russia). The concentration of melittin stock solutions was measured spectrophotometrically at 280 nm using a molar extinction coefficient of 5400 $M^{-1}$ $cm^{-1}$.

### *2.1. Isolation of microsomal fraction and purified Na,K-ATPase from duck salt glands*

Microsomal fractions enriched by Na,K-ATPase and purified Na,K-ATPase were prepared from nasal (supraorbital) salt glands of domestic ducks following a protocol of Smith [23]. The final pellets were suspended in 0.25 M sucrose and 25 mM imidazole, pH 7.4, frozen in liquid nitrogen, and stored at −70 °C for use within 1–2 months. According to data from SDS-PAGE, the content of the α-subunit of Na,K-ATPase (a protein with a molecular mass of about 100 kDa) in microsomes enriched by Na,K-ATPase was about 30%. Na,K-ATPase activity in the microsomal fractions in the presence of alamethicin (3 μg/μg of protein) was equal to 100–200 μmol/mg of protein per hour; latent Na,K-ATPase activity was between 10% and 20% of total ATPase activity. Ouabain-independent Mg-ATPase does not exceed 20% of total ATPase. Activity of the purified enzyme was 1000–1600 μmol/mg of protein per hour at 37 °C; there was no Mg-ATPase in these preparations. Protein concentration was measured according to the method Lowry et al. [24] using BSA as a standard.

### *2.2. Measurement of melittin effect on Na,K-ATPAse activity*

To study the time dependence of Na,K-ATPase inhibition by melittin, microsomes enriched by Na,K-ATPase (1 mg/ml) or purified enzyme (0.3 mg/ml) were incubated at 20 °C for 0–30 min in a mixture of 25 mM imidazole, pH 7.4 and 250 mM sucrose with melittin at concentrations of 4, 10, 20 and 60 μM that provided melittin/Na,K-ATPase molar ratios of 2:1, 5:1, 10:1 and 30:1, respectively. Aliquots of 10 and 50 μl were taken from the samples containing purified ATPase and microsomes, respectively, at different time intervals and were added to the assay medium (1 ml). As result of this procedure, the concentration of melittin was decreased 20–100-fold, respectively. In control experiments, purified Na,K-ATPase or mircosomes were incubated in the same medium without melittin.

Na,K-ATPase activity was measured at 37 °C using an assay that coupled ATP hydrolysis to NADH oxidation [25]. Na,K-ATPase activity in microsomal fraction was determined as the difference between total ATPase activity and ouabain-insensitive (Mg-ATPase) activity measured under the same conditions but in the presence of 1 mM ouabain. Melittin did not affect Mg-ATPase activity. The assay medium (1 ml) consisted of 30 mM imidazole, pH 7.4, 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 3 mM ATP, 1.5 mM phosphoenolpyruvate, 0.2 mM NADH, 5 IU of lactate dehydrogenase, and 15 IU pyruvate kinase. In all cases, the activities of lactate dehydrogenase and pyruvate kinase did not limit total ATPase activity. It was also shown that melittin in the concentrations used did not affect the activity of lactate dehydrogenase and pyruvate kinase. Reaction was started by the addition of 2–5 μg of purified enzyme or 10–15 μg of microsomes enriched by Na,K-ATPase. The absorbance at 340 nm was continuously recorded on a Hitachi 200-20 spectrophotometer (Japan). Enzyme activity was calculated from the slope of the traces using a value 6220 $M^{-1}$ $cm^{-1}$ for the NADH absorption coefficient.

### *2.3. Measurement of melittin effect on Na,K-ATPase aggregation*

Cupric phenanthroline was used as a chemical cross-linking agent to reveal the formation of Na,K-ATPase oligomers [26]. Microsomes enriched in Na,K-ATPase (1 mg/ml) or purified Na,K-ATPase (0.3 mg/ml) were incubated at 20 °C in a solution of 25 mM imidazole, pH 7.4, 0.25 M sucrose and melittin at a concentration providing melittin/Na,K-ATPase ratios of 2:1, 5:1, 10:1 and 30:1. After incubation for 0–30 min, a solution of cupric phenanthroline (5 mM $CuSO_4$ and 15 mM 1,10-phenanthroline) was added to a final $CuSO_4$ concentration of 0.1 mM. After 2-min incubation, the cross-linking reaction was stopped by the addition of solution containing EDTA, *N*-ethylmalei-

mide, and SDS to final concentrations 1 mM, 16 mM, and 10 mg/ml, respectively. Samples were mixed with sample loading buffer that did not contain 2-mercaptoethanol and then proteins were separated using the Laemmli procedure of SDS-PAGE [27] with 3% stacking and 3–20% gradient running gels. After electrophoresis, the gels were fixed with solution of 25% isopropanol and 10% acetic acid, stained by Coomassie brilliant blue R-250, and washed with a mixture of isopropanol–ethanol. Then gels were scanned on an UltroScan XL laser densitometer (LKB, Sweden). The Na,K-ATPase α-subunit peak areas were calculated using the GelScanXL program (LKB).

### 2.4. Measurement of lipid content in membranes

Lipid phosphorus was measured according to the method of Bartlett [28] after ashing the preparations with $H_2SO_4$. Lipid content was calculated with the assumption that the mean molecular weight of phospholipid molecule is equal to 750 Da.

## 3. Results

### 3.1. Melittin effect on the cross-linking of the proteins in the microsomal fraction and in preparations of purified Na,K-ATPase

A method of chemical cross-linking by cupric phenanthroline was earlier successfully used to reveal the aggregation of Na,K-ATPase [26] and Ca-ATPase of the SR [21] induced by the interaction of these P-type ATPases with different ligands. In order to study melittin effects on the aggregation of Na,K-ATPase, microsomal fractions enriched in this enzyme and preparations of purified Na,K-ATPase from duck salt glands were incubated with melittin for different time intervals and then were treated by cupric phenanthroline for 2 min. Membrane proteins and newly formed protein aggregates in which individual protein chains were cross-linked by covalent S–S bonds were separated by SDS-PAGE. To prevent the disruption of S–S bonds, the samples were treated before electrophoresis by sample loading buffer without 2-mercaptoethanol.

Fig. 1 illustrates melittin effects on the cross-linking of proteins in preparations of Na,K-ATPase by cupric phenanthroline. It can be seen that at a melittin/protein molar ratio of about 10:1, the treatment of microsomal fractions by cupric phenanthroline after 2–32-min incubation of these fractions with melittin resulted in the progressive decrease of 100-kDa protein peak area (Fig. 1A). This treatment led simultaneously to the appearance of new protein bands corresponding to proteins with high molecular masses (about 120, 240, and higher). We did not observe a decrease in the amount of any other proteins present in the microsomal fraction except the 100-kDa protein that appears to be Na,K-ATPase α-subunit.

A similar effect was observed when preparations of purified Na,K-ATPase were treated by melittin (Fig. 1B). In these preparations two main proteins were present with molecular masses of about 100 and 65 kDa (Na,K-ATPase α- and β-subunits, respectively). At a melittin/protein molar ratio of 2:1, the treatment of purified Na,K-ATPase with cupric phenanthroline after 2–30-min incubation with melittin also resulted in a decrease in the peak area of the α-subunit and in the appearance of new protein bands corresponding to proteins with molecular masses of about 120, 240, and higher (in addition, some amount of protein did not enter into the gel). We did not observe a decrease in

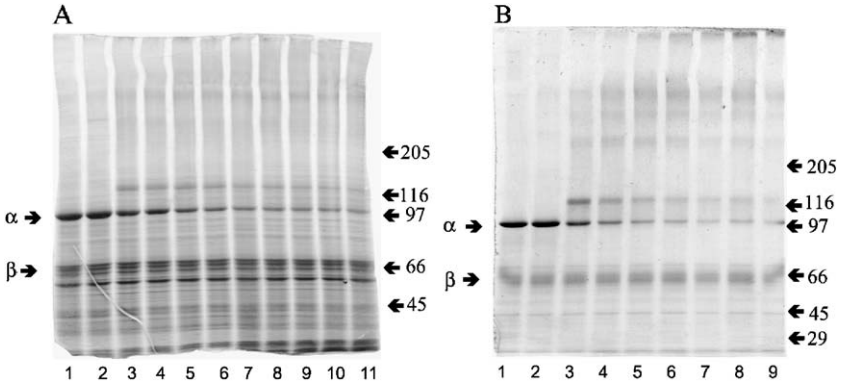


Fig. 1. SDS-PAGE of microsomal fractions enriched by Na,K-ATPase (A) and purified Na,K-ATPase (B) from duck salt glands incubated with melittin at melittin/Na,K-ATPase molar ratios of 10:1 and 2:1, respectively, and then treated by cupric phenenthroline for 2 min. Microsomal fractions were incubated with melittin for 0 min (lane 3), 2 min (lane 4), 7 min (lane 5), 10 min (lane 6), 13 min (lane 7), 17 min (lane 8), 22 min (lane 9), 27 min (lane 10), and 32 min (lane 11). Purified Na,K-ATPase was incubated with melittin for 0 min (lane 3), 2 min (lane 4), 5 min (lane 5), 10 min (lane 6), 15 min (lane 7), 20 min (lane 8), and 30 min (lane 9). Then, cupric phenanthroline was added to each sample for additional 2 min (see Materials and methods). Aliquots of 18 μg and 4 mg of protein were added to each lane in gels A and B, respectively. Lane 1, microsomal fraction (A) and purified Na,K-ATPase (B) without treatment by melittin and cupric phenenthroline, lane 2, microsomal fraction and purified Na,K-ATPase treated for 2 min with cupric phenenthroline. Arrows on the left side of gels indicate the positions Na,K-ATPase α and β subunits and arrows on the right show molecular mass protein standards (from top to bottom of gel): myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase B, 97 kDa; BSA, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa.

the amount of protein corresponding to β-subunit (molecular mass, 65 kDa) induced by melittin in purified preparation of Na,K-ATPase as well as in microsomal fraction after cross-linking of proteins by cupric phenenthroline (Fig. 1).

It should be noted that the origin of band corresponding to a 120-kDa protein that appeared after the treatment of Na,K-ATPase preparations by melittin and then by cupric phenanthroline is not completely clear. Potentially, 120-kDa protein aggregate may be the result of the cross-linking of two or more proteins with lower molecular masses or interaction of melittin tetramer with Na,K-ATPase α-subunit. However, we did not observe a decrease in the amount of any protein with molecular mass less than 100 kDa. It is important to note that 120-kDa protein band was seen even in the case if cupric phenanthroline was added immediately after the addition of melittin (Fig. 1A and B, lines 3). However, we did not see this band after treatment of preparations by cupric phenanthroline without melittin (Fig. 1A and B, lines 2) or after treatment of Na,K-ATPase preparations by melittin without subsequent addition of cupric phenanthroline (data not shown). It means that this band appeared as a result of the effect of cupric phenanthroline on the enzyme when Na,K-ATPase preparation was pretreated by melittin.

The appearance of the band corresponding to a 120-kDa protein was earlier observed when SR Ca-ATPase was treated by cupric phenanthroline [21]. It was explained by intramolecular cross-linking of the enzyme with cupric phenanthroline that caused the change in its electrophoretic mobility. We suggested that the 120-kDa protein band appeared also as result of intramolecular cross-linking of the Na,K-ATPase α-subunit. This band does not appear in the absence of melittin probably because under these conditions cross-linking agent has no access to corresponding SH-groups. The addition of melittin to Na,K-ATPase preparations appears to change very quickly the properties of the membrane or conformation of the enzyme that, in turn, increases the accessibility of particular SH-groups of enzyme α-subunit to cupric phenanthroline.

It can be seen that an increase in time of Na,K-ATPase incubation with melittin results not only in a decrease in the amount of the α-subunit but also in a decrease in the peak areas of the newly formed cross-linked protein of 120 and 240 kDa. By contrast, an increase in cross-linking products with higher molecular masses occurred with some lag phase. All these results demonstrate that incubation of Na,K-ATPase with melittin induces a time-dependent conversion of small aggregates of the enzyme into the larger aggregates.

### *3.2. Relationship between melittin-induced aggregation and inhibition of Na,K-ATPase*

We have shown earlier that melittin induced an irreversible time-dependent inhibition of Na,K-ATPase [20]. Fig. 1 illustrates that aggregation of Na,K-ATPase is also time-dependent in both purified and crude preparations. All aggregates are formed from the molecules of Na,K-ATPase and cupric phenanthroline cross-links the α-subunits in these aggregates by S–S bonds. Therefore, the loss of the α-subunit in 100-kDa peak area in our experiments is due to by the process of aggregation. It means that we can follow the aggregation estimating the decrease in the amount of α-subunit in the corresponding protein band. In order to understand the relationship between melittin-induced aggregation of Na,K-ATPase and the loss of its enzyme activity, we compared time courses of these two processes at different melittin/protein ratios. Results of these experiments are presented in Figs. 2 and 3.

Incubation of microsomal fractions enriched by Na,K-ATPase with melittin at melittin/protein ratios 5:1, 10:1, and 30:1 with subsequent cross-linking of proteins by cupric phenanthroline results in the progressive loss of protein in a band corresponding to the α-subunit (Fig. 2A). The decrease of the amount of α-subunit as result of a 30-min incubation

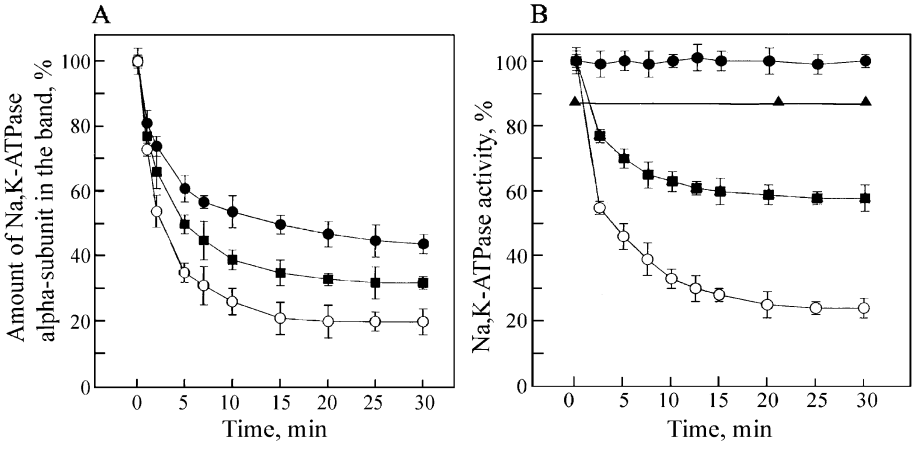


Fig. 2. Time courses of melittin-induced loss of Na,K-ATPase α-subunit as a result of enzyme aggregation (A) and loss of Na,K-ATPase activity (B) after incubation of microsomal fractions from duck salt glands with the peptide at melittin/Na,K-ATPase molar ratios of 5:1 (●), 10:1 (■), and 30:1 (○). Control without the addition of alamethicin and melittin is shown by triangles.

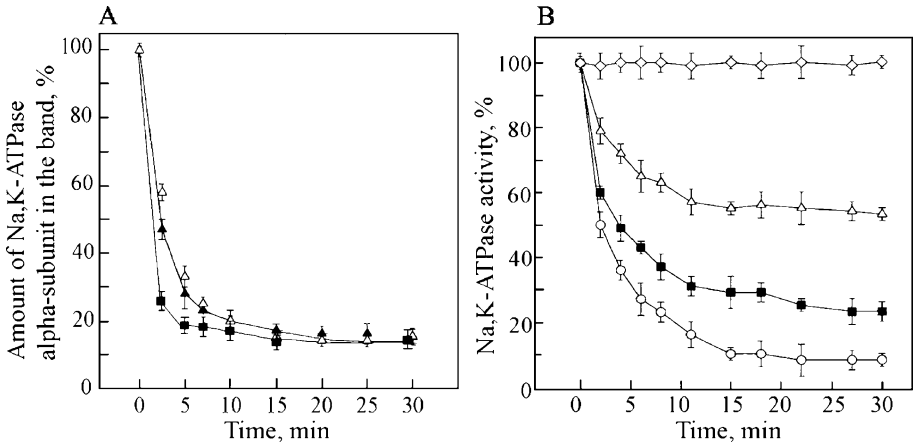


Fig. 3. Time courses of melittin-induced loss of Na,K-ATPase α-subunit as a result of enzyme aggregation (A) and loss of Na,K-ATPase activity (B) after incubation of purified Na,K-ATPase from duck salt glands without melittin (◇) and with the peptide at melittin/Na,K-ATPase molar ratios of 2:1 (△), 10:1 (■), and 30:1 (○). The lipid/protein molar ratio in the preparation of Na,K-ATPase used for aggregation studies was 0.6 (△) or 0.1(▲).

with melittin at a melittin/protein ratio of 5:1 was about 50%, increasing to about 70% and 80% at melittin/protein ratios of 10:1 and 30:1, respectively.

Over the same 30-min time period, we did not observe any inhibition of Na,K-ATPase activity in microsomal fractions at the 5:1 melittin/protein ratio, compared with the control sample containing alamethicin (Fig. 2B). Therefore, the addition of melittin to microsomal fractions at this ratio during 2 min increases the activity of Na,K-ATPase up to level that was observed in the presence of alamethicin. These data show that melittin at the melittin/protein ratio 5:1, during the first 2 min after addition of the microsomal fraction, increases permeability of the membrane and provides "demasking" of latent Na,K-ATPase. However, at this melittin/protein molar ratio (5:1), melittin does not inhibit Na,K-ATPase activity in the microsomal fraction.

Inhibition of Na,K-ATPase activity after 30 min of incubation of microsomal fractions with melittin at melittin/protein ratios 10:1 and 30:1 was about 40% and 80%, respectively (compared with controls containing alamethicin). Thus, the time courses of the loss of Na,K-ATPase activity and aggregation of 100-kDa protein in microsomal fractions look similar only at the highest melittin/protein ratio of 30:1, and we did not observe a similarity between these two processes at lower melittin/protein ratios (5:1 and 10:1). Analysis of the time dependence of inhibition and aggregation of Na,K-ATPase at a melittin/protein ratio of 30:1 showed that inhibition may be described by the sum of two exponents with a residual term (melittin-insensitive activity), whereas aggregation is described by monexponential curve with a residual term (portion of protein that does not aggregate in the presence of melittin). These data are presented in Table 1. We can see that melittin at the melittin/protein ratio of 30:1 provided for 85.5% of Na,K-ATPase activity inhibition, with the fast phase contributing 39% of the total inhibition, and the slow phase contributing 46.5%. The values of pseudo-first order rate constants for these two phases were equal to 2.7 and 0.14 $min^{-1}$. Under the same conditions, melittin induced aggregation of 81% of the total amount of α-subunit with the value of the pseudo-first order rate constant equal to 0.15 $min^{-1}$. Therefore, precise analysis demonstrates that there is no correlation between melittin-induced aggregation and inhibition of Na,K-ATPase even at melittin/protein ratio of 30:1.

Similar experiments were carried out with purified preparations of Na,K-ATPase from duck salt glands and the results are presented in Fig. 3. Na,K-ATPase in these preparations did not change after the addition of alamethicin (data not shown). Incubation of purified Na,K-ATPase with melittin for 30 min at a melittin protein molar ratio of 2:1 led to a progressive decrease of the amount of the α-subunit to less than 20% (Fig. 3A). However, the loss of Na,K-ATPase activity after the 30-min incubation of the preparation with melittin at the same melittin/protein ratio was less than 50%. A further increase of melittin/protein ratios to 10:1 and 30:1 resulted in an increase in enzyme inhibition over the 30-min incubation to about 80% and 95%, respec-

Table 1
Kinetic parameters of melittin-induced inhibition and aggregation of Na,K-ATPase in microsomal fractions of duck salt glands at a melittin/protein ratio of 30:1[a]

| Parameters | $A_1$, % | $A_2$, % | $A_0$, % | $k_1$, $min^{-1}$ | $k_2$, $min^{-1}$ |
|---|---|---|---|---|---|
| Inhibition | 39.0 | 46.5 | 14.5 | 2.7 | 0.15 |
| Aggregation | 81.8 | | 19.2 | | 0.15 |

[a] The time courses of melittin-induced Na,K-ATPase inhibition and aggregation were fitted to a single exponential (aggregation) or two exponentials (inhibition). $A_1$ and $A_2$, the amount of Na,K-ATPase activity (in the case of inhibition) or the amount of α-subunit (in the case of aggregation) lost during fast and slow phases, respectively; $A_0$, residual activity that is insensitive to melittin (in the case of inhibition) or portion of α-subunit that does not aggregate in the presence of melittin (in the case of aggregation); $k_1$ and $k_2$, the pseudo-first order rate constants of fast and slow phases, respectively.

tively (Fig. 3B). The increase in melittin/protein molar ratio to 5:1 (Fig. 3A) and 10:1 (data not shown) increased the rate of aggregation of the α-subunit but did not affect the total amount of aggregated protein: in both cases, this was about 80%. These data clearly show that in purified preparations of Na,K-ATPase, there is no correlation between melittin-induced aggregation of the enzyme and melittin-induced inhibition of its activity. Moreover, the curves describing the aggregation were the same when the lipid/protein ratio in purified preparations of the enzyme were decreased from 0.6 to 0.1. This means that the melittin effect on the aggregation of the enzyme is not determined by the melittin/lipid ratio.

## 4. Discussion

It was shown previously that melittin strongly restricted rotational diffusion of band 3 protein in erythrocytes [16], bacteriorodopsin in reconstituted vesicles from dimirystoylphosphatidylcholine [17], and Ca-ATPase in SR membranes [15]. This immobilization of integral membrane proteins was suggested to be due to the association of these proteins in the presence of melittin with creation of protein aggregates [15–17]. Analysis of data obtained by time-resolved phosphorescence anisotropy decay of SR Ca-ATPase labeled with erythrosine isothiocyanate has shown that conversion of highly mobile molecules into large aggregates occurred not immediately but step by step. At first, mobile molecules were converted into less mobile aggregates, and then into an immobile, very aggregated species [15]. Shorina et al. [21] confirmed that melittin induced increasing aggregation of Ca-ATPase in SR membrane using the method of chemical cross-linking with cupric phenanthroline. According to the molecular model proposed by Clague and Cherry [16], and supported by Voss et al. [15], for melittin-induced protein aggregation, the hydrophobic portion of melittin partitions into the bilayer, thereby anchoring the basic terminal part of the molecule close to the membrane surface. The basic part of the melittin molecule neutralized repulsion between negatively charged phospholipid headgroups and integral proteins that resulted in the aggregation of molecules of integral proteins.

In the present work using the method of cross-linking by cupric phenanthroline, we have found that melittin also induced the aggregation of Na,K-ATPase, another member of the P-type ATPase family. The aggregation was observed both in microsomal membranes and in membranes that were obtained after treatment of microsomal fractions with SDS and the subsequent purification of membranes by centrifugation in sucrose gradient. In contrast to Ca-ATPase, which consists of one polypeptide chain with molecular mass of about 100 kDa, the molecule of Na,K-ATPase is composed of two subunits: α- ($M_r \sim 100$ kDa) and β-subunit ($M_r \sim 55$–65 kDa) that are tightly attached to each other [29]. We found that after treatment of membranes with melittin, the cupric phenanthroline cross-linked only the 100-kDa protein corresponding to the α-subunit of Na,K-ATPase and did not cross-link the β-subunits. Because β-subunits are bound to the α-subunits very tightly (subunits may be separated only by treatment with high concentrations of detergents, such as SDS), it is difficult to imagine that melittin can separate the subunits and induce the aggregation of only α-subunits. A more reasonable explanation is that melittin induces aggregation of the αβ-dimers but the β-subunits do not cross-link by cupric phenanthroline, perhaps because they do not have access for oxidation of SH-groups. At the present time, the primary structure of the duck salt gland β-subunit is unknown. However, all Na,K-ATPase β-subunits, whose primary structures have been deduced from cDNA sequences, contain seven cysteine residues. Six of them form thee disulfide bonds and one is located in the middle of a single transmembrane domain and is resistant to sulfhydryl-labeling reagents in the absence of denaturing reagents [30]. Thus, in aggregates formed by Na,K-ATPase subunits in the presence of melittin, only the α-subunits are attached to each other by covalent S–S bonds after the addition of cupric phenanthroline. Treatment of such aggregates with SDS before electrophoresis will remove the β-subunits from the aggregates.

Earlier we have shown that melittin irreversibly inhibited Na,K-ATPase activity from duck salt glands and SR Ca-ATPase activity from rabbit skeletal muscle. At high melittin/ATPase molar ratios (30:1 or 50:1), the time dependence of the inhibition was described by the sum of two exponential curves [20]. The pseudo-first order rate constants for the fast and slow phases of inhibition of both enzymes are more than 10-fold different. We can see from Table 1 that the values of these constants for Na,K-ATPase at a melittin/protein ratio of 30:1 are 2.7 and 0.15 $min^{-1}$. These data indicate that there are two processes with different kinetic parameters, which lead to the inhibition of Na,K-ATPase by melittin. Because the value of the rate constant for the slow phase of inhibition at the 30:1 melittin/protein ratio is equal to the value of the rate constant for enzyme aggregation, one can suggest that under these conditions the slow phase of inhibition may be induced by enzyme aggregation. However, this suggestion contradicts the results presented in Figs. 1 and 2: at a melittin/protein ratio of 5:1 we observed aggregation of Na,K-ATPase in microsomal fractions but did not observe any loss of enzyme activity. In addition, we did not see any correlation between the loss of activity and the aggregation of Na,K-ATPase in purified preparations at melittin/protein ratios of 2:1 and 10:1. The data suggest that inhibition of Na,K-ATPase and its aggregation are two independent processes that proceed simultaneously. Earlier, using a similar approach, we have shown that melittin-induced inhibition of SR Ca-ATPase also is not due to the aggregation of the enzyme molecules [21].

Melittin has been shown to affect the function of many proteins including enzymes and their regulators. The interaction of melittin with Ca-binding proteins is now very well

characterized. Calmodulin and troponin C bind the peptide with a high affinity in a Ca-dependent manner and undergo significant changes in conformation [5]. The binding of melittin to calsequestrin (a calcium-binding protein of the SR lumen) results in a folding of the protein molecule into a more compact and trypsin-resistent structure [6]. Some calmodulin target proteins, for example, myosin light chain kinase, also bind melittin and they compete with calmodulin for specific binding site [9]. These findings raised the hypothesis that melittin might be similar to a certain protein determinant (module) participating in protein–protein interactions. It means that melittin-binding sites of the proteins might be sites for the binding of protein partners.

Another group of proteins whose function is affected by melittin are membrane embedded P-type ATPases. The mechanism of melittin effects on these proteins is not completely understood. There are at least two plausible explanations of melittin effect on their activity. One suggests that these membrane proteins, like Ca-binding proteins, contain a specific melittin-binding site, which serves as a macromolecular interaction site. Another explanation suggests that melittin affects the properties of the membrane lipid bilayer that, in turn, affects the conformation and the activity of the enzyme. Photoaffinity labeling has shown that a melittin analog interacts directly with the catalytic subunits of H,K-, Na,K- and SR Ca-ATPases [13,31,32]. Using this analog, two melittin-binding sites were identified in H,K-ATPase [32]. One of them is located in nucleotide-binding domain at sequence MI(603)DPPRAT that is highly conserved for all isoforms of Na,K-ATPase α-subunit and all SERCA isoforms. These data are consistent with the hypothesis assuming that these ATPases contain specific binding site for melittin and are located far from the surface of membrane lipid bilayer. It seems likely that inhibition of Na,K-ATPase and other P-type ATPases by melittin is due to its binding to this site.

It is unclear now why melittin binding results in time-dependent two-phase inhibition of Na,K- and Ca-ATPases. It reasonable to propose that if one of these phases is connected with melittin binding to molecules of ATPases, another one might be the result of the change in the membrane lipid bilayer caused by melittin. This, in turn, affects the conformation and the activity of the enzymes. We did not study melittin effects on the lipid bilayer of preparations of Na,K-ATPase. Therefore, a question on the cause of two-phase inhibition of Na,K- and SR Ca-ATPase by melittin should be addressed in future studies. However, we demonstrated that melittin-induced inhibition and aggregation of these two enzymes occur independently.

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