Melittin-Induced Inhibition and Aggregation of Ca-ATPase in Skeletal Muscle Sarcoplasmic Reticulum: A Comparative Study†

Ekaterina A. Shorina, Nataliya V. Mast, Olga D. Lopina, and Alexander M. Rubtsov*

Department of Biochemistry, School of Biology, Lomonosov Moscow State University, 119899 Moscow, Russia

Received April 22, 1997; Revised Manuscript Received August 4, 1997⊗

ABSTRACT: Incubation of melittin with sarcoplasmic reticulum membranes at pH 7.0 and different melittin:Ca-ATPase molar ratios results in the progressive loss of enzyme activity. At high melittin:Ca-ATPase molar ratios (10:1 and 30:1), enzyme inhibition may be described by a biexponential curve. At pH 7.0, the values of the pseudo-first-order rate constants are 1.0 and 0.1 min^{-1} for the fast and slow phases of inhibition, respectively, at a melittin:Ca-ATPase molar ratio of 30:1. At pH 6.0 and a melittin:Ca-ATPase molar ratio of 30:1, melittin does not inhibit Ca-ATPase. Melittin-induced aggregation of Ca-ATPase molecules was studied using cupric phenanthroline as a chemical cross-linking agent. At a melittin:Ca-ATPase molar ratio of 5:1, aggregation of Ca-ATPase protein was not observed; however, the loss of enzyme activity was about 30% after 30 min. At melittin:Ca-ATPase molar ratios of 10:1 and 30:1, significant aggregation of Ca-ATPase protein takes place. The rate of Ca-ATPase aggregation is much lower than the rate of enzyme inhibition. At melittin:Ca-ATPase molar ratios of 10:1 and 30:1, the rate of Ca-ATPase protein aggregation is close to that for the slow phase of enzyme inhibition. At pH 6.0 and a melittin:Ca-ATPase molar ratio of 30:1, significant aggregation of Ca-ATPase occurs. It is concluded that melittin induces both Ca-ATPase inhibition and aggregation. These two processes may occur simultaneously, but under some conditions either inhibition or aggregation takes place independently of each other. Therefore, the aggregation of Ca-ATPase induced by melittin is not necessary for enzyme inhibition.

Melittin, a basic 26-amino acid residue amphiphilic peptide from bee venom, is widely used as a tool for the study of protein−lipid and protein−protein interactions in artificial and biological membranes [for review, see (*1*, *2*)]. Melittin has a high positive charge at its C-terminus, and because of this charge and its amphiphilic properties, it can interact with many proteins and negatively charged phospholipids (*1*). In particular, melittin activates protein kinase C, Ca/calmodulin-dependent protein kinase II (*3*), and phospholipase A_2 (*2*); interacts with calmodulin (*4*), myosin light chains (*5*), and calsequestrin (*6*); and inhibits P-type ion-motive ATPases including the gastric H,K-ATPase (*7*, *8*), Na,K-ATPase (*3*, *9*), plasma membrane Ca-ATPase (*10*), and sarcoplasmic reticulum (SR)[1] Ca-ATPase (*11*−*14*).

Inhibition of ion-motive ATPases by melittin has been extensively studied in recent years. However, the mechanism of the melittin inhibitory action is not fully understood. Direct binding of melittin with the catalytic subunit of Na,K-ATPase and with SR Ca-ATPase was demonstrated using a photoactivatable analogue of melittin (*9*, *15*). In the H,K-ATPase molecule, two melittin binding sites were identified in the nucleotide binding and phosphorylation domains using the same approach (*16*). It has been suggested that the hydrophilic cytoplasmic domain of the SR Ca-ATPase also contains a melittin binding site(s), and occupation of this (these) site(s) by melittin results in enzyme inhibition (*14*−*17*).

An alternative point of view on the mechanism of melittin inhibitory action based on melittin-induced restriction of enzyme rotational mobility was put forward in studies of the SR Ca-ATPase (*11*−*13*, *18*). According to this view, the hydrophobic portion of the melittin molecule partitions at the bilayer surface with its hydrophilic and basic portions free to interact with acidic residues of the enzyme molecule on the membrane−water interface, thus electrostatically cross-linking Ca-ATPase into large aggregates. This aggregation restricts drastically Ca-ATPase rotational and/or conformational mobility and, therefore, decreases enzyme activity because a strong correlation between enzyme activity and protein rotational mobility was shown (*19*, *20*). The ability of melittin to induce large-scale aggregation of bacteriorhodopsin (*21*) and band 3 protein in red blood cells (*22*) was also described.

Recently we have found that the inhibition of Na,K-ATPase and SR Ca-ATPase by melittin depends on the time of enzyme incubation with the peptide (*17*). The time-course of enzyme inhibition at high melittin:ATPase molar ratio may be described by a biexponential curve suggesting the existence of two classes of melittin binding sites. In the present study, we have compared the time dependencies of melittin-induced Ca-ATPase inhibition and aggregation to elucidate the relationships between these processes. Evidence has been obtained that Ca-ATPase inhibition and aggregation induced by melittin occur independently.

† This work was supported by a grant to O.D.L. from the Russian Foundation for Basic Research (95-04-11918).

* To whom correspondence should be addressed.

⊗ Abstract published in *Advance ACS Abstracts,* September 15, 1997.

[1] Abbreviations: SR, sarcoplasmic reticulum; MOPS, 3-(*N*-morpholino)propanesulfonic acid; ATP, adenosine triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; NADH, nicotinamide adenine dinucleotide, reduced form; BSA, bovine serum albumin.

S0006-2960(97)00934-3 CCC: $14.00

MATERIALS AND METHODS

Reagents and Solutions. ATP, EGTA, EDTA, imidazole, Tris, sucrose, histidine, NADH, phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase were from "Sigma" (USA); SDS was from "Serva" (Heidelberg, Germany). Melittin from bee venom free of phospholipase A_2 activity, obtained by HPLC chromatography as described by Wille (*23*) and Voss et al. (*11*), 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} (*1*).

Computer processing of the data was done using the "Biochemical Utilities" program package designed by Dr. S. N. Fedosov (Department of Biochemistry, Moscow State University). Each experimental point was the result of three independent measurements; the mean values of them are presented on plots as a symbol. When the standard error is larger than the size of the symbol, it is indicated by the vertical bar.

Preparations and Assays. The light SR fraction was isolated from rabbit white skeletal muscle by differential centrifugation (*24*). The final pellet was suspended in 0.25 M sucrose and 25 mM imidazole, pH 7.0, frozen in liquid nitrogen, and stored at −70 °C for use within one month. Protein concentration was measured according to Lowry et al. (*25*) using BSA as a standard. According to SDS−PAGE data, the content of Ca-ATPase protein in these preparations was about 80%.

To study the time dependence of Ca-ATPase inhibition by melittin, the SR vesicles (1 mg/mL) were incubated at 20 °C for 0−30 min in medium containing 25 mM imidazole, pH 7.0, or 25 mM MOPS, pH 6.0, 250 mM sucrose, and melittin at concentrations of 40, 80, and 240 μM which provided melittin:Ca-ATPase molar ratios of 5:1, 10:1, and 30:1, respectively. Aliquots (5 or 10 μL) were taken at different time intervals and added to the assay medium (1 mL). As a result of this procedure, the concentration of melittin was decreased 200- or 100-fold, respectively. In control experiments, SR vesicles were incubated in the same medium lacking melittin.

Ca-ATPase activity was measured at 25 °C by an assay that coupled ATP hydrolysis to NADH oxidation (*26*). The assay medium (1 mL) consisted of 50 mM MOPS, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 3 mM ATP, 1 mM EGTA, 0.2 mM phosphoenolpyruvate, 5 IU of lactate dehydrogenase, 15 IU of pyruvate kinase, and 0.1 mg of NADH. The reaction was started by the addition of 5−10 μg of SR protein into the assay medium to record the base line (Ca-independent ATPase activity). The next step was the addition of $CaCl_2$ to 1 mM final concentration; then Ca ionophore A23187 (0.4 μg) was added. The absorbance at 340 nm was continuously recorded on a "Hitachi 200−20" spectrophotometer (Japan). Enzyme activities were calculated from the slopes of the traces using a value 6220 M^{-1} cm^{-1} for the NADH absorption coefficient. The presence of A23187 in the assay medium rendered the SR vesicles freely permeable to Ca^{2+}, so the measured ATPase activity represented the steady-state hydrolysis rate in the absence of a Ca^{2+} concentration gradient across the SR membrane. It was shown in a separate experiments that melittin in the concentrations used does not inhibit the activity of pyruvate kinase and lactate dehydrogenase.

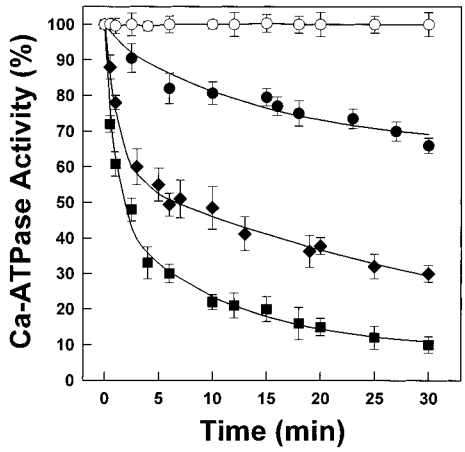

FIGURE 1: Time dependence of the inhibition of Ca-ATPase activity by melittin during incubation of SR vesicles with peptide at pH 6.0 (○) and 7.0 (●, ◆, ■). Incubation was carried out at melittin:Ca-ATPase molar ratios of 5:1 (●), 10:1 (◆), and 30:1 (○ and ■). The solid lines represent fits to single or double exponential decay with the parameters given in Table 1.

Cross-Linking and Electrophoresis. Cupric phenanthroline was used as a chemical cross-linking agent to evaluate the formation of Ca-ATPase oligomers in the SR membranes (*27*−*29*). The SR vesicles (1 mg/ml) were incubated at 20 °C in medium containing 25 mM imidazole, pH 7.0, or 25 mM MOPS, pH 6.0, 250 mM sucrose, and melittin in the concentrations 40, 80, and 240 μM which provided melittin:Ca-ATPase molar ratios of 5:1, 10:1, and 30:1, respectively. After 0−30 min of incubation, a stock 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, and $CaCl_2$ and MOPS, pH 7.0, were added to final concentrations of 1 mM and 25 mM, respectively. After 2 min of incubation, the cross-linking reaction was stopped by adding EDTA to 1 mM, *N*-ethylmaleimide to 16 mM, and SDS to 10 mg/mL. The Laemmli procedure for SDS−PAGE was used (*30*) with 3% stacking and 3−20% gradient running gels. After electrophoresis, the gels were fixed, stained by Coomassie Brilliant Blue R-250, and destained by a standard procedure; then they were scanned on an UltroScan XL laser densitometer (LKB, Sweden). The Ca-ATPase peak areas were calculated using the GelScanXL program (LKB, Sweden).

RESULTS

Time Dependence of SR Ca-ATPase Inhibition by Melittin. We recently demonstrated that inhibition of Ca-ATPase by melittin depends on the time of enzyme incubation with the peptide (*17*). At high melittin:Ca-ATPase molar ratio (50:1), inhibition of Ca-ATPase may be considered as a pseudo-first-order reaction. The time-course of enzyme inhibition is fitted by the sum of two exponentials characterizing fast and slow phases of the inhibitory process. Analysis of enzyme inhibition at melittin:Ca-ATPase molar ratios of 30:1 and 10:1 shows that under these conditions the inhibitory process is also biphasic (Figure 1, Table 1). The fast phase of inhibition results in about 50% and 40% loss of enzyme activity at melittin:Ca-ATPase molar ratios of 30:1 and 10:1, respectively, with the corresponding values of the rate constants being 1.00 and 0.80 min^{-1}. The slow phase of inhibition results in an additional loss of about 40% and 50% of enzyme activity at melittin:Ca-ATPase molar ratios of 30:1

Table 1: Inhibition of Sarcoplasmic Reticulum Ca-ATPase by Melittin[a]

melittin:Ca-ATPase molar ratio; pH	A_1, %	A_2, %	A_r, %	k_1, min^{-1}	k_2, min^{-1}
5:1; pH 7.0	29.9	–	70.1	0.11	–
10:1; pH 7.0	43.1	53.8	3.1	0.80	0.03
30:1; pH 7.0	52.7	38.7	8.6	1.00	0.10
30:1; pH 6.0	–	–	–	–	–

[a] The time-courses of Ca-ATPase inhibition by melittin were fitted to a single exponential (melittin:Ca-ATPase molar ratio of 5:1) or two exponentials (melittin:Ca-ATPase molar ratios of 10:1 and 30:1). A_1 and A_2, the amount of Ca-ATPase activity lost during fast and slow phases of inhibition, respectively; A_r, residual activity; k_1 and k_2, rate constants of fast and slow phases of inhibition, respectively.

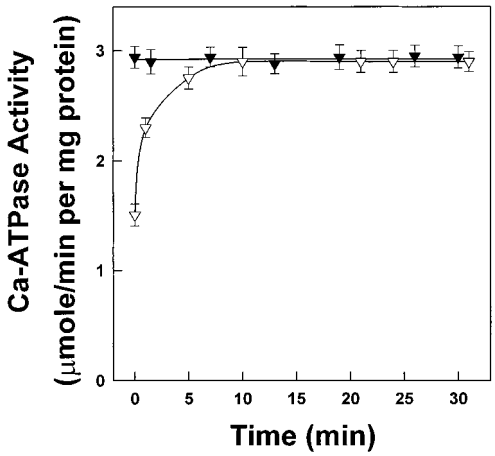

FIGURE 2: Effect of melittin on the Ca-ATPase activity of SR vesicles in the presence (▼) and in the absence (▽) of the Ca ionophore A23187. Vesicles were incubated with melittin at pH 6.0 (for details, see Materials and Methods).

and 10:1, respectively. Less than 10% of the Ca-ATPase activity is melittin-insensitive. At a melittin:Ca-ATPase molar ratio of 5:1 Ca-ATPase inhibition by melittin may be approximated by a single exponential with the maximal inhibition being about 30% (Table 1). These data show that inhibition of the enzyme is a time-dependent process at all of the melittin:Ca-ATPase molar ratios which were used.

Incubation of Ca-ATPase with melittin at pH 6.0 for at least 30 min did not lead to loss of the enzyme activity (Figure 1). However, under these conditions melittin strongly increased the rate of ATP hydrolysis by the Ca-ATPase in the absence of the Ca ionophore A23187 (Figure 2). Presumably, at pH 6.0, melittin interacts with SR membranes, and penetrates into the phospholipid bilayer, increasing the permeability of SR membranes for Ca^{2+}. The interaction of melittin with SR membranes at pH 6.0 does not result in Ca-ATPase inhibition.

Effect of Melittin on Cross-Linking of the Ca-ATPase in SR Membranes by Cupric Phenanthroline. A method of chemical cross-linking by cupric phenanthroline was used to evaluate melittin-induced aggregation of Ca-ATPase molecules in the membrane. SR membranes were incubated with melittin for different time intervals and then were treated with cupric phenanthroline for 2 min. Membrane proteins were separated by SDS−PAGE.

The effect of melittin on cross-linking of the Ca-ATPase by cupric phenanthroline is illustrated in Figure 3. At low melittin:Ca-ATPase molar ratio (5:1), the treatment of SR vesicles with cupric phenanthroline after 2−30 min incubation of samples with melittin did not decrease the Ca-ATPase peak area (Figure 3A, the data are presented quantitatively in Figure 4). Similar results were obtained in the absence of melittin (data not shown). Increasing the melittin:Ca-ATPase molar ratio to 10:1 led to the appearance of new protein bands with high molecular masses (about 120, 240, 330, 400, and 500 kDa) on the electrophoretograms (Figure 3B) and to the time-dependent decrease of the Ca-ATPase peak area (Figure 4). The appearance of the 120-kDa protein band seems to be connected with intramolecular cross-linking of the Ca-ATPase. The protein bands with molecular masses of about 240, 330, 400, and 500 kDa probably correspond to dimers, trimers, and tetramers of the Ca-ATPase. Their electrophoretic mobilities seem to be connected with the different number of intramolecular bonds in individual Ca-ATPase molecules forming these aggregates as established by Ross and McIntosh (*31*).

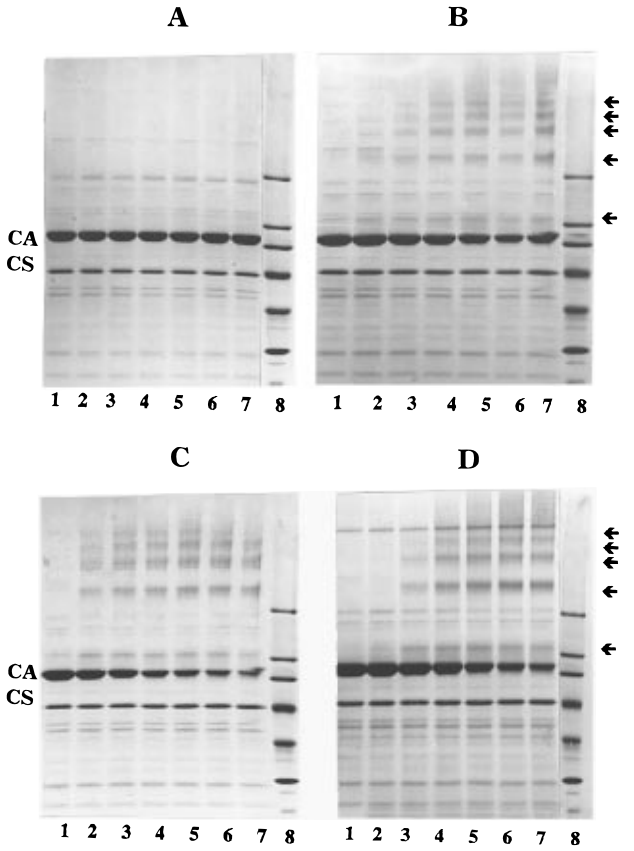

FIGURE 3: SDS−PAGE of SR vesicles incubated with melittin and treated by cupric phenanthroline. (A) Melittin:Ca-ATPase molar ratio of 5:1, pH 7.0. (B) Melittin:Ca-ATPase molar ratio of 10:1, pH 7.0. (C) Melittin:Ca-ATPase molar ratio of 30:1, pH 7.0. (D) Melittin:Ca-ATPase molar ratio of 30:1, pH 6.0. For each gel, SR vesicles were incubated with melittin for 0 min (lane 1), 2 min (lane 2), 5 min (lane 3), 10 min (lane 4), 15 min (lane 5), 20 min (lane 6), and 30 min (lane 7) after which cupric phenanthroline was added for 2 more min (for details, see Materials and Methods). Lane 8, molecular mass protein standards (from top to bottom of each gel, respectively: myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase *b*, 97.4 kDa; BSA, 66 kDa; ovalbumin, 45 kDa; and carbonic anhydrase, 29 kDa). CA, Ca-ATPase monomer; CS, calsequestrin. Arrows indicate new protein bands (cross-linked Ca-ATPase aggregates) with molecular masses (from top to bottom) of 500, 400, 330, 240, and 120 kDa, respectively.

Therefore, 2 min treatment of SR membranes with cupric phenanthroline after their incubation with melittin at a melittin:Ca-ATPase molar ratio of 10:1 results in cross-linking of the Ca-ATPase aggregates which were formed

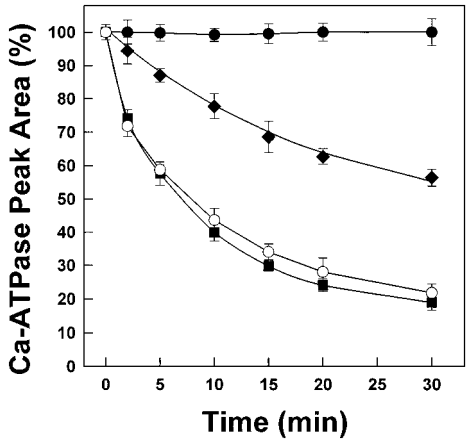

FIGURE 4: Time dependence of Ca-ATPase protein cross-linking by cupric phenanthroline after incubation of SR vesicles with melittin at pH 6.0 (○) and 7.0 (●, ◆, ■). Incubation was carried out at melittin:Ca-ATPase molar ratios of 5:1 (●), 10:1 (◆), and 30:1 (○ and ■). The solid lines represent fits to single exponentials with the parameters given in Table 2.

during the incubation. These protein aggregates were not found at the melittin:Ca-ATPase molar ratio of 5:1. An increase in the melittin:Ca-ATPase molar ratio to 30:1 increased Ca-ATPase oligomerization. The rate of decrease of Ca-ATPase peak area under these conditions was much higher (Figure 4), and the newly formed protein bands have the same high molecular masses (Figure 3C). Thus, only at high melittin:Ca-ATPase molar ratio (10:1 and 30:1) did melittin induce the formation of Ca-ATPase protein aggregates which were detected after 2 min treatment with cupric phenanthroline.

Incubation of SR vesicles with melittin at pH 6.0 and at a high melittin:Ca-ATPase molar ratio (30:1) which does not inhibit the enzyme activity (Figure 1) leads to the formation of Ca-ATPase protein aggregates (Figure 3D). The rate of decrease of the Ca-ATPase peak area at pH 6.0 is relatively high and is comparable to that at pH 7.0 and the same melittin:Ca-ATPase molar ratio (Figure 4). The new protein bands formed at pH 6.0 (Figure 3D) and 7.0 (Figure 3C) after treatment of SR membranes with cupric phenanthroline have the same molecular masses. Thus, the incubation of SR membranes with melittin at pH 6.0 induced large-scale oligomerization of Ca-ATPase protein. In contrast to pH 7.0, Ca-ATPase oligomerization at pH 6.0 was not coupled with the loss of enzyme activity.

Therefore, at the melittin:Ca-ATPase molar ratio of 5:1, Ca-ATPase oligomerization was not observed but the enzyme activity was inhibited by melittin. At pH 6.0, melittin did not inhibit Ca-ATPase activity despite significant enzyme molecule aggregation.

Analysis of the time-course of the Ca-ATPase oligomerization process shows that the rate of decrease of the Ca-ATPase peak area on electrophoretograms at pH 7.0 was increased with increase in the melittin:Ca-ATPase molar ratio from 10:1 to 30:1 (Figure 4). However, the rate of this process is much slower than the rate of enzyme inhibition. At the melittin:Ca-ATPase molar ratio of 30:1, the half-time for the decrease of Ca-ATPase peak area is about 7 min in comparison with 2 min half-time for the Ca-ATPase inhibition (compare Figures 1 and 3). To compare the inhibition and aggregation processes, the time dependence of the decrease of the Ca-ATPase peak area to a first approximation

Table 2: Parameters of Melittin-Induced Ca-ATPase Aggregation[a]

melittin:Ca-ATPase molar ratio; pH	A_1, %	A_r, %	k, min^{-1}
5:1; pH 7.0	–	–	–
10:1; pH 7.0	58.3	41.7	0.05
30:1; pH 7.0	80.6	19.4	0.14
30:1; pH 6.0	96.8	3.2	0.13

[a] The time-courses of the decrease of Ca-ATPase monomer peak area during melittin-induced protein aggregation were fitted to a single exponential. A_1, the amount of Ca-ATPase protein cross-linked by melittin; A_r, residual amount of Ca-ATPase; k_1, rate constant of the decrease of Ca-ATPase peak area.

can be described by monoexponential curve. It is evident that the rate constant for the decrease of Ca-ATPase peak area is much lower than the rate constant for enzyme inhibition in the fast phase and comparable with that for the slow phase of inhibition (Table 2).

DISCUSSION

It has been shown by many investigators that melittin is a potent inhibitor of SR Ca-ATPase (*11*−*13*) as well as other P-type ATPases (*7*−*9*). In these studies, usually SR Ca-ATPase and Na,K-ATPase preparations were incubated with melittin for 20−30 min before measurements of enzyme activity were made. However, the time-course of the melittin inhibitory effect was not previously studied in detail. We have shown recently that inhibition of SR Ca-ATPase and Na,K-ATPase by melittin is a time-dependent process (*17*).

It should be noted also that different investigators have used in their experiments a wide range of melittin and Ca-ATPase concentrations that complicates comparison of the data. Moreover, at a constant melittin concentration, the inhibition of Ca-ATPase depends on protein concentration. Thus, an increase in the Ca-ATPase concentration in the incubation medium leads to a decrease in enzyme inhibition (*14*). This is probably related to a relatively fast redistribution of melittin between the aqueous phase and SR membranes. According to Voss et al. (*11*), after 30 min of incubation melittin binds completely with SR membranes. To compare the data obtained in different experiments, these authors proposed to use a constant melittin:Ca-ATPase molar ratio over a wide range of protein concentrations. In the present study, we used the same approach, working with SR protein concentrations close to those used by other investigators (*11, 13*).

An analysis of the time-course of the melittin inhibitory action on the SR Ca-ATPase shows that at a high melittin:Ca-ATPase molar ratio, enzyme inhibition may be described by a sum of two exponentials (Table 1). This probably reflects the existence of two processes with different kinetic parameters which result in Ca-ATPase inhibition. It should be noted that the kinetic parameters of SR Ca-ATPase inhibition by melittin obtained in the present study are very close to those obtained with the use of much lower melittin and Ca-ATPase concentrations but with the same melittin:Ca-ATPase molar ratio (*17*).

As mentioned above, there are two alternative points of view on the mechanism of SR Ca-ATPase inhibition by melittin. According to one of these, melittin binds from the solution with a specific binding site on the cytoplasmic domain of the Ca-ATPase molecule without penetration into

the lipid bilayer (*15, 14*). According to another, melittin first penetrates into the lipid bilayer, interacts with negatively charged amino acid residues of the Ca-ATPase at the membrane−water interface, and induces protein aggregation that results in enzyme inhibition (*11*−*13, 18*). However, the time-courses of Ca-ATPase inhibition and aggregation induced by melittin were not studied by these investigators. Because two phases of Ca-ATPase inhibition by melittin were found in our experiments, we have compared the time-courses of Ca-ATPase inhibition and aggregation in the presence of melittin.

To study the process of aggregation of Ca-ATPase molecules, we used a method of cross-linking with cupric phenanthroline. This compound cross-links protein molecules located in close proximity to each other by inducing the formation of S−S bridges. As we have demonstrated previously, cross-linking of the Ca-ATPase with cupric phenanthroline is a relatively slow process (*28, 29*). Incubation of SR vesicles with cupric phenanthroline for 2−30 min results in a slow and gradual decrease of the peak area of the Ca-ATPase monomer and in the appearance of new protein bands with high molecular masses (cross-linked enzyme oligomers) on the electrophoretograms. We have also demonstrated earlier that short-term heat treatment of SR vesicles at 45 °C induces the formation of stable aggregates consisting of Ca-ATPase molecules in SR membranes (*28, 29, 32*). After such treatment, even 1−2 min incubation of SR preparations with cupric phenanthroline is sufficient for cross-linking of all the Ca-ATPase protein aggregates. The peak area of the monomeric form of the Ca-ATPase decreased sharply during the first 2 min of incubation (about 70% of enzyme molecules are cross-linked), and new protein bands with high molecular masses appear on electrophoretograms. Further increases in the incubation time of heat-treated SR preparations with cupric phenanthroline lead to a further slow and gradual decrease of Ca-ATPase peak area (*28, 29*). Therefore, these data show that 2 min incubation with cupric phenanthroline is enough for cross-linking all the stable Ca-ATPase protein aggregates existing in SR membranes.

A study of the time-course of melittin-induced Ca-ATPase aggregation using this chemical cross-linking method demonstrated that the process of aggregation is relatively slow, and its kinetic parameters correlate satisfactorily with the kinetic parameters of the slow phase of Ca-ATPase inhibition (Table 2). Therefore, there are two processes which lead to Ca-ATPase inhibition by melittin, and the slow process is probably connected with the aggregation of enzyme molecules.

Ca-ATPase aggregation induced by melittin is very well documented in studies where enzyme inhibition was compared with the changes of the Ca-ATPase oligomeric state using saturation transfer electron paramagnetic resonance and time-resolved phosphorescence anisotropy measurements (*11*−*13*). It was shown that 30 min incubation of SR membranes with melittin leads to a decrease of the content of Ca-ATPase monomers and to the formation of large protein aggregates. An analysis of protein aggregation at different melittin:Ca-ATPase molar ratios shows relatively good correlation of this process with the loss of enzyme activity. It was shown earlier (*20*) that the restriction of Ca-ATPase mobility connected with protein aggregation leads to a decrease of enzyme activity. Therefore, melittin-induced Ca-ATPase aggregation was suggested to be the main reason for melittin inhibition of enzyme activity (*11*−*13*).

Regulation of Ca-ATPase activity by aggregation−disaggregation induced by different peptides is probably universal. It was shown recently that in cardiac SR membranes, Ca-ATPase interaction with the endogenous membrane regulatory peptide phospholamban results in protein aggregation and inhibition *(33, 34*). Phosphorylation of phospholamban by cAMP- and/or Ca/calmodulin-dependent protein kinases quenches its positive charge and weakens its interaction with Ca-ATPase. As a result, enzyme molecules are disaggregated, and Ca-ATPase activity is increased, in good correlation with the increase of protein mobility (*33, 34*). Melittin-induced large-scale aggregation was reported not only for SR Ca-ATPase but also for bacteriorhodopsin (*21*) and band 3 protein in red blood cells (*22*). Therefore, it was concluded that melittin induces the aggregation of a number of membrane proteins, and Ca-ATPase aggregation leads to enzyme inhibition.

There is some evidence that inhibition of SR Ca-ATPase is not obligatorily connected with protein aggregation. For example, melittin inhibits Ca-ATPase after enzyme reconstitution into liposomes with high lipid:protein ratio where only a single Ca-ATPase molecule is present in each liposome, and protein aggregation cannot occur (*14*). Inhibition of Ca-ATPase by melittin was observed also after binding of two antibodies to the cytoplasmic domain of the enzyme, sterically hindering protein−protein interaction (*14*). On the other hand, melittin-induced Ca-ATPase aggregation does not necessarily lead to enzyme inhibition. At high ionic strength, inhibition of Ca-ATPase at melittin:Ca-ATPase molar ratios of 3:1 and 17:1 is practically the same but the oligomeric state of the enzyme differs drastically [see Figures 2, 5, and Table 1 in (*11*)]. At the melittin:Ca-ATPase molar ratio of 17:1 the content of enzyme monomers is decreased and the amount of protein aggregates is increased in comparison with the melittin:Ca-ATPase molar ratio of 3:1. We have recently found (*28, 29*) that short-term heating of SR membranes at 45 °C induces large-scale aggregation of Ca-ATPase protein without any loss of enzyme hydrolytic activity. Therefore, we can suggest that Ca-ATPase aggregation does not necessarily lead to its inhibition, and that the melittin inhibitory effect may take place without Ca-ATPase aggregation.

The data obtained in the present study are in good agreement with this suggestion. At the melittin:Ca-ATPase molar ratio of 5:1, there was no protein aggregation, but there was a significant loss of enzyme activity. In contrast, at pH 6.0 and a melittin:Ca-ATPase molar ratio of 30:1, large-scale Ca-ATPase aggregation occurred which did not lead to enzyme inhibition. At pH 7.0 and melittin:Ca-ATPase molar ratios of 10:1 and 30:1, melittin-induced Ca-ATPase aggregation and inhibition took place simultaneously. A comparison of the time-courses of Ca-ATPase aggregation and inhibition suggests that under these conditions protein aggregation correlates with the slow, but not with the fast, phase of enzyme inhibition. Therefore, another type of melittin interaction with the Ca-ATPase protein should exist which is not connected with the peptide-induced protein aggregation. Such an interaction seems to be connected with a specific melittin binding site on the protein molecule.

Using a radioactive photoaffinity analog of melittin, [^{125}I]-azidosalicylylmelittin, two melittin binding sites were identi-

fied in H,K-ATPase: at sequences MI(603)DPPRAT and Y(480)RERFP in the nucleotide binding and phosphorylation domains, respectively (*16*). This photoaffinity analog was also incorporated into the catalytic subunit of the Na,K-ATPase (*9*) and into Ca-ATPase (*15*). The motif MI(L)-DPPR is highly conserved, and the sequences MI(591)-DPPRAA and ML(600)DPPRKE are present in all isoforms of Na,K-ATPase α-subunits and in all SERCA isoforms, respectively (*35*, *36*). All of these sequences are located in the nucleotide binding domains of the corresponding enzymes far from the surface of the membrane. Therefore, it is likely that the putative melittin binding site has a similar structure and is located in the nucleotide binding domains of the H,K-ATPase, Na,K-ATPase, and Ca-ATPase. We have demonstrated earlier that ATP protects both the Ca-ATPase and Na,K-ATPase against inhibition by melittin (*17*). With the Ca-ATPase, this protection is connected with the elimination of the fast phase of enzyme inhibition without any significant effect on the slow phase of the inhibition. These data support the suggestion that the melittin binding site is located in the nucleotide binding domain of the Ca-ATPase. We suggest also that the direct interaction of melittin (from the solution) with this site is responsible for the fast phase of Ca-ATPase inhibition.

The slow phase of Ca-ATPase inhibition by melittin, at least under certain conditions, is probably connected with protein aggregation as suggested by the good correlation of the time-course of protein aggregation with the slow phase of Ca-ATPase inhibition. Nevertheless, our data clearly show that Ca-ATPase aggregation is not essential for inhibition of the enzyme. Some additional still unknown factors may be involved in the inhibition of SR Ca-ATPase by melittin connected with protein aggregation.

ACKNOWLEDGMENT

We thank Dr. R. Lozier for critical revision of the manuscript and helpful discussion. We are grateful to Drs. J. Cuppoletti and D. H. Malinowska for the linguistic improvement of the paper. Vasilii Betin provided excellent technical assistance. In addition, we thank two anonymous reviewers for their very thoughtful and helpful comments and suggestions.

REFERENCES

1. Dempsey, C. E. (1990) *Biochim. Biophys. Acta 1031*, 143−161.
2. Fletcher, J. F., and Jiang, M.-S. (1993) *Toxicon 31*, 669−695.
3. Raynor, R. J., Zheng, B., and Kuo, J. F. (1991) *J. Biol. Chem. 266*, 2753−2758.
4. Kataoka, M., Head, J. F., Seaton, B. A., and Engelman, D. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6944−6948.
5. Malencik, D. A., and Anderson, S. R. (1988) *Biochemistry 27*, 1941−1949.
6. He, Z., Dunker, A. K., Wesson, C. R., and Trumble, W. R. (1993*) J. Biol. Chem. 268*, 24635−24641.
7. Cuppoletti, J., Blumenthal, K. E., and Malinowska, D. H. (1989) *Arch. Biochem. Biophys. 275*, 263−270.
8. Cuppoletti, J. (1990) *Arch. Biochem. Biophys. 278*, 405−415.
9. Cuppoletti, J., and Abbot, A. J. (1990) *Arch. Biochem. Biophys. 283*, 249−257.
10. James, P., Maeda, M., Fisher, R., Verma, A. K., Krebs, J., Penniston, J. T., and Carafoli, E. (1988) *J. Biol. Chem. 263*, 2905−2910.
11. Voss, J., Birmachu, W., Hussey, D. M., and Thomas, D. D. (1991) *Biochemistry 30*, 7498−7506.
12. Mahaney, J. E., and Thomas, D. D. (1991) *Biochemistry 30*, 7171−7180.
13. Voss, J. C., Mahaney, J. E., and Thomas, D. D. (1995) *Biochemistry 34*, 930−939.
14. Baker, K. J., East, J. M., and Lee, A. G. (1995) *Biochemistry 34*, 3596−3604.
15. Cuppoletti, J., and Malinowska, D. H. (1992) *Mol. Cell. Biochem. 114*, 57−63.
16. Huang, P., Malinowska, D. H., Clark, M., Blumenthal, K. E., and Cuppoletti, J. (1996) *Biophys. J. 66*, A235.
17. Murtazina, D. A., Mast, N. V., Rubtsov, A. M., and Lopina, O. D. (1997) *Biochemistry (Moscow) 67*, 54−61.
18. Mahaney, J. E., Kleinschmidt, J., Marsh, D., and Thomas, D. D. (1992) *Biophys. J. 63*, 1513−1522.
19. Squier, T. C., and Thomas, D. D. (1988) *J. Biol. Chem. 263*, 9171−9177.
20. Birmachu, W., and Thomas, D. D. (1990) *Biochemistry 29*, 3904−3914.
21. Hu, K.-S., Duffon, M. J., Morrison, J., and Cherry, R. J. (1985) *Biochim. Biophys. Acta 816*, 358−364.
22. Clague, M. J., and Cherry, R. J. (1989) *Biochem. J. 252*, 791−794.
23. Wille, B. (1989) *Anal. Biochem. 178*, 118−120.
24. Ritov, V. B., Mel'gunov, V. I., Komarov, P. G., Alekseeva, O. M., and Akimova, E. I. (1977) *Dokl. Akad. Nauk SSSR 233*, 727−733.
25. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951*) J. Biol. Chem. 195*, 265−275.
26. Anderson, K. W., and Murphy, A. J. (1983) *J. Biol. Chem. 258*, 14276−14278.
27. Murphy, A. J. (1976) *Biochem. Biophys. Res. Commun. 70*, 160−166.
28. Geimonen, E. R., Rubtsov, A. M., and Boldyrev, A. A. (1993) *Biochemistry (Moscow) 58*, 1296−1306.
29. Geimonen, E., Batrukova, M. A., and Rubtsov, A. M. (1994) *Eur. J. Biochem. 225*, 347−354.
30. Laemmli, U. K. (1970) *Nature (London) 227*, 680−685.
31. Ross, D. C., and McIntosh, D. B. (1987) *J. Biol. Chem. 262*, 2042−2049.
32. Rubtsov, A. M., Boldyrev, A. A., Yang, L., McStay, D., and Quinn, P. J. (1994) *Biochemistry (Moscow) 59*, 1263−1268.
33. Voss, J., Jones, L. R., and Thomas, D. D. (1994) *Biophys. J. 67*, 190−196.
34. Voss, J. C., Mahaney, J. E., Jones, L. R., and Thomas, D. D. (1995) *Biophys. J. 68*, 1987−1795.
35. Sweadner, K. J. (1989) *Biochim. Biophys. Acta 988*, 185−220.
36. Carafoli, E. (1991) *Physiol. Rev. 71*, 129−153.

BI9709349