Mechanism of Inhibition of the Ca^{2+}-ATPase by Melittin†

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ABSTRACT: The Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum is inhibited by melittin at pH 7.4. Melittin has no effect on the rate of phosphorylation of the ATPase or on the rate of the Ca^{2+} transport step, but melittin inhibits dephosphorylation of the phosphorylated ATPase at pH 7.3. At pH 6.0, melittin has no effect on ATPase activity or on the rate of dephosphorylation. At pH 7.4, inhibition of ATPase activity fitted to a K_d of 0.4 μM for melittin. Analogues of melittin in which the two Arg residues were replaced by Gln [melittin(RR to QQ)] or the two Lys residues were replaced by Gln [melittin-(KK to QQ)] also inhibited ATPase activity, but with an increased K_d value of 3.4 μM. Analogues of melittin containing an extra Lys residue at the C-terminus [melittin(+K)] or in which the Trp residue had been replaced with a Leu residue [melittin(W to L)] had the same effect on activity as melittin. Melittin and all the analogues increased the permeability of the SR membrane to Ca^{2+} with equal potency at pH 6.4, as shown by a reduction in level of Ca^{2+} accumulation. Melittin and all the analogues also shifted the E2−E1 equilibrium of the ATPase toward E1 with equal potency at pH 7.2, consistent with stronger binding to the E1 conformation. It is suggested that effects on Ca^{2+} permeability and on the E2−E1 equilibrium could follow from binding of the N-terminus of melittin at the membrane−water interface, and that effects on ATPase activity could follow from binding of the positively charged C-terminus between the phosphorylation and nucleotide binding domains. Inhibition of ATPase activity by melittin is observed in reconstituted vesicles containing single ATPase molecules. Binding of monoclonal antibodies to the ATPase does not prevent inhibition of ATPase activity by melittin. We conclude that inhibition does not require aggregation of the ATPase molecules.

The activity of the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum is modified by interaction with a variety of peptides. Binding of phospholamban, a 52 amino acid peptide, to the SERCA2 isoform of Ca^{2+}-ATPase in cardiac muscle sarcoplasmic reticulum (SR)[1] inhibits ATPase activity (Tada, 1992). Experiments in which the SERCA1 isoform of the Ca^{2+}-ATPase, purified from skeletal muscle, has been reconstituted with phospholamban (Szymanska et al., 1990; Tada, 1992; Vorherr et al., 1992) or in which the ATPase has been coexpressed with phospholamban in COS cells (Toyofuku et al., 1993) have shown that the SERCA1 isoform can also be inhibited by phospholamban. Binding of the hydrophilic domain of phospholamban to the ATPase reduces the maximal rate of ATP hydrolysis by the ATPase by slowing the rate of dissociation of Ca^{2+} from the phosphorylated ATPase ($E1PCa_2 \rightarrow E2P$) (Hughes et al., 1994a).

Inhibition of the Ca^{2+}-ATPase by peptide toxins has also been reported. Myotoxin from rattlesnake venom has been shown to bind to the Ca^{2+}-ATPase and reduce Ca^{2+} uptake by SR vesicles (Mori et al., 1988; Utaisincharoen et al., 1991). Melittin from bee venom is also a potent inhibitor of the Ca^{2+}-ATPase (Voss et al., 1991; Mahaney & Thomas, 1991), the (H^++K^+)-ATPase (Cuppoletti et al., 1989; Cuppoletti, 1990), and the (Na^++K^+)-ATPase (Cuppoletti & Abbott, 1990). Melittin is a 26 amino acid amphipathic peptide with a high positive charge at the C-terminus (Dempsey, 1990). In crystals, it adopts an α-helical structure; in water, it exists either as a monomer with no defined structure or as a tetramer with α-helical structure (Dempsey, 1990). Melittin has strong hemolytic activity, and has been shown to bind to lipid bilayers, causing micellization at high concentrations (Dempsey, 1990). Melittin also causes aggregation of membrane proteins, including band 3 in red blood cells (Clague & Cherry, 1989), bacteriorhodopsin (Hu et al., 1985), and the Ca^{2+}-ATPase (Voss et al., 1991; Mahaney & Thomas, 1991; Mahaney et al., 1992). It has been suggested that aggregation of membrane proteins results from binding of melittin to the lipid bilayer with the N-terminal region embedded in the bilayer, with the positively charged C-terminus binding electrostatically to negative charges on the membrane protein (Hu et al., 1985). It has further been suggested that protein rotational mobility is required for the function of the Ca^{2+}-ATPase, and that aggregation, by preventing this motion, inhibits ATPase activity (Voss et al., 1991; Mahaney & Thomas, 1991; Mahaney et al., 1992).

More specific binding of melittin to membrane proteins is suggested by a recent study using a photoactivatable analogue of melittin. This has identified a binding site for melittin on the (H^++K^+)-ATPase at the sequence I^{603}DPPRAT in the nucleotide binding domain, and Y^{480}RERFP in the phosphorylation domain (Huang et al., 1994).

Here we characterize the effects of melittin on the kinetics of the Ca^{2+}-ATPase, and provide evidence using analogues of melittin for the existence of two classes of binding sites on the ATPase.

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[1] Abbreviations: SR, sarcoplasmic reticulum; NBD, 7-nitro-2,1,3-benzoxadiazole; FITC, fluorescein isothiocyanate.

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Table 1: Melittin and Its Analogues

	peptide sequence
melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2
melittin(RR to QQ)	GIGAVLKVLTTGLPALISWIKQKQQQ-NH_2
melittin(KK to QQ)	GIGAVLKVLTTGLPALISWIQRQRQQ-NH_2
melittin(+K)	GIGAVLKVLTTGLPALISWIKRKRQQK-NH_2
melittin(W to L)	GIGAVLKVLTTGLPALISLIKRKRQQ-NH_2

MATERIALS AND METHODS

Melittin and its analogues (Table 1) were synthesized by Dr. R. Sharma. All were purified by HPLC and shown to be pure using Vestec MALDI-TOF mass spectrometry, with sinapinic acid as matrix.

Sarcoplasmic reticulum from rabbit skeletal muscle and the purified Ca^{2+}-ATPase were prepared as described in Michelangeli et al. (1991). ATPase activities were determined at 25 °C as described (Michelangeli et al., 1991). Concentrations of ATPase were estimated using the extinction coefficient (1.2 L g^{-1} cm^{-1} for a solution in 1% SDS) given by Hardwicke and Green (1974). Free concentrations of Ca^{2+} were calculated using the binding constants for Ca^{2+}, Mg^{2+}, and H^+ to EGTA given by Godt (1974). Ca^{2+} uptake by sealed SR vesicles (0.17 mg of protein/mL) was monitored spectrophotometrically by using the dye murexide in 40 mM Hepes/KOH, pH 6.3, containing 5 mM Mg^{2+}, 100 mM KCl, and 50 μM Ca^{2+}. Spectra were run on an Aminco DW2000 dual-wavelength spectrometer with a wavelength pair of 507 and 542 nm, as described in McWhirter et al. (1987).

Measurements of equilibrium levels of phosphorylation of the ATPase by [^{32}P]P_i were carried out in 150 mM Mes/Tris, pH 6.3, containing 5 mM EGTA and the required concentration of P_i and Mg^{2+}, at 25 °C and a protein concentration of 0.1 mg/mL. Samples were incubated for 10 s and then quenched with 12% trichloroacetic acid, 0.2 M phosphoric acid. The precipitate was collected on Whatman GF/C filters, washed 3 times with quenching solution, and then counted.

The time dependence of phosphorylation of the ATPase by [γ-^{32}P]ATP at 25 °C was determined using a Biologic QFM-5 system as described in Starling et al. (1994). Melittin at the required concentration was present in both the ATPase and ATP containing syringes. The time dependence of dephosphorylation of the ATPase phosphorylated with [γ-^{32}P]ATP was determined using the triple-mixing capability of the Biologic QFM-5 system (Starling et al., 1994) with melittin present, when required, in all buffers. The time dependence of dephosphorylation of the ATPase phosphorylated with [^{32}P]P_i was determined as described by Henao et al. (1991). The time dependencies of Ca^{2+} dissociation and of phosphorylation-induced Ca^{2+} release were determined using a Biologic rapid filtration system at room temperature as in Starling et al. (1993, 1994).

Binding of ATP to the ATPase was determined as described by Dupont (1977). The ATPase (2 mg/mL) was incubated with [^{14}C]ATP in 150 mM MOPS/Tris, pH 7.2, containing 100 mM KCl, 5 mM Mg^{2+}, and 0.5 mM EGTA. The solution was filtered through a 0.45 μm Millipore filter under vacuum and the filter counted.

The ATPase was labeled with 7-nitro-2,1,3-benzoxadiazole (NBD) as described (Wictome et al., 1992a). Measurements of NBD and tryptophan fluorescence were performed at 25 °C using an SLM Amino 8000C fluorometer with excitation and emission wavelengths of 430 and 520 nm, and 290 and 340 nm, respectively.

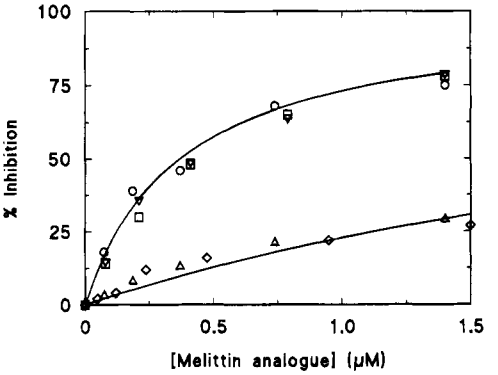


FIGURE 1: Inhibition of ATPase activity by melittin and its analogues. Shown are the effects of melittin (○), melittin(RR to QQ) (◇), melittin(KK to QQ) (△), melittin(+K) (□), and melittin-(W to L) (▽) on the ATPase activity of the purified ATPase (0.07 μM) measured at pH 7.4 and 25 °C in 40 mM Hepes/KOH, pH 7.4, at 2.1 mM ATP and 25 μM Ca^{2+}. Results are expressed as the percent inhibition of activity measured in the absence of melittin (2.3 IU/mg of protein).

Reconstitution of the ATPase into sealed vesicles in a monomeric form was performed as described by Heegaard et al. (1990). SR (0.3 mg) was solubilized with $C_{12}E_8$ (0.6 mg) in buffer (75 μL; 30 mM Tris/HCl, pH 7.1, 0.4 M KCl, 0.4 M sucrose, 4 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol, and 1 mM sodium azide). Phospholipid (21 mg) was suspended in the same buffer (300 μL) containing 11 mg of cholate and sonicated to clarity in a bath sonicator. The phospholipid and ATPase samples were then mixed and dialyzed for 60 h at 5 °C against 0.1 M Na_2HPO_4, pH 7.1, 4 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol, and 1 mM sodium azide, changing the dialysis buffer every 10 h.

RESULTS

Effects of Melittin and Its Analogues on ATPase Activity and Accumulation of Ca^{2+}. Addition of melittin to the purified Ca^{2+}-ATPase (0.07 μM; present as unsealed, membrane fragments) results in inhibition of ATPase activity (Figure 1). The extent of inhibition as a function of melittin concentration fits to a simple binding curve for melittin, with a K_d of 0.37 ± 0.03 μM, and a maximum inhibition of 100%. In contrast to our results with synthetic samples of melittin, Voss et al. (1991) reported biphasic inhibition of ATPase activity under comparable conditions using melittin purified from bee venom. The level of inhibition observed with 0.5 μM melittin was found to decrease from 60% at 0.07 μM ATPase to 20% at 0.4 μM ATPase, suggesting depletion of the melittin by binding to the membrane. Depletion of melittin occurred as a result of binding to the lipid component of the membrane, since addition of vesicles of di(C18:1)PC or of extra ATPase to 0.07 μM ATPase resulted in the same reduction in inhibition by 0.5 μM melittin when the amount of added lipid (calculated for the ATPase on the basis of a lipid:ATPase molar ratio of 30:1) was the same (data not shown). Greco and Hakala (1979) have shown that for a tight binding inhibitor the true inhibition constant K_i can be calculated from the equation:

$$K_i = 0.66I_{75} - I_{50} \quad (1)$$

where I_{75} and I_{50} are the concentrations of inhibitor resulting

in 75 and 50% inhibition of activity, respectively, at any given enzyme concentration. The data in Figure 1 give a K_i value of 0.37 μM, identical to that obtained by fitting the data to a simple binding curve. Thus, at the low ATPase concentrations used in these experiments (0.07 μM), depletion of melittin was not significant. Inhibition by melittin was fully reversed by addition of sonicated vesicles of di-(C18:1)PC to a final concentration of 1 mM.

Inhibition by the analogues melittin(W to L) and melittin-(+K) was very similar to that observed with melittin, but melittin(RR to QQ) and melittin(KK to QQ) were much less efficient at inhibiting ATPase activity, the data fitting to a K_d value of 3.4 ± 0.4 μM, with a maximum inhibition of 100% (Figure 1). Fitting the data to eq 1 gave a K_i value of 3.4 μM, again suggesting no significant depletion of the melittin analogues under these conditions.

The effect of melittin on ATPase activity was pH-dependent, with no inhibition being observed at pH 6 at concentrations of melittin up to 1.5 μM; inhibition at pH 8.0 was comparable to that shown in Figure 1 at pH 7.4 (data not shown). As reported previously by Voss et al. (1991), inhibition of ATPase activity by melittin at pH 7.4 is abolished in the presence of 500 mM LiCl. The effect, however, is not simply one of ionic strength. Thus, addition of 500 mM KCl or 500 mM choline chloride only reduced the inhibition caused by 2 μM melittin from 85% to 64%. LiCl itself had a greater inhibitory effect on the ATPase than KCl; 500 mM LiCl and KCl inhibited ATPase activity by 60% and 42%, respectively (data not shown).

In sealed SR vesicles, steady-state ATPase activity is low because of the buildup of a high concentration of Ca^{2+} within the vesicles; the Ca^{2+} concentration gradient can be collapsed by addition of the Ca^{2+} ionophore A23187, allowing the expression of full ATPase activity (de Meis, 1981). Effects of melittin and melittin(RR to QQ) on the ATPase activity of SR vesicles in the presence of A23187 are very similar to those observed for the purified ATPase (Figure 2B). However, in the absence of A23187, an increase in ATPase activity is observed (Figure 2A). As shown, addition of the weakly inhibitory analogues melittin(RR to QQ) and melittin-(KK to QQ) leads to a 5-fold increase in activity at 0.8 μM, with no further stimulation at higher concentrations. Melittin(W to L) and melittin(+K) also increase the activity of sealed SR vesicles, with maximal stimulation being observed at 0.2 μM, with higher concentrations leading to inhibition.

Melittin and all the analogues reduced the level of accumulation of Ca^{2+} by SR vesicles measured at pH 6.4, with very similar potencies (Figure 3). A fit for all the data to a single binding site with maximum inhibition of 100% is shown in Figure 3, giving a K_d value of 0.6 ± 0.1 μM. Fits to the data for the individual melittin analogues gave K_d values between 0.58 and 0.79 μM. Although more complex models would give better fits to the data, that shown in Figure 3 is judged acceptable given the difficulties in measuring accurately the low levels of accumulation of Ca^{2+} observed at high concentrations of the melittin analogues. At pH 7.4, melittin(W to L) and melittin(+K) were found to have a slightly greater potency (K_d = 0.4 ± 0.1 μM) than melittin(RR to QQ) and melittin(KK to QQ) (K_d = 0.6 ± 0.1 μM), presumably because of inhibition of ATPase activity at pH 7.4 by melittin(W to L) and melittin(+K) (data not shown).

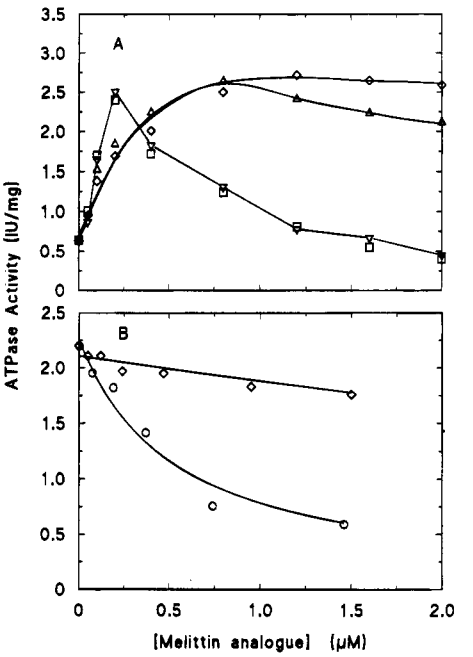


FIGURE 2: Effect of melittin and its analogues on the ATPase activity of SR vesicles. Shown are the effects of the analogues of melittin on the ATPase activity of SR (0.07 μM ATPase) in the absence (A) or presence (B) of 4 μg/mL A23187: (○) melittin; (◇) melittin(RR to QQ); (△) melittin(KK to QQ); (□) melittin-(+K); (▽) melittin(W to L). Activities were measured at pH 7.4 and 25 °C in 40 mM Hepes/KOH, pH 7.4, at 2.1 mM ATP and 25 μM Ca^{2+}.

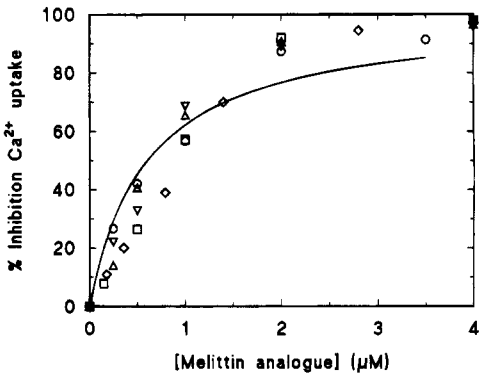


FIGURE 3: Effect of melittin and its analogues on accumulation of Ca^{2+} by SR vesicles. Shown are the effects of melittin (○), melittin-(RR to QQ) (◇), melittin(KK to QQ) (△), melittin(+K) (□), and melittin(W to L) (▽) on the maximal level of accumulation of Ca^{2+} by SR vesicles (1.5 μM ATPase) measured in 40 mM Hepes/KOH, pH 6.3, 5 mM Mg^{2+}, 100 mM KCl, 50 μM Ca^{2+}, and 50 μM ATP. Results are expressed as the percent inhibition of uptake measured in the absence of melittin (87.1 nmol of Ca^{2+}/mg of protein). The curve shows a fit to a single binding site for the melittin analogues with a K_d of 0.61 μM, maximal inhibition being fixed at 100%.

Effects of Melittin on Ca^{2+} and ATP Binding to the ATPase. Measurements of ATPase activity as a function of Ca^{2+} concentration show an increase in activity with increasing Ca^{2+} concentration in the micromolar range followed by a decrease in activity with increasing Ca^{2+} concentration in the millimolar range (Hughes et al., 1994b). The level of inhibition of ATPase activity by melittin was found to be independent of Ca^{2+} concentration (data not shown). Binding of Ca^{2+} to the high-affinity, transport sites on the ATPase can be monitored by observation of the resulting changes in tryptophan fluorescence intensity (Dupont & Leigh, 1978; Fernandez-Belda et al., 1984; Orlowski & Champeil, 1991b;

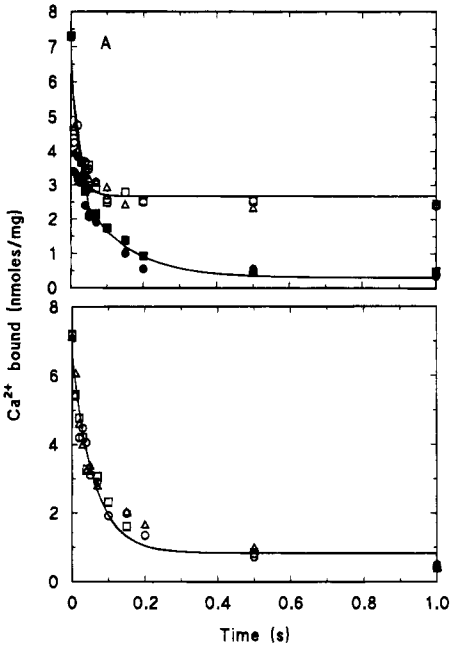


FIGURE 4: (A) Rapid filtration measurement of the rate of Ca^{2+} dissociation from the ATPase in the absence (○, ●) or presence of 2 μM melittin (□, ■) or melittin(RR to QQ) (△, ▲). The ATPase was incubated with $^{45}Ca^{2+}$ and then perfused with buffer (150 mM MOPS/Tris, pH 7.2, 100 mM KCl, and 20 mM Mg^{2+}) containing either 2 mM EGTA (●, ■, ▲) or 1 mM $^{40}Ca^{2+}$ (○, □, △). Curves are fits to single- and double-exponential decays, with the parameters given in the text. (B) ATP-induced release of $^{45}Ca^{2+}$ from the ATPase. ATPase (0.4 mg/mL) was first equilibrated in pH 7.2 buffer (150 mM MOPS/Tris, 100 mM KCl, and 20 mM Mg^{2+}) containing 100 μM $^{45}Ca^{2+}$ and 0.5 mM [^{3}H]sucrose, and then 0.2 mg of ATPase was adsorbed onto Millipore filters. The loaded filter was then perfused for the given periods with the same buffer containing 100 μM $^{40}Ca^{2+}$, 0.5 mM [^{3}H]sucrose, and 2 mM ATP, in the absence (○) or presence of 2 μM melittin (□) or melittin-(RR to QQ) (△). The lines represent fits to single-exponential decays with the parameters given in the text.

Henderson et al., 1994a). The change in fluorescence intensity on removal of Ca^{2+} is larger by a factor of 1.5 in the presence of 1.5 μM melittin or melittin(RR to QQ) than in its absence, and the concentration of Ca^{2+} resulting in half-maximal changes is increased from 0.8 μM in the absence of melittin or melittin(RR to QQ) to 2.5 μM in their presence (data not shown).

Neither melittin nor melittin(RR to QQ) had any effect on the rate of Ca^{2+} dissociation from the ATPase (Figure 4A). It has been found that if the $^{45}Ca^{2+}$-bound ATPase is adsorbed onto Millipore filters and washed with EGTA, then essentially all the bound $^{45}Ca^{2+}$ is lost, whereas on washing with 1 mM $^{40}Ca^{2+}$, only half the Ca^{2+} is lost (Dupont, 1984; Inesi, 1987; Michelangeli et al., 1990b; Orlowski & Champeil, 1991b). The data for the dissociation of $^{45}Ca^{2+}$ in the presence of $^{40}Ca^{2+}$ fit well to a single exponential (Figure 4A) with a rate of 40.0 ± 10.3 s^{-1}. The data obtained on washing with EGTA did not fit to a single exponential, but, as found previously (Starling et al., 1993), good fits could be obtained to the sum of two exponential processes of equal amplitude and rates of 84.3 ± 29.6 and 8.0 ± 1.2 s^{-1}.

At pH 8.0, Mg^{2+} has been shown to inhibit ATPase activity, probably by binding to Ca^{2+} binding sites on the phosphorylated ATPase (Bishop & Al-Shawi, 1988; Michelangeli et al., 1990a; Hughes et al., 1994b). The level of inhibition by melittin was independent of Mg^{2+} concentration at pH 8.0 (data not shown).

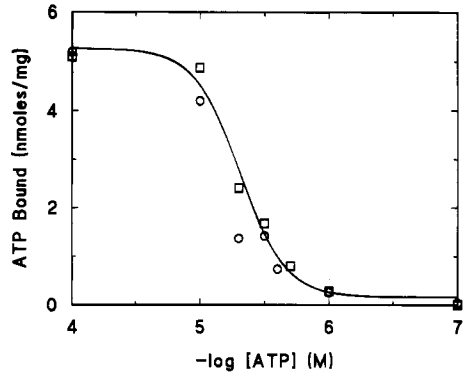


FIGURE 5: Effect of melittin on ATP binding to the ATPase. The ATPase was incubated with [^{14}C]ATP in 150 mM MOPS/Tris, pH 7.2, containing 100 mM KCl, 5 mM Mg^{2+}, and 0.5 mM EGTA in the absence (○) or presence (□) of 4 μM melittin, respectively. The line shows a fit to a single binding site for MgATP with a K_d value of 6.8 μM in the presence of melittin.

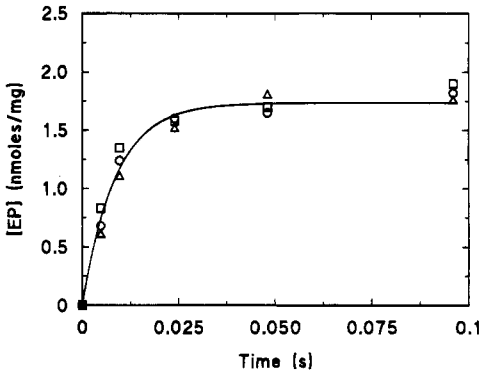


FIGURE 6: Rate of phosphorylation of the ATPase by [γ-^{32}P]ATP in the absence (○) or presence of 2 μM melittin (□) or melittin-(RR to QQ) (△). The ATPase (0.2 mg/mL) incubated in buffer (pH 7.2, 150 mM MOPS/Tris, 5 mM Mg^{2+}, 100 mM KCl, and 100 μM Ca^{2+}) was mixed in a 1:1 ratio with the same buffer containing 50 μM [γ-^{32}P]ATP to give a final ATP concentration of 25 μM. The line represents a fit to a single-exponential process with a rate of 116 s^{-1}.

Inhibition of ATPase activity by melittin was independent of ATP concentration in the ATP concentration range from 1 μM to 3.0 mM (data not shown). Binding of ATP to the ATPase was measured directly by filtration as described by Dupont (1977) and gave very similar K_d values (ca 7.0 μM) in the absence or presence of 4.0 μM melittin at pH 7.2, in the presence of 5 mM Mg^{2+} (Figure 5).

Effects of Melittin and Its Analogues on Partial Reactions of the ATPase. The rate of phosphoenzyme formation when the ATPase incubated in the presence of Ca^{2+} was mixed with 25 μM ATP at pH 7.2 was 116 ± 12 s^{-1} (Figure 6). In the presence of 2 μM melittin or melittin(RR to QQ), the rates of phosphorylation were not significantly changed (Figure 6).

The effect of melittin on the rate of dephosphorylation of the ATPase was investigated by first phosphorylating the ATPase with [^{32}P]P_i at pH 6.0 in the absence of Ca^{2+} and the presence of 14% dimethyl sulfoxide to give a high level of phosphoenzyme formation, followed by mixing with an excess of a pH 7.5 medium containing KCl and ATP. As shown in Figure 7A, an initial level of phosphorylation of ca. 1.8 nmol of [EP]/mg of protein was observed under these conditions, with dephosphorylation fitting to a single-exponential process with a rate constant of 17.3 ± 1.3 s^{-1}.

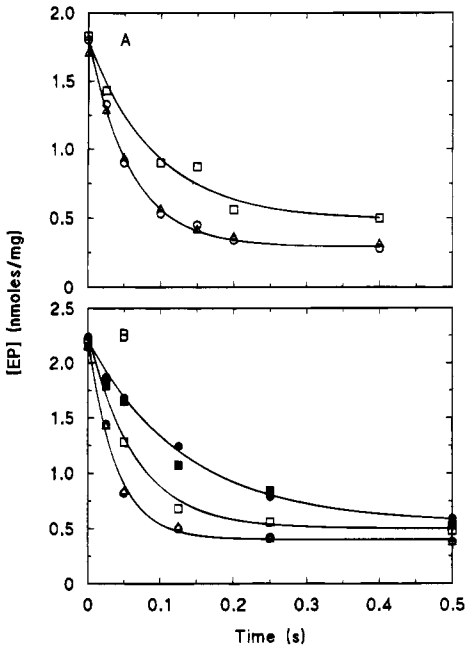


FIGURE 7: Effect of melittin and melittin(RR to QQ) on the rate of dephosphorylation of the ATPase in the absence (A) or presence (B) of Ca^{2+}. (A) The enzyme syringe contained ATPase (4.0 mg/mL) in 12.5 mM Mes/Tris, pH 6.0 containing 10 mM EGTA, 1 mM [^{32}P]P_i, 20 mM Mg^{2+}, and 14% (v/v) dimethyl sulfoxide. The second syringe contained 100 mM Mes/Tris, pH 7.5, containing 100 mM KCl, 4 mM Mg^{2+}, and 5.3 mM ATP. Syringes also contained either no melittin (○) or 2 μM melittin (□) or melittin-(RR to QQ) (△). The contents of the enzyme syringe were mixed in a 1:16 volume ratio with the dephosphorylation mixture (the pH after mixing was 7.3), and the reaction was quenched at the given times with 25% trichloroacetic acid, 0.2 M phosphoric acid. The solid lines represent fits to single exponentials with the parameters given in the text. (B) The enzyme syringe contained ATPase (0.2 mg/mL) in 150 mM MOPS/Tris, pH 7.2, 5 mM Mg^{2+}, 100 mM KCl, and 100 μM Ca^{2+} (○, □, △) or 130 mM Mes/Tris, pH 6.0, 5 mM Mg^{2+}, 100 mM KCl, and 100 μM Ca^{2+} (●, ■). This was mixed in a 1:1 ratio with a solution containing 100 μM [γ-^{32}P]ATP in the same buffer. The mixture was incubated for 200 ms, and then mixed in a 1:1 ratio with the same buffer containing 5.0 mM unlabeled ATP. The syringes also contained either no melittin (○, ●) or 2 μM melittin (□, ■) or melittin(RR to QQ) (△). The reaction was quenched at the given times with 25% trichloroacetic acid, 0.2 M phosphoric acid. The solid lines represent fits to single exponentials with the parameters given in the text.

In the presence of 2 μM melittin, the rate of dephosphorylation was reduced to 10.9 ± 2.6 s^{-1}, but the rate observed in the presence of melittin(RR to QQ) (16.1 ± 0.7 s^{-1}) was not significantly different than that measured in its absence (Figure 7A).

The effect of melittin on the rate of dephosphorylation of the ATPase phosphorylated by [γ-^{32}P]ATP in the presence of Ca^{2+} was determined in a triple mixing experiment. ATPase in the presence of Ca^{2+} was first incubated with 25 μM [γ-^{32}P]ATP for 200 ms to phosphorylate the ATPase which was then mixed in a 1:1 ratio with 5.0 mM cold ATP (Figure 7B). As shown, dephosphorylation under these conditions at pH 7.2 fits to a single exponential, with a rate constant of 25.6 ± 2.0 s^{-1}. The rate of dephosphorylation was again unaffected by 2 μM melittin(RR to QQ) but was reduced to 15.3 ± 1.8 s^{-1} by 2 μM melittin (Figure 7B). At pH 6.0, the presence of 2 μM melittin had no effect on the rate of dephosphorylation, which was 7.5 ± 0.7 s^{-1} (Figure 7B).

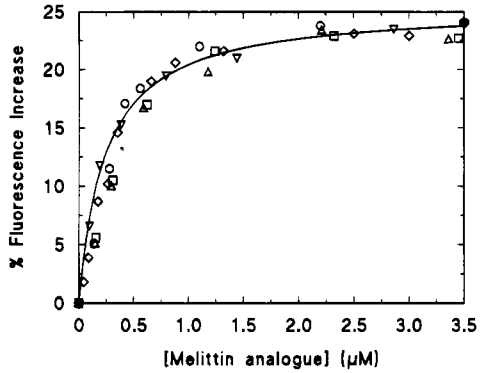


FIGURE 8: Effect of melittin and its analogues on the fluorescence intensity of NBD-labeled ATPase. Shown are the effects of melittin (○), melittin(RR to QQ) (◇), melittin(KK to QQ) (△), melittin-(+K) (□), and melittin(W to L) (▽) on the fluorescence of NBD-labeled ATPase measured at pH 7.2 in 150 mM MOPS/Tris, 0.3 mM EGTA, and 100 mM choline chloride. The solid line is a fit to a single binding site with a K_d of 0.2 μM.

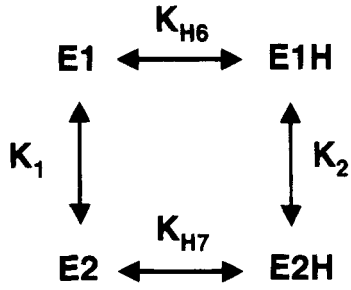
The effect of 2 μM melittin on the rates of dephosphorylation of the ATPase phosphorylated with P_i or ATP were the same if 2 μM melittin was contained in both the dephosphorylation medium and the medium containing the ATPase, or if the dephosphorylation medium contained 4 μM melittin, and the ATPase was incubated in medium containing no melittin (data not shown); we conclude that binding of melittin to the ATPase was fast on the time scale of the dephosphorylation experiment.

Melittin and melittin(RR to QQ) at 2 μM had no effect on the level of phosphorylation of the ATPase by P_i, measured at pH 6.0 in the presence of 10 mM Mg^{2+}, at concentrations of P_i between 1 and 20 mM (data not shown).

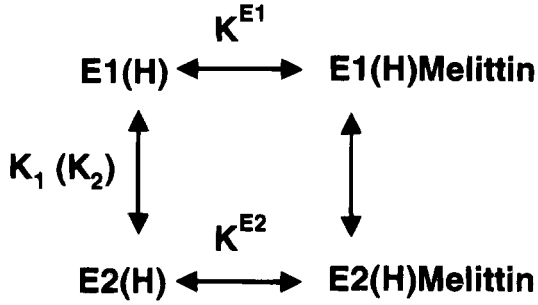
The time dependence of Ca^{2+} release from the ATPase on phosphorylation was determined by incubating the ATPase with 100 μM $^{45}Ca^{2+}$ in buffer containing 20 mM Mg^{2+} and 100 mM KCl, at pH 7.2 and then perfusing with the same medium containing 100 μM unlabeled Ca^{2+} and 2 mM ATP. The rate of dissociation of $^{45}Ca^{2+}$ from the ATPase under these conditions gives the rate of dissociation of Ca^{2+} from the phosphorylated ATPase (Orlowski & Champeil, 1991a; Henderson et al., 1994b). The data fit to single-exponential processes with rate constants of 16.5 ± 2.8, 21.3 ± 3.5, and 16.6 ± 2.8 s^{-1} in the absence and presence of 2 μM melittin(RR to QQ) and melittin, respectively (Figure 4B).

Effects of melittin on the E2−E1 equilibrium of the ATPase can be studied by making use of the conformational sensitivity of the fluorescence of NBD-labeled ATPase (Wakabayashi et al., 1990; Wictome et al., 1992b; Henderson et al., 1994a). Addition of melittin and all the analogues to NBD-labeled ATPase at pH 7.2 result in identical increases in fluorescence intensity, which fit to an effective K_d value of 0.2 μM (Figure 8). Effects of pH on NBD fluorescence have been shown to be consistent with Scheme 1, with values of K_{H6}, K_{H7}, and K_1 of 5 × 10^5 M^{-1}, 3.0 × 10^8 M^{-1}, and 4.0, respectively (Henderson et al., 1994a), and fluorescence intensities of 9.2 and 13.7 for the E1 and E2 states, respectively. The maximal fluorescence increase observed with melittin (Figure 8) is consistent with Scheme 1, with an increased value for the equilibrium constant E1/E2 of 40 in the presence of saturating concentrations of melittin.

Scheme 1



Scheme 2



From Scheme 2, the effective dissociation constant K^{eff} for binding of melittin to the ATPase is given by

$$1/K^{eff} = [(1/K^{E2}) + K_1(1/K^{E1})]/(1 + K_1)$$

where K^{E2} and K^{E1} are dissociation constants for melittin for E2 and E1 respectively. In terms of Scheme 2, a 10-fold increase in the equilibrium constant E1/E2 in the presence of excess melittin puts $K^{E1} = 10.0K^{E2}$, and with $K^{eff} = 0.2$ μM, K^{E2}, and K^{E1} become 1.64 and 0.16 μM, respectively.

Effect of Reconstitution of the ATPase in Monomeric Form. To test for possible effects of aggregation of the ATPase, we reconstituted the ATPase from solution in $C_{12}E_8$ into phospholipid vesicles at 10 000 phospholipid molecules per ATPase molecule, conditions which give sealed vesicles each containing a single ATPase molecule (Heegaard et al., 1990). It is necessary in these experiments to consider the effects of binding of melittin to the lipid in the system. If vesicles of dioleoylphosphatidylcholine prepared by dialysis from cholate solution are added to the purified ATPase, then the level of inhibition observed with melittin is reduced, presumably because of depletion of the melittin by binding to the lipid (Table 2). However, if the same experiment is repeated but with vesicles of dioleoylphosphatidylcholine containing a 1:1 molar ratio of cholesterol, then higher levels of inhibition are observed (Table 2).

The ATPase activity measured for the ATPase reconstituted in dioleoylphosphatidylcholine plus cholesterol is lower than that observed for the unreconstituted ATPase (Table 2), because of the formation of sealed vesicles in which ca. 50% of the ATPase molecules will be oriented with their ATP binding sites facing the lumen of the vesicles (Gould et al., 1987). Addition of 6 μM melittin(RR to QQ) to the reconstituted ATPase increases ATPase activity to a level comparable to that observed for the unreconstituted ATPase (Table 2), presumably by making the membrane permeable to ATP. Addition of 6 μM melittin to the reconstituted ATPase results in a lower activity than that observed with melittin(RR to QQ); the activity is comparable to that observed on addition of melittin to the unreconstituted ATPase in the presence of dioleoylphosphatidylcholine/cholesterol vesicles (Table 2). We have confirmed that the ATPase is in monomeric form when reconstituted in a 1:1 molar ratio of dioleoylphosphatidylcholine to cholesterol by showing that glutaraldehyde is unable to cross-link the ATPase (data not shown). We conclude that melittin is able to inhibit the ATPase when reconstituted in the monomeric form in sealed vesicles.

Table 2: Effects of Melittin on the Activity of the ATPase Reconstituted in Monomeric Form[a]

system	ATPase activity (IU/mg of protein) − melittin	+6 μM melittin-(RR to QQ)	+6 μM melittin
ATPase + di(C18:1)PC[b]	3.0	2.9	2.4
ATPase + di(18:1)PC + cholesterol[c]	2.9	2.7	1.9
reconstituted SR/di(C18:1)PC + cholesterol[d]	1.9	2.5	1.7

[a] ATPase activities measured at 2.1 mM ATP, 25 μM Ca^{2+}, 25 °C, in 40 mM Hepes/KOH, pH 7.2. [b] Reconstituted vesicles of di(C18:1)PC added to the purified ATPase at a molar ratio of phospholipid to ATPase of 10 000:1. [c] Reconstituted vesicles of a 1:1 molar ratio of di(C18:1)PC to cholesterol added to the purified ATPase at a molar ratio of phospholipid to ATPase of 10 000:1. [d] ATPase reconstituted in monomeric form with a 1:1 molar ratio of di(C18:1)PC to cholesterol, at a molar ratio of phospholipid to ATPase of 10 000:1.

Table 3: Effects of Monoclonal Antibodies on Inhibition of ATPase Activity by Melittin[a]

	ATPase activity (IU/mg)	% inhibition by 2 μM melittin
ATPase	2.95	90
ATPase + Y/1H12	2.77	84
ATPase + Y/1H12+1/2H7	2.71	80

[a] ATPase activities measured in 40 mM Hepes/KOH, pH 7.4, at 2.1 mM ATP, 25 μM Ca^{2+}, and 25 °C.

Effect of Melittin on ATPase Activity in the Presence of Monoclonal Antibodies. The ATPase was incubated for 30 min with two affinity-purified monoclonal antibodies raised against the ATPase, Y/1H12 and 1/2H7, at a molar ratio of antibody to ATPase of 3:1; it has been shown that a molar ratio of antibody to ATPase of 3:1 is sufficient to cause maximum inhibition with monoclonal antibodies which are inhibitory (Colyer et al., 1989). As shown previously (Colyer et al., 1989), neither of these antibodies affected the activity of the ATPase (Table 3). The presence of the antibodies had no significant effect on the inhibition of ATPase activity observed with melittin (Table 3).

DISCUSSION

Effects of melittin on the Ca^{2+}-ATPase are specific and different than those of other peptides such as phospholamban. Binding of melittin to the Ca^{2+}-ATPase results in inhibition of activity with a K_d value of 0.4 μM. Melittin had no significant effect on the affinity of the ATPase for Ca^{2+} as determined by the Ca^{2+} dependence of ATPase activity (data not shown). However, measurements of tryptophan fluorescence intensity as a function of Ca^{2+} concentration detected a 3-fold decrease in affinity for Ca^{2+} in the presence of 1.5 μM melittin or melittin(RR to QQ) (data not shown). Decreases in the affinity of the ATPase for Ca^{2+} have

previously been detected on binding a variety of positively charged molecules to the SR membrane, and have been attributed to effects of the buildup of positive charge at the membrane surface (Lee et al., 1983). Melittin does not affect the rate of dissociation of $^{45}Ca^{2+}$ from the ATPase either on washing with excess $^{40}Ca^{2+}$, when dissociation is observed only from the outer of the two Ca^{2+} binding sites, or on washing with EGTA, when dissociation is observed from both Ca^{2+} binding sites (Figure 4).

Melittin had little effect on the affinity of the ATPase for ATP (Figure 5), on the rate of phosphorylation of the ATPase by ATP (Figure 6), or on the rate of dissociation of Ca^{2+} from the phosphorylated ATPase (Figure 4B); inhibition of ATPase activity by phospholamban has been shown to follow from a decrease in the rate of this later step (Hughes et al., 1994a). Melittin does, however, have a marked effect on the rate of dephosphorylation. In the presence of 2 μM melittin, the rate of dephosphorylation of the ATPase phosphorylated with P_i was reduced from 17.3 to 10.9 s^{-1} (Figure 7A). The rate of dephosphorylation of the ATPase phosphorylated with ATP decreased from 25.6 to 15.3 s^{-1} in the presence of 2 μM melittin (Figure 7B).

The decrease in steady-state ATPase activity observed at 2 μM melittin (Figure 1) is greater than the decrease in the rate of dephosphorylation (Figure 7). This can be attributed to the higher concentration of ATPase used in the dephosphorylation experiment (0.4 μM) compared to that used in the measurement of ATPase activity (0.07 μM). Significant partitioning of melittin into the phospholipid bilayer portion of the membrane results in an increase in the apparent K_d for inhibition with increasing protein concentration (data not shown, but see below). We conclude that the observed inhibition of ATPase activity follows from reduction in the rate of dephosphorylation of the phosphorylated ATPase.

The C-terminus of melittin is highly positively charged (Table 1), so that charge effects are likely to be involved in the interaction between melittin and the Ca^{2+}-ATPase. We have found that at pH 6.0, melittin up to 1.5 μM had no effect on ATPase activity (data not shown), and melittin at pH 6.0 also had no effect on the rate of dephosphorylation of the phosphorylated ATPase (Figure 7B). A pK value of 6.5 has been suggested for Lys-21 in melittin [see discussion in Dempsey (1990)], which would be consistent with our results if this Lys was involved in binding; binding of melittin to the ATPase could also be affected by protonation at the binding site on the ATPase. The importance of charge interactions is shown more definitively by experiments with melittin analogues. The measured K_d value for inhibition of ATPase activity by the analogues melittin(RR to QQ) or melittin(KK to QQ) (Table 1) was much higher than for melittin itself (3.4 μM for the analogues compared to 0.4 μM for melittin) (Figure 1). As shown in Figure 7, 2 μM melittin(RR to QQ) had no detectable effect on the rate of dephosphorylation of the phosphorylated ATPase. Inhibition of the ATPase by the analogue melittin(+K) containing an extra lysine residue at the C-terminus was, however, identical to that observed with melittin (Figure 1), suggesting that the effects of melittin show some structural specificity and do not depend simply on the total number of positively charged residues. Inhibition of ATPase activity by the analogue melittin(W to L) in which the single tryptophan residue is replaced by leucine was identical to that observed with melittin (Figure 1); it has been reported that removal of this tryptophan residue results in a very marked reduction in the hemolytic potency of melittin (Perez-Paya et al., 1994).

Comparison of the effects of melittin and of the analogues melittin(RR to QQ) and melittin(KK to QQ) allows us to distinguish between the inhibitory effects described above and effects that are likely to follow from binding to the lipid bilayer portion of the membrane. Melittin has been shown to increase the permeability of lipid bilayers to a wide variety of small molecules (Dempsey, 1990). As shown in Figure 3, accumulation of Ca^{2+} by SR vesicles is inhibited with comparable potency by melittin and by the analogues melittin(RR to QQ) and melittin(KK to QQ) which show a much lower inhibitory potency against ATPase activity (Figure 1). It has been shown that the hemolytic potency of melittin is little affected by substitution of individual charged amino acids in the C-terminal region by leucine (Blondelle & Houghten, 1991).

An increase in the permeability of the membrane to Ca^{2+} caused by binding of the melittin analogues is also suggested by studies of the ATPase activity of sealed SR vesicles. The steady-state ATPase activity of sealed SR vesicles is low (Figure 2B) because of inhibition of the ATPase by the high concentrations of Ca^{2+} reached within the vesicles by the Ca^{2+}-pumping action of the ATPase. In the presence of the Ca^{2+} ionophore A23187 which prevents accumulation of Ca^{2+} by the SR vesicles, the measured ATPase activity is comparable to that seen for the purified ATPase present as unsealed membrane fragments (Figures 1 and 2A). Addition of melittin(RR to QQ) or melittin(KK to QQ) increases the activity of sealed SR vesicles, although not to the level observed on addition of A23187 (Figure 2B). Low concentrations of melittin also increase the ATPase activity observed for sealed SR vesicles, but the activity decreases at higher concentrations of melittin (Figure 2B), due to inhibition of the ATPase (Figure 1).

These experiments suggest two modes of binding of melittin to the SR membrane. The first is critically dependent on the polar C-terminal region, and accounts for inhibition of ATPase activity. The second, accounting for the increased permeability of the SR membrane to Ca^{2+}, is independent of the C-terminal region, and is thus likely to follow from binding of the amphipathic N-terminal region at the lipid−water interface of the membrane.

Two distinct modes of binding are also suggested by experiments on the effects of melittin and its analogues on the fluorescence intensity of NBD-labeled ATPase (Figure 8). The fluorescence of the NBD-labeled ATPase is believed to reflect the E2−E1 equilibrium for the ATPase (Wakabayashi et al., 1990; Wictome et al., 1992b; Henderson et al., 1994a), and effects of melittin at pH 7.2 are consistent with binding constants of 1.6 and 0.16 μM for E2 and E1, respectively (Figure 8). All the analogues of melittin had identical effects on the E2−E1 equilibrium (Figure 8), showing that the effects are independent of the nature of the C-terminal region. These effects could therefore follow from binding of the amphipathic N-terminal region of melittin and its analogues to the ATPase close to the membrane−water interface.

It has been shown that melittin causes aggregation of a wide variety of membrane proteins and it has been suggested that this aggregation could follow from binding of melittin at the membrane−water interface (Hu et al., 1985; Clague & Cherry, 1989; Voss et al., 1991; Mahaney & Thomas,

1991; Mahaney et al., 1992). It has also been suggested that the aggregation observed with the Ca^{2+}-ATPase was responsible for the observed inhibition of ATPase activity (Voss et al., 1991; Mahaney & Thomas, 1991; Mahaney et al., 1992). To test this possibility, we reconstituted the ATPase into sealed phospholipid vesicles, using conditions where it has been shown that each vesicle contains a small number of isolated, monomeric ATPase molecules (Heegaard et al., 1990). We reconstituted the ATPase into a 1:1 molar ratio of dioleoylphosphatidylcholine and cholesterol, since cholesterol reduced the partitioning of melittin into the phospholipid and thus gave a higher level of inhibition of ATPase activity by melittin, as shown in experiments in which the purified ATPase was simply mixed with phospholipid vesicles (Table 2).

Lower levels of ATPase activity were observed for the reconstituted ATPase than for the unreconstituted ATPase (Table 2) due to the formation of sealed vesicles in which ca. 50% of the ATPase molecules oriented with their ATP binding sites facing the lumen (Gould et al., 1987); addition of melittin(RR to QQ), by making the membrane leaky to ATP, unmasked the activity of these ATPase molecules (Table 2). The ATPase activity observed for the reconstituted vesicles was less in the presence of melittin than in the presence of melittin(RR to QQ) (Table 2), indicating inhibition of activity by melittin. We conclude that inhibition is unrelated to aggregation of the ATPase.

We also studied the effects of binding of monoclonal antibodies on inhibition by melittin. Monoclonal antibodies Y/1H12 and 1/2H7 have been shown to bind to the phosphorylation and nucleotide binding domains of the ATPase, respectively, in a noncompetitive manner (Colyer et al., 1989; Tunwell et al., 1991). The simultaneous presence of two antibody molecules bound to the surface of the ATPase might be expected to reduce the likelihood of aggregation of the ATPase. However, the presence of the antibodies had little effect on the degree of inhibition of the ATPase by melittin (Table 3).

A photoactivatable analogue of melittin has been shown to label the $(H^+ + K^+)$-ATPase at sequences I^{603}DPPRAT and Y^{480}RERFP, located in the nucleotide binding and phosphorylation domains, respectively (Huang et al., 1994). Since melittin inhibits a number of P-type ATPases (Cuppoletti, 1990; Cuppoletti et al., 1989; Cuppoletti & Abbott, 1990), and since the motif DPPR is highly conserved, it is likely that melittin binds to the corresponding region (L^{600}-DPPRKE) in the Ca^{2+}-ATPase. Antibody binding studies have suggested that this is a surface-exposed region of the ATPase (Mata et al., 1992). Fluorescence energy transfer experiments have located the nucleotide binding domain on the top surface of the ATPase, ca. 70 Å above the surface of the membrane (Stefanova et al., 1993). The length of the melittin molecule in an α-helical conformation is ca. 40 Å, so that a melittin molecule inserted into the lipid bilayer portion of the membrane is unlikely to be able to bind to such a site on the ATPase. Separate binding sites for melittin in this region of the ATPase and at the membrane−water interface are therefore probable. Binding of melittin to the ATPase, possibly between the nucleotide binding and phosphorylation domains, would lead to a reduction in the rate of dephosphorylation of the ATPase. Binding to the membrane−water interface would lead to an increase in permeability of the membrane to Ca^{2+}. Effects of melittin on the E2−E1 equilibrium of the ATPase could also follow from binding at the membrane−water interface. A precedent for two such classes of binding site for melittin occurs with protein kinase C. Melittin has been shown both to bind to the ATP binding domain of protein kinase C and to prevent phosphatidylserine-dependent activation, possibly by binding at a lipid binding site on the kinase (Raynor et al., 1991; Gravilt et al., 1994).

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