Effects of melittin on lipid–protein interactions in sarcoplasmic reticulum membranes

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ABSTRACT To investigate the physical mechanism by which melittin inhibits Ca-adenosine triphosphatase (ATPase) activity in sarcoplasmic reticulum (SR) membranes, we have used electron paramagnetic resonance spectroscopy to probe the effect of melittin on lipid-protein interactions in SR. Previous studies have shown that melittin substantially restricts the rotational mobility of the Ca-ATPase but only slightly decreases the average lipid hydrocarbon chain fluidity in SR. Therefore, in the present study, we ask whether melittin has a preferential effect on Ca-ATPase boundary lipids, i.e., the annular shell of motionally restricted lipid that surrounds the protein. Paramagnetic derivatives of stearic acid and phosphatidylcholine, spin-labeled at C-14, were incorporated into SR membranes. The electronic paramagnetic resonance spectra of these probes contained two components, corresponding to motionally restricted and motionally fluid lipids, that were analyzed by spectral subtraction. The addition of increasing amounts of melittin, to the level of 10 mol melittin/mol Ca-ATPase, progressively increased the fraction of restricted lipids and increased the hyperfine splitting of both components in the composite spectra, indicating that melittin decreases the hydrocarbon chain rotational mobility for both the fluid and restricted populations of lipids. No further effects were observed above a level of 10 mol melittin/mol Ca-ATPase. In the spectra from control and melittin-containing samples, the fraction of restricted lipids decreased significantly with increasing temperature. The effect of melittin was similar to that of decreased temperature, i.e., each spectrum obtained in the presence of melittin (10:1) was nearly identical to the spectrum obtained without melittin at a temperature ~ 5°C lower. The results suggest that the principal effect of melittin on SR membranes is to induce protein aggregation and this in turn, augmented by direct binding of melittin to the lipid, is responsible for the observed decreases in lipid mobility. Protein aggregation is concluded to be the main cause of inactivation of the Ca-ATPase by melittin, with possible modulation also by the decrease in mobility of the boundary layer lipids.

INTRODUCTION

Lipid and protein dynamics are important physical determinants of biological membrane function. In skeletal muscle sarcoplasmic reticulum (SR)[1] membranes, it has been demonstrated that optimal calcium uptake correlates directly with the ability of the Ca-adenosine triphosphatase (ATPase), an integral membrane protein, to undergo microsecond rotational motion in a fluid lipid bilayer. For example, reducing the rotational mobility of the ATPase by lowering temperature, selectively cross-linking the Ca-ATPase, or decreasing the lipid:protein ratio has a profound inhibitory effect on both adenosine triphosphate (ATP) hydrolysis and calcium uptake by SR (Bigelow et al., 1986; Squier and Thomas, 1988; Squier et al., 1988*a, b;* Birmachu and Thomas, 1990). Conversely, increasing the fluidity (decreasing the viscosity) of the SR lipids adjacent to the enzyme using diethyl ether results in an increased rate of Ca-ATPase rotation and a concomitant increase in Ca-ATPase activity (Bigelow and Thomas, 1987). These studies and many more (reviewed by Hidalgo, 1985, 1987) show that lipid-protein interactions play an important role in mediating molecular dynamics and enzymatic activity in the SR membrane.

Melittin, a 26-residue peptide isolated from honey bee venom (reviewed by Dempsey, 1990), is a member of a class of amphipathic, membrane-binding basic peptides known to alter the physical state of proteins and/or lipids in membranes. Melittin binds to a variety of proteins, including calmodulin (Malencik and Anderson, 1985; Kataoka et al., 1989; Raynor et al., 1991), protein kinase C (O'Brian and Ward, 1989; Raynor et al., 1991), and myosin light chains (Malencik and Anderson, 1988) and to membranes containing H,K-ATPase (Cuppoletti et al., 1989; Cuppoletti, 1990; Raynor et al., 1991), Na,K-ATPase (Cuppoletti and Abbott, 1990), bacteriorhodopsin (Hu et al., 1985), and erythrocyte band 3 (Dufton et al., 1984*a, b;* Clague and Cherry, 1988, 1989; Hui et al., 1990). Melittin recently has been shown to interact with SR membranes (Mahaney and Thomas, 1991; Voss et al., 1991), resulting in nearly complete abolition of Ca-ATPase activity.

Thomas and co-workers have studied the physical interaction of melittin with SR membranes to characterize the molecular mechanism of melittin-induced Ca-ATPase inhibition. Using time-resolved phosphorescence anisotropy, Voss et al. (1991) demonstrated that melittin binding to SR membranes restricts the rotational mobility of the Ca-ATPase by inducing large-scale protein aggregation. Similar aggregation effects have been observed in studies of bacteriorhodopsin (Hu et al., 1985) and erythrocyte band 3 (Dufton et al., 1984*b;* Hui

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[1] *Abbreviations used in this paper:* ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; EPR, electron paramagnetic resonance; MOPS, 3-(*N*-morpholino)propanesulfonic acid; 14-PCSL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine; 14-SASL, 14-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid; SR, sarcoplasmic reticulum.

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et al., 1990). In a complementary electron paramagnetic resonance (EPR) study, Mahaney and Thomas (1991) confirmed that melittin restricts protein mobility in SR but showed that the slight decrease in overall (bulk) lipid fluidity was not sufficient to explain the protein immobilization. They showed that the restriction in lipid chain motion was significant for C-5 stearic acid spin label but not for a C-16 label, suggesting that melittin interacts mainly with the membrane surface, consistent with studies in other systems (Batenburg et al., 1987; Schulze et al., 1987; Altenbach and Hubbell, 1988; Altenbach et al., 1989; Maurer et al., 1991).

Structural analyses of melittin (reviewed by Dempsey, 1990) have shown that the success of the peptide in binding to and perturbing membrane proteins and/or lipids lies both in its extreme amphipathic character and the high density of basic (positively charged) residues in its sequence, which enable the peptide to interact effectively both with surface anions (from protein or lipid) and with lipid hydrocarbon chains. Based on the physical features of melittin and the peptide's ability to aggregate membrane proteins, Clague and Cherry (1989) proposed a model describing melittin-induced membrane protein aggregation, in which the hydrophobic portion of melittin partitions into the bilayer, thereby anchoring the basic moieties close to the membrane surface. The basic groups on the peptide are then positioned properly to neutralize the repulsion from negative charges on the membrane surface, both between phospholipid headgroups and integral proteins and/or between integral proteins themselves. This model, which is supported by the direct physical studies provided by Mahaney and Thomas (1991) and Voss et al. (1991), depends on both the hydrophobic (lipid associating) and hydrophilic (protein associating) properties of melittin and suggests that the peptide may exert its effects specifically at the interface between protein and lipid, i.e., at the integral protein's boundary lipids. However, until now, no quantitative study addressing the effect of melittin on lipid-protein interactions of any integral protein has been reported.

In a previous study, Bigelow and Thomas (1987) used EPR spectroscopy of spin-labeled lipid analogues to study selectively the effects of diethyl ether on both Ca-ATPase boundary lipids and the bulk lipids of SR. They found that diethyl ether has no effect on the number of lipids occupying the annular shell of the Ca-ATPase, but it does fluidize selectively the boundary lipids of the enzyme by increasing the rotational mobility of their hydrocarbon chains while leaving the chain mobility of the bulk lipids relatively unaffected. This result suggests that ether has a specific mechanism of action at the protein-lipid interface, whereby fluidization of the lipids adjacent to the protein is responsible for the ether-induced increase in Ca-ATPase rotational motion and concomitant increase in enzyme activity. A similar study of the physical effects of melittin on the boundary lipids of the Ca-ATPase would determine whether the peptide interacts with the enzyme specifically at the protein-lipid interface to promote large-scale aggregation with concomitant inhibition of enzymatic activity.

In the present study, we have used EPR spectroscopy to investigate the effects of melittin on protein-lipid interactions in SR membranes. In particular, we have probed melittin's effects on the hydrocarbon chain dynamics of the boundary lipids of the Ca-ATPase to determine whether these lipids are involved in, or affected by, the inhibitory interaction of the peptide with the Ca-ATPase. The results of this study provide new insight into the interaction of melittin with a functioning membrane system and help to define the role of boundary lipids in the peptide's mechanism of action. On a more general level, the interaction of amphipathic peptides and other surface-binding proteins with membranes is of great interest, and defining their effects on integral protein boundary lipids should help to clarify our physical understanding of these interactions.

METHODS

Reagents and solutions

The spin labels used in this study were synthesized according to Marsh and Watts (1982). ATP, 3-(*N*-morpholino)propanesulfonic acid (MOPS), and melittin were obtained from Sigma (St. Louis, MO). All other reagents were obtained from Mallinckrodt (Darmstadt, Germany) and were of the highest purity available. All enzymatic assays and EPR experiments were carried out in 60 mM KCl, 6 mM $MgCl_2$, 0.1 mM $CaCl_2$, 20 mM MOPS, pH 7.0, unless otherwise stated.

Preparations and assays

SR vesicles were prepared from the fast-twitch skeletal muscle of New Zealand white rabbits (Fernandez et al., 1980). The vesicles were purified on a discontinuous sucrose gradient (Birmachu et al., 1989) to remove heavy SR vesicles (junctional SR containing calcium-release proteins). All preparation was done at 4°C. SR vesicle pellets were resuspended in 0.3 M sucrose, 20 mM MOPS (pH 7.0), rapidly frozen, and stored in liquid nitrogen until use. SR vesicles prepared in this fashion contained typically 80 ± 5% Ca-ATPase protein (i.e., 7.2 nmol Ca-ATPase/mg SR protein) and ~80 phospholipids per Ca-ATPase (i.e., 580 nmol phospholipid/mg SR protein) (Bigelow et al., 1986). The ATP hydrolysis of the SR vesicles was fully coupled to calcium transport (Squier and Thomas, 1989). SR lipids were extracted by a modification (Hidalgo et al., 1976) of the method of Folch et al. (1957), using nitrogen-saturated solvents to prevent lipid peroxidation. The lipids were stored in a chloroform:methanol (2:1 vol/vol) mixture under nitrogen at −20°C.

Calcium-dependent ATPase activity was measured as described by Squier and Thomas (1989). The molar concentration of phospholipids in SR and SR lipid extract samples was determined from phosphorus assays (Chen et al., 1956). SR protein concentrations were determined by the biuret assay (Gornall et al., 1949) using bovine serum albumin as a standard.

Melittin was purified according to Wille (1989), with modifications described by Voss et al. (1991). The purified melittin preparations used in this study contained no detectable phospholipase activity, as assayed by the procedure of Wille (1989). Stock solutions of melittin were prepared in the experimental buffer using lyophilized melittin. The concentration of these stock solutions was determined from the absorbance at 280 nm using $\epsilon_{280} = 5{,}400\ M^{-1}\ cm^{-1}$.

Spin labeling and sample preparation

Lipid hydrocarbon chain rotational mobility was measured using either a stearic acid spin label (14-(4,4-dimethyloxazolidine-*N*-oxyl)-stearic acid [14-SASL]) or a phosphatidylcholine spin label (1-acyl-2-[14-4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine [14-PCSL]). Before incorporation into SR, the spin labels were diluted from a dimethylformamide stock solution into ethanol (due to the greater miscibility of ethanol with water), usually to 10 mM for SASL and 2.25 mM (≈2 mg/ml) for PCSL. To incorporate 14-SASL into SR, the label was added to the membranes (25 mg/ml in 0.3 M sucrose, 20 mM MOPS, pH 7.0, 25°C), the sample was vortexed well, diluted by a factor of 10 with experimental buffer, and pelleted in a low-speed table-top centrifuge to remove any aqueous label. To incorporate 14-PCSL into SR, the label was rapidly added to the membranes (3 mg/ml in 0.3 M sucrose, 20 mM MOPS, pH 7.0, 25°C) while vortexing, and the mixture was incubated for 30 min at 25°C with intermittent vortexing. The SR was diluted into 0.3 M sucrose, 20 mM MOPS, pH 7.0, washed by centrifugation at 100,000 × *g* for 50 min at 4°C, and resuspended in the desired experimental buffer. Incorporation of 14-SASL or 14-PCSL into protein-free lipid samples (vesicles or aqueous dispersions) was accomplished by adding the spin label to the extracted lipids in a chloroform:methanol (2:1 vol/vol) mixture before drying with nitrogen. All samples contained 1 mol percent spin label, and sample concentrations were kept sufficiently high (>50 mg/ml SR; >50 mM SR lipids) to minimize the spectral contribution from unbound, aqueous labels. Labeling of SR was carried out at 4°C, whereas labeling of SR lipids was carried out at 25°C.

SR membrane samples containing melittin were prepared by first diluting melittin from a stock solution (>10 mM melittin) into experimental buffer, followed by the addition of 2 mg of spin-labeled SR (final volume 1 ml). The melittin-containing SR membranes were pelleted in a low-speed table-top centrifuge to concentrate the vesicles in preparation for EPR analysis. Control samples without melittin were treated identically, but substituting buffer for the melittin solution. All SR membrane samples were prepared at 25°C, and sample concentrations were 50 mg SR/ml or higher. Melittin binding to SR membranes under identical experimental conditions was quantified by Voss et al. (1991), who demonstrated clearly that ≥95% of the melittin added to the SR samples binds to the membranes. Electron micrographs of control SR membranes and SR membranes in the presence of melittin (up to 20 mol melittin/mol Ca-ATPase) show that the SR vesicles remain intact and have the same size distribution in the presence of melittin (data not shown).

Protein-free SR lipid samples (which consisted of aqueous dispersions of extracted SR lipids) containing melittin were prepared by adding melittin directly to the rehydrated lipids under vortex, followed by additional vortexing. To preserve the integrity of the protein-free lipid bilayers in these studies, the amount of melittin added to the protein-free lipid samples was kept low (Dempsey, 1990), i.e., at or below 5 mol melittin/80 mol SR lipids (equivalent to the melittin:Ca-ATPase mol ratio of 5:1). None of the protein-free lipid spectra contained evidence of melittin-induced spectral heterogeneity, which would arise from the formation of melittin-lipid-probe micelles in our samples (cf. Mahaney and Thomas, 1991). The spectra from the extracted lipids all consisted of a single axially anisotropic component that is characteristic of lipid spin labels in fluid bilayers; no second component with the reduced anisotropy characteristic of micelles was present.

EPR spectroscopy

EPR spectra recorded at the Max-Planck Institute were acquired using a spectrometer (model E-12 Century Line, 9 GHz; Varian, Sunnyvale, CA) equipped with a rectangular cavity (TE_{102}; Varian). Temperature was controlled to within ±0.5°C with a nitrogen gas flow temperature regulation system developed at the Max-Planck Institute. Spectra were digitized using an IBM microcomputer and downloaded to a PDP 11/10 computer system with interactive graphics for spectral analysis. The spectral acquisition and analysis software were developed by Dr. M. D. King and Dr. W. Möller (Max-Planck Institute). Spectra were obtained using 100-kHz field modulation with a peak-to-peak modulation amplitude of 1.6 G. The microwave power for all samples was set to 10 mW. Samples were contained in sealed 1-mm-diam glass capillaries accommodated within standard 4-mm-diam quartz tubes containing light silicone oil for thermal stability. At the University of Minnesota, EPR spectra were acquired with a spectrometer (model ESP-300; Bruker Instruments, Billerica, MA) equipped with a TE_{102} cavity (ER4201; Bruker) and digitized with the spectrometer's built-in microcomputer using OS-9-compatible ESP 1600 spectral acquisition software (Bruker). Spectra were downloaded to an IBM-compatible microcomputer and analyzed with software developed by R. L. H. Bennett (University of Minnesota). Spectra were obtained using 100-kHz field modulation with a peak-to-peak modulation amplitude of 2 G. Sample temperature was controlled to within 0.5°C with a variable temperature controller (model ER4111; Bruker). The microwave power for all samples was set to provide a microwave field intensity at the sample (H_1) of 0.07 g, usually 5–10 mW (Squier and Thomas, 1986). Samples were contained in 1-mm-diam glass capillaries. Equivalent experiments performed on the two spectrometers resulted in virtually identical EPR spectra.

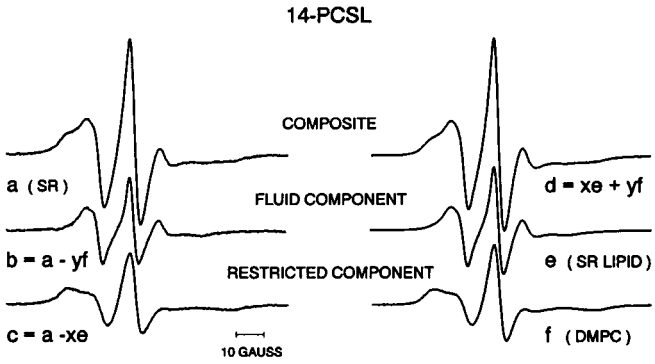

FIGURE 1 Procedure for separating the composite EPR spectra from SR membranes into motionally restricted and fluid components. (*a*) The experimental composite spectrum of 14-PCSL incorporated in SR membranes at 0°C; (*b*) a fluid component spectrum obtained by subtracting 59% ($y = 0.59$) of the intensity of spectrum *f* from spectrum *a*; (*c*) the restricted component spectrum obtained by subtracting 41% ($x = 0.41$) of the intensity of spectrum *e* from spectrum *a*; (*d*) summed composite spectrum obtained by combining 41% ($x = 0.41$) of the intensity of spectrum *e* with 59% ($y = 0.59$) of the intensity of spectrum *f*; (*e*) the experimental spectrum of 14-PCSL in an aqueous dispersion of extracted SR lipids at −2°C; (*f*) the experimental spectrum of 14-PCSL incorporated into sonicated DMPC liposomes at 6°C. Spectra were normalized to the same spin concentration before analysis.

EPR spectral analysis

EPR spectra of 14-SASL or 14-PCSL in SR membranes contain contributions from two distinct motional populations of probes (reviewed by Hidalgo, 1985; Marsh, 1985; Bigelow and Thomas, 1987). One component corresponds to labels in a fluid lipid environment, similar to that of spectra obtained from labels in aqueous dispersions of extracted lipids. The other component, which is most evident in the outer wings of the spectra, corresponds to labels in a considerably more restricted environment. This component has been shown to arise from labels whose motion is restricted by interaction with the integral membrane proteins of SR (Marsh, 1985). As demonstrated in Fig. 1, these composite spectra were resolved into single component spectra by subtraction, essentially as described by Marsh (1982).

Difference spectra corresponding to the motionally restricted component (e.g., Fig. 1 *c*) of SR composite spectra (e.g., Fig. 1 *a*) at low

temperatures (0–5°C) matched the 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) model spectra (e.g., Fig. 1 *f*) nearly identically and were virtually free from irregularities caused by spectral mismatch during the subtractions. However, subtraction of composite spectra at temperatures above 20°C was severely hampered due to lack of adequate matching between the fluid component of the composite and any model spectrum of extracted SR lipids. Such mismatch resulted in large spectral irregularities in the fluid region of the restricted-component difference spectra, which made accurate selection of the restricted endpoint nearly impossible. This is probably due to exchange between fluid and motionally restricted components in the membrane, which partially averages the two spectral components and thus prevents their precise resolution (cf. Davoust and Devaux, 1982). Therefore, quantitative analysis was carried out only for spectra obtained at or below 20°C.

Single-component spectra were analyzed by measuring the inner ($2T_\perp'$) and outer ($2T_\parallel'$) spectral splittings (where resolved) (Squier and Thomas, 1989), which are sensitive both to the rotational amplitude and rotational rate (Moser et al., 1989) of the spin label, and the half-width at half-height of the low-field peak (Δ_L), which is sensitive mainly to the rate of motion of the spin label. However, changes in the parameters $2T_\parallel'$ and $2T_\perp'$ and Δ_L cannot be related linearly to the changes in lipid chain mobility because of the complications introduced by slow motional effects and inhomogeneous broadening, respectively. Therefore, to facilitate comparison of the effects of melittin on these and other parameters, a melittin-induced change in a given parameter is converted to an "effective temperature change" (cf. Bigelow et al., 1986; Bigelow and Thomas, 1987), defined as the temperature change required to produce the same parameter value in a control sample without melittin. This allows the comparison of changes in different spectral parameters with each other and with changes in enzymatic activity.

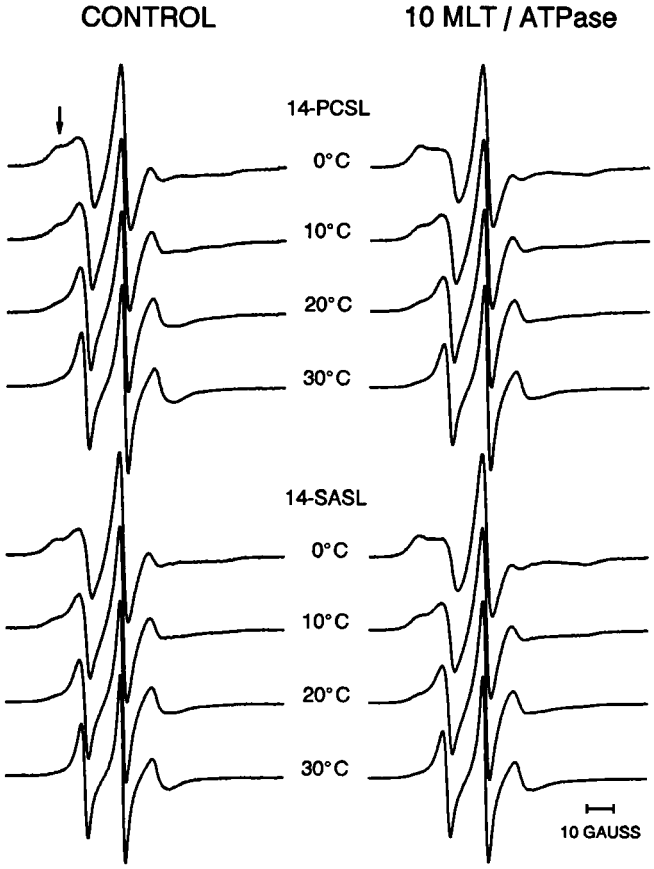

FIGURE 2 Effects of melittin and temperature on conventional EPR spectra of 14-PCSL and 14-SASL incorporated in SR membranes. The arrow indicates the presence of the restricted population of probes. SR membranes were incubated with 10 mol melittin/mol ATPase for 30 min at 25°C and prepared for EPR analysis as described in Methods. Spectra were separated into restricted and mobile component spectra and analyzed as described in Methods. Spectra are shown normalized to the same spin concentration.

RESULTS

Effects of melittin and temperature on the EPR spectra of spin-labeled SR membranes

Typical EPR spectra of 14-PCSL and 14-SASL incorporated into control SR membranes without melittin are shown in Fig. 2 (*left*). Direct overlay comparison shows that the control spectra of the two labels are qualitatively similar and contain only slight differences due to the slightly broader linewidths in the PCSL spectra. Incubation of SR membranes with melittin affects the spectrum of each label in a manner similar to that of lowering the sample temperature; i.e., with progressive addition of melittin, the inner spectral features (due primarily to fluid lipids) broaden and the splitting of the resolved outer wings (due primarily to restricted lipids) increases slightly (Fig. 2, *right*). For each label, the maximum effect occurs at a level of 10 mol melittin/mol Ca-ATPase. At a molar ratio of 5:1 melittin:ATPase (not shown), the spectra are intermediate between those of the control and 10:1 samples (Fig. 2), and spectra obtained at 20:1 are nearly identical to those obtained at 10:1. Although the control spectra of the two labels are similar, the magnitude of the spectral change induced by melittin is somewhat greater for 14-SASL than for 14-PCSL. This is confirmed by comparing the effects of melittin with the effects of temperature variation. For 14-PCSL, each spectrum from the melittin-containing (10:1) sample was virtually identical to the control (no melittin) spectrum recorded at a temperature 5°C lower, whereas the 14-SASL spectra require a temperature difference of nearly 10°C. With increasing sample temperature, however, the differences between the spectra from control and melittin-containing samples diminish slightly.

Spectral subtractions

The EPR spectra shown in Fig. 2 all consist of two components. One component is similar to that of dispersions of the extracted lipids (cf. Fig. 1 *e*). The other component, which is resolved in the outer wings of the spectrum and is indicated by the arrow in Fig. 2, corresponds to a more motionally restricted environment than that of the extracted membrane lipids at all except very low temperatures (cf. Fig. 1 *c*). As previously demonstrated in this system (Bigelow and Thomas, 1987) and a wide range of other membrane systems (Marsh, 1985), this component arises from lipids interacting directly with

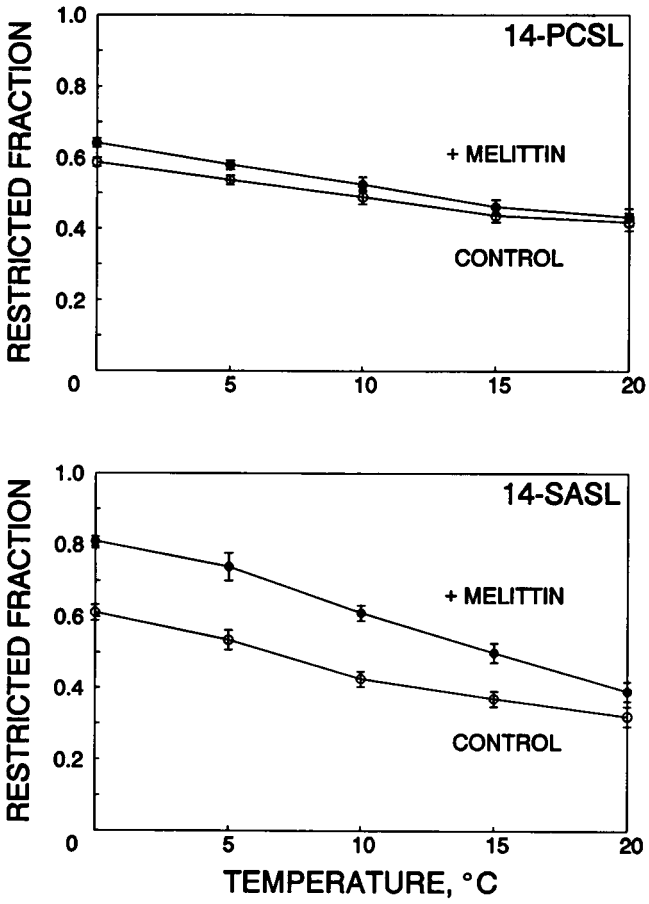

FIGURE 3 Effects of melittin and temperature on the fraction of motionally restricted spin labels in the EPR spectra of 14-PCSL (*upper*) and 14-SASL (*lower*) in SR membranes. The fractions were obtained by spectral subtraction as described in Methods. The open symbols correspond to control samples without melittin and the filled symbols correspond to samples containing 10 mol melittin/mol Ca-ATPase. For each label, data obtained from samples containing 5 mol melittin/mol Ca-ATPase (not plotted) fell directly between the control and 10:1 melittin samples at each temperature. Error bars represent the results of duplicate measurements.

the integral membrane proteins and demonstrates that both the 14-PCSL and the 14-SASL spin labels partition into the boundary layer of the Ca-ATPase in SR membranes.

To quantify the effects of melittin on the two motional populations of lipids in the composite spectra, we used spectral subtraction to separate the spectra into the two single components (see Methods). The most important parameter obtained from the subtractions is the mole fraction (f) of the restricted component in the composite spectra of each label (Fig. 3). These results agreed with the qualitative spectral overlay comparisons described above, in that the values of f obtained for the melittin-containing samples (10 mol melittin/mol Ca-ATPase) were similar to those obtained for the control samples without melittin recorded at temperatures nearly 5°C lower for 14-PCSL and nearly 10°C lower for 14-SASL. Values of f obtained from samples with 5 mol melittin/mol Ca-ATPase (not shown) fell directly between the control values and the values obtained with 10 melittin per ATPase (mol/mol) at each temperature. The fraction of labels that are restricted is slightly temperature dependent, consistent with several previous studies of SR boundary lipids (McIntyre et al., 1982; East et al., 1985). The values of f obtained from the 14-PCSL spectra change by 18% between 0 and 20°C, for both the control and melittin-containing samples. This is in contrast to the values of f obtained from the 14-SASL spectra, where the control sample without melittin changes by 30% and the melittin-containing sample changes by nearly 42% between 0 and 20°C. The most likely explanation why 14-SASL is more sensitive than 14-PCSL to changes in temperature and melittin concentration lies in a limited selectivity between 14-PCSL and 14-SASL for interaction with both melittin and the Ca-ATPase and possibly also to changes in the protonation state of 14-SASL (compare, for example, Esmann and Marsh, 1985).

Table 1 lists the spectral parameters obtained from the 14-PCSL difference spectra. The individual spectral components were characterized by the outer splitting, $2T_{\parallel}'$, the inner splitting, $2T_{\perp}'$, and the outer half-width at half-height of the low-field peak, Δ_L (cf. Figs. 2 and 4 of Mahaney and Thomas, 1991, for definition). $2T_{\perp}'$ was not measurable for the spectra of the restricted component because these lie in the slow motional regime of conventional nitroxide EPR spectroscopy (cf. Marsh, 1981, 1982). Addition of melittin, as well as decreasing temperature, increases $2T_{\parallel}'$ (Fig. 4) in both spectral components and decreases $2T_{\perp}'$ in the fluid component spectrum (not shown), consistent with a reduction in lipid mobility. The trends in the data for Δ_L with increasing melittin concentration are in agreement with those for the spectral splitting data: Δ_L decreases for the motionally restricted spectra and increases in the motionally fluid spectra, consistent with a decreased lipid mobility in the presence of melittin. The values of Δ_L become less reliable at higher temperature, primarily due to inaccuracies in the difference spectra arising from the generally poorer subtraction endpoints, and therefore they report only qualitative effects. Similar spectral parameters obtained from the 14-SASL difference spectra (not shown) were in agreement with those obtained from the 14-PCSL difference spectra, and the trends in the data were nearly identical (Fig. 4). In general, the spectral parameter data quantitatively support the qualitative assessment (given above) that the effect of melittin on the spectra of 14-PCSL or 14-SASL in SR membranes is similar to the effect of a decrease in temperature for each of the two resolved motional components.

DISCUSSION

We have used EPR spectroscopy to quantify the effects of melittin on (*a*) the fraction, f, of motionally restricted

TABLE 1 **Effects of melittin on the EPR spectrum of the restricted and fluid components of 14-PCSL in SR membranes***

Component	Temperature				
	0°C	5°C	10°C	15°C	20°C
Restricted					
f (0)	0.59 ± 0.01	0.54 ± 0.01	0.49 ± 0.02	0.44 ± 0.02	0.42 ± 0.03
f (10)	0.64 ± 0.01	0.59 ± 0.01	0.52 ± 0.02	0.46 ± 0.02	0.43 ± 0.03
$2T_{\parallel}'$ (0)	58.0 ± 0.3	56.5 ± 0.5	56.0 ± 0.3	55.6 ± 0.4	54.0 ± 0.5
$2T_{\parallel}'$ (10)	59.0 ± 0.2	58.0 ± 0.5	57.4 ± 0.3	56.8 ± 0.3	55.0 ± 0.5
Δ_L (0)	3.7 ± 0.2	4.2 ± 0.2	5.0 ± 0.2	6.0 ± 0.2	6.2 ± 0.2
Δ_L (10)	3.6 ± 0.2	3.6 ± 0.2	3.7 ± 0.1	3.9 ± 0.3	5.0 ± 0.2
Fluid					
$2T_{\parallel}'$ (0)	40.8 ± 0.2	38.4 ± 0.2	36.8 ± 0.2	36.0 ± 0.3	35.0 ± 0.2
$2T_{\parallel}'$ (10)	41.2 ± 0.5	40.0 ± 0.2	38.0 ± 0.1	37.1 ± 0.3	36.0 ± 0.3
$2T_{\perp}'$ (0)	19.9 ± 0.2	20.7 ± 0.2	21.4 ± 0.2	21.7 ± 0.2	21.9 ± 0.2
$2T_{\perp}'$ (10)	19.5 ± 0.3	20.2 ± 0.2	20.7 ± 0.2	21.3 ± 0.2	21.6 ± 0.2
Δ_L (0)	4.1 ± 0.2	3.4 ± 0.2	3.1 ± 0.2	2.9 ± 0.2	2.8 ± 0.2
Δ_L (10)	4.5 ± 0.3	3.9 ± 0.2	3.4 ± 0.2	3.1 ± 0.2	2.5 ± 0.2

* Mole fraction of the motionally restricted component, f, determined by spectral subtractions (see Methods). The EPR parameters outer splitting, $2T_{\parallel}'$, inner splitting, $2T_{\perp}'$, and half-width at half-height of the low-field peak, Δ_L, are in Gauss. Values in parentheses correspond to either control samples without melittin (0) or samples with 10 mol melittin/mol Ca-ATPase (10). Errors represent the variation of duplicate measurements.

lipids in SR and (*b*) the hydrocarbon chain dynamics of this and the fluid (bulk) SR lipid populations. The effects of melittin on the EPR spectra are remarkably similar to those of decreasing temperature; f increases and lipid mobility in both environments decreases. These results, combined with those from previous studies of the interaction of melittin with SR membranes, are consistent with a melittin-induced protein aggregation that is facilitated by the lipids in the Ca-ATPase annulus.

Temperature effects on lipid chain dynamics and the boundary lipid population

Although there have been a number of EPR studies investigating the temperature dependence of lipid-protein interactions (SR, Hidalgo, 1985, 1987; other systems, Watts et al., 1979; Marsh et al., 1981, 1982; Esmann et al., 1985; see also Marsh and Watts, 1982, 1988; Devaux and Seigneuret, 1985), the effects of temperature on the motional parameters in the component spectra of each motional population of lipids in SR have so far not been quantified in detail. Therefore, in addition to monitoring the fraction of boundary lipids, we have measured the motional parameters of the component spectra as a function of temperature. The fraction of restricted lipids, obtained from spectra of both 14-PCSL and 14-SASL, decreases with increasing temperature over the range from 0 to 20°C (Fig. 3), consistent with previous studies on SR membranes (McIntyre et al., 1982; East et al., 1985). Increasing temperature decreases the rotational mobilities of both restricted and fluid components, as indicated by the splitting and linewidth parameters (Table 1, Fig. 4). Although the changes in the spectral parameters for restricted and fluid components appear to be similar, the parameters cannot be compared directly for the two components because the spectra lie in different motional regimes. However, the temperature dependences (i.e., the effective temperature change) of these parameters for the two components provide a means of comparing effects of other perturbations, such as anesthetic (Bigelow and Thomas, 1987) or melittin (discussed below). The present study extends the results of a previous study of the temperature dependence of SR lipid hydrocarbon chain mobility (Bigelow et al., 1986), in which protein-free lipid extracts were studied to avoid the problems of analyzing composite spectra. The present study provides new insight by separating the two components in intact SR and by showing that the chain dynamics in both populations of lipids respond similarly to changes in temperature.

Melittin effects on lipid chain dynamics and the boundary lipid population

The rationale of the present study is similar to that used by Bigelow and Thomas (1987), who studied the molecular basis of Ca-ATPase activation by diethyl ether (Table 2). These authors found that the addition of 8% diethyl ether to SR membranes increases protein rotational mobility twofold (effective temperature increase of 10°C) and activates the enzyme twofold (effective temperature increase of 10°C). They found that ether does not affect the fraction of restricted lipids in SR and has only a slight effect on the rotational dynamics of the motionally fluid lipids (effective temperature increase of 1°C) but significantly mobilizes the hydrocarbon chains of the restricted boundary lipids of the Ca-ATPase (effective temperature increase of 10°C). Bigelow and Thomas (1987) concluded that this selective mobilization of the boundary lipid hydrocarbon chains facilitates

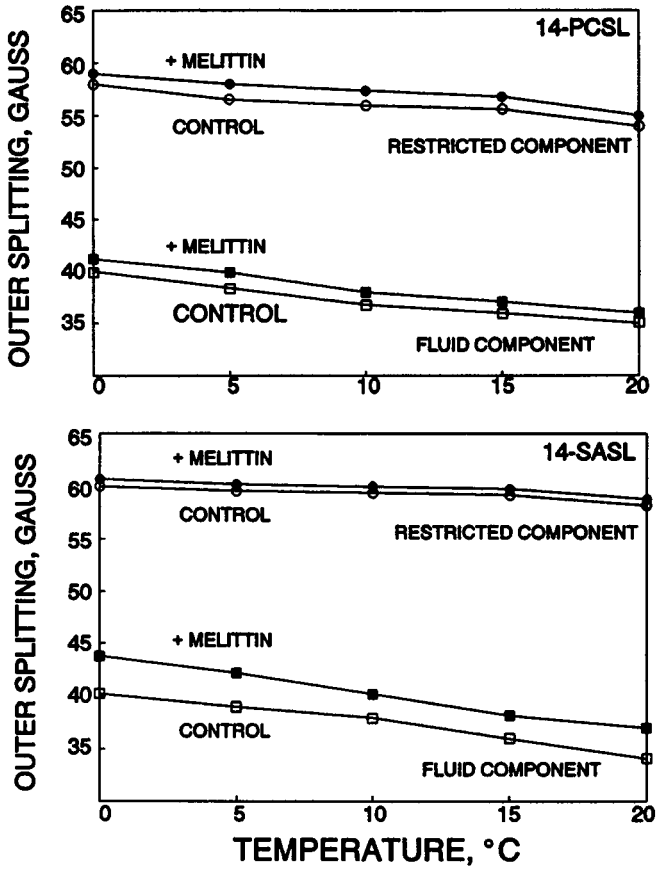

FIGURE 4 Effects of melittin and temperature on 14-PCSL (*upper*) and 14-SASL (*lower*) outer hyperfine splitting in the motionally restricted (*circles*) and fluid (*squares*) component spectra. The open symbols correspond to control samples without melittin and the filled symbols correspond to samples containing 10 mol melittin/mol Ca-ATPase. For each label, data obtained from samples containing 5 mol melittin/mol Ca-ATPase (not plotted) fell directly between the control and 10:1 melittin samples at each temperature. Errors are smaller than the symbols.

protein rotational motions that are essential for optimal enzymatic function. In the present study, we ask whether melittin has a similar, but opposite, effect, i.e., does melittin preferentially affect the boundary lipid chains such that overall protein rotation is restricted, giving rise to enzyme inhibition? Unlike diethyl ether, melittin (molar ratio 10:1 melittin:ATPase) does affect the fraction of motionally restricted lipids (effective temperature decrease of 5°C) and decreases the mobilities of both the motionally restricted and fluid components (effective temperature decreases of 7 and 4°C, respectively). The effects of lipid mobility on enzyme activity are likely to be complex and, in this case, can best be compared by equivalent temperature shifts. Whereas an exact one-to-one correspondence is not to be expected between the effects of temperature and of melittin binding, such an approach is most likely to give a correct impression of the relative sizes of the different effects. Although the data on lipid mobility suggest a slight preferential effect of melittin on the restricted lipids, melittin's effect on protein mobility and enzymatic activity (effective temperature decreases of 22 and 21°C, respectively) (cf. Bigelow et al., 1986; Mahaney and Thomas, 1991) are much greater than its effect on either lipid component (Table 2), suggesting that melittin exerts its functional effects primarily by inducing protein–protein interaction. Even for the 14-SASL probe, the effects of melittin are equivalent to a temperature decrease of 10°C, which is still considerably less than the effect on activity. Therefore, in contrast to the activating effects of diethyl ether, the effect of melittin on the fluidity of Ca-ATPase boundary lipids alone cannot account for the melittin-induced loss of Ca-ATPase rotational mobility and enzymatic activity (Table 2). We conclude that the loss of enzymatic activity most likely results from melittin-induced aggregation of the Ca-ATPase, which is facilitated by melittin's interaction with the lipids adjacent to the protein.

Whereas both the 14-SASL and 14-PCSL spin labels record similar fractions, f, of motionally restricted lipid in control SR membranes at low temperatures (Fig. 3), both the temperature dependence and the effects of melittin are found to be greater with 14-SASL than with 14-PCSL. As stated in the Results, the most likely reason for this is a limited selectivity in the interaction of 14-SASL with the Ca-ATPase, relative to that of 14-PCSL. In particular, it is to be anticipated that the binding of melittin will alter the thermodynamic state of lipids associated with the protein relative to those in the bulk lipid regions, and this effect is likely to be larger for charged lipids, hence explaining the greater effect of melittin in the case of 14-SASL.

Ca-ATPase aggregation effects

Previous studies have demonstrated that one of the physical effects of lowering temperature (Birmachu and

TABLE 2 **Effective temperature changes equivalent to the effects of diethyl ether and melittin on SR membranes***

Perturbant	Ca-ATPase activity	Ca-ATPase mobility	f	Restricted component, $2T_{\parallel}'$	Fluid component, $2T_{\parallel}'$
			°C		
8% Ether	+10	+11	0	+10	+1
10 Melittin:ATPase	−21	−22	−5	−7	−4

* Ether data from Bigelow and Thomas (1987). Ca-ATPase activity and mobility data (+10 melittin:ATPase) from Mahaney and Thomas (1991). f is the mole fraction of motionally restricted lipids and $2T_{\parallel}'$ is the outer splitting of the component spectra (see Methods).

Thomas, 1990) or increasing melittin concentration (Mahaney and Thomas, 1991; Voss et al., 1991) is to induce large-scale Ca-ATPase aggregation. This aggregation might be expected to decrease the population of boundary lipids, if these lipids are excluded from the region of protein–protein interfacial contact (cf. Marsh et al., 1982). However, Blasie et al. (1990) and Stokes and Green (1990) have reported x-ray structures of the ATPase in which the cytoplasmic "headpiece" of the protein is somewhat larger than the intramembranous domain, suggesting that protein aggregation may not result in the exclusion of lipids from the boundary shell, since intramembranous protein–protein interfacial contact may be prevented by the larger extramembranous portion of the protein. This is supported by studies in which the protein was extensively aggregated by glutaraldehyde cross-linking but the fraction of restricted lipids remained unchanged (Thomas et al., 1982). Since the present study shows that the population of restricted lipids effectively increases with melittin-induced protein aggregation, it is very unlikely that boundary lipids are excluded or that melittin acts simply by cross-linking the cytoplasmic headpiece of the Ca-ATPase, without involving boundary lipids at all. On the contrary, our data suggest that the complex of melittin, Ca-ATPase, and lipid effectively extends the restricted lipid region between the aggregated proteins and results in protein aggregation. An alternative interpretation is that melittin restricts lipid chain mobility by direct interaction with the lipids themselves. In a series of control experiments on dispersions of extracted SR lipids alone (not shown), we found that lowering temperature (between 0 and −5°C) and adding melittin (3 mol% or less) gives rise to the appearance of a slightly restricted lipid component, but these effects were much less than observed in intact SR. It is therefore unlikely that the increased size of the motionally restricted population in SR membranes, both on decreasing temperature and on adding melittin, is due to changes in the lipids alone.

The exchange between bulk lipids and boundary lipids can be an important consideration in the interpretation of EPR data from lipid spin labels. If the exchange rate is high enough ($\gtrsim 10^7\ s^{-1}$), the resolution of the two spectral components is reduced, and the apparent fraction restricted is decreased (Davoust and Devaux, 1982). Therefore, the decreasing fraction of restricted lipids with increasing temperatures observed in the present study could arise partly from increasing exchange rates. However, we expect exchange effects to be small since (*a*) even in fluid membranes, significant exchange effects are observed mainly at temperatures ≥ 20°C, whereas our measurements were made between 0 and 20°C (Davoust and Devaux, 1982), and (*b*) East et al. (1985) concluded that exchange effects in SR membranes are not large below 20°C.

In conclusion, the results of the present study are consistent with the formation of an ATPase-melittin-lipid complex at the lipid-protein interface, because melittin increases the fraction, f, of motionally restricted lipids and decreases the mobility of the restricted lipid population. Since melittin also decreases slightly the fluidity of the fluid lipid population, the results do not rule out a direct interaction between melittin and these lipids. Nevertheless, the magnitude of changes in f and lipid fluidity (based on the effective temperature change) are significantly smaller than the melittin-induced changes in Ca-ATPase mobility and activity observed in previous studies. Therefore, we conclude that the loss of enzymatic activity most likely results from melittin-induced aggregation of the Ca-ATPase, which is facilitated by melittin's interaction with the lipids adjacent to the protein. These results may provide insight into the action of amphipathic membrane-binding peptides in general by illustrating the potential role of boundary lipids in the interaction of such peptides with integral membrane proteins.

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