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The Effect of a Phase Transition on Penetration of Phospholipid Monolayers by Melittin and Glucagon[1]

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The penetration of melittin and glucagon into phospholipid monolayers was studied by measuring compression isotherms of phospholipids in the absence and presence of various concentrations of protein in the subphase. Differences in molecular area were calculated as a function of protein concentration at constant pressure. Area change as a function of surface pressure at constant protein concentration was also calculated. Melittin showed greater affinity for penetration into phosphatidylglycerol (PG) than into phosphatidylcholine (PC) monolayers. The cutoff pressure for melittin penetration was 45 mN/m with PC and 60 mN/m (extrapolated) with PG. Dipalmitoyl PC and PG monolayers show phase transitions upon compression at 25°C. Both melittin and glucagon showed increased penetration as measured by area change within the region of the phase transition with both lipids. Glucagon showed a cutoff pressure of 25 mN/m for penetration into dimyristoyl PC. The preference of glucagon for interaction with lipid bilayers in the gel phase is discussed with respect to monolayer penetration as a function of surface pressure.

Melittin and glucagon are peptides of 26 and 29 amino acid residues, respectively. Both peptides have segments of amphiphilic α-helix and interact strongly with phospholipids. The 20 amino-terminal residues of melittin are arranged as two amphiphilic α-helical segments with a nonhelical segment at residues 11 and 12 (1). The six carboxy-terminal residues are nonhelical and contain four positive charges contributed by lysine and arginine residues. Glucagon contains two segments of amphiphilic α-helix in its crystal structure (2). The small size of these peptides and knowledge of their crystal structures make them attractive models for the study of lipid-protein interactions.

Melittin is highly surface active even at an air-water interface, attaining an equilibrium pressure of 24.5 mN/m (3). In the presence of a lipid monolayer, melittin will penetrate to even higher pressures. Bougis *et al.* (4) recently reported on the penetration of melittin into phospholipid monolayers at constant area as followed by the increase in surface pressure. Here we report on the penetration of melittin and glucagon into phospholipid monolayers at constant surface pressure as followed by the increase in surface area. Of particular interest is the penetration of these peptides into phospholipid monolayers where the phospholipid is undergoing a phase transition. A zwitterionic phospholipid, phosphatidylcholine, and an anionic phospholipid, phosphatidylglycerol, were used in this study. Monolayers of the dipalmitoyl homologs of these two lipids undergo phase transitions upon compression at 25°C. The phase transition has been shown to en-

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hance various phenomena in lipid bilayers. The passive permeability of phospholipid bilayers is increased at the phase transition (5) as is also phospholipase A_2 activity in liposomes (6) and plasma-induced dissolution of multilamellar vesicles (7), to mention just a few examples.

Of additional interest with respect to glucagon is its reported preference for interaction with phospholipid bilayers in the gel phase (8, 9). The advantage of a monolayer system is the ability to study interactions over a range of surface pressures. Results of this study should thus complement studies of glucagon interactions with phospholipid bilayers.

MATERIALS AND METHODS

Proteins. Purified melittin was obtained as a generous gift from Dr. Franklyn G. Prendergast, The Mayo Clinic, Rochester, Minnesota. A stock solution of about 1 mg/ml was prepared in buffer. The exact concentration was determined by its absorption at 280 nm using an extinction coefficient of 1.6 ml mg^{-1} cm^{-1}. This solution was stored at 4°C. Glucagon was obtained from Sigma Chemical Company, St. Louis, Missouri. Glucagon (1–3 mg) was dissolved in 1 ml of 0.01 M NaOH by brief sonication in a sonic-cleaner bath. Tris buffer, 0.5 M (pH 7), was then added to a final volume of 10 ml. If absorption at 320 nm due to light scattering was detected, the solution was centrifuged. The concentration of glucagon was determined by its absorption at 278 nm using an extinction coefficient of 2.21 ml mg^{-1} cm^{-1} (10). The solution was stored at 4°C and used within 3 days. Each day the light scattering at 320 nm was measured and the solution centrifuged and reassayed if necessary.

Phospholipids. Didecanoyl phosphatidylcholine (DDPC)[3] and dimyristoyl phosphatidylcholine (DMPC) were synthesized as described by Jensen and Pitas (11). Didecanoyl phosphatidylglycerol (DDPG) was synthesized by phospholipase *D*-catalyzed transphosphatidylation of DDPC (12). Dipalmitoyl phosphatidylcholine (DPPC) and dipalmitoyl phosphatidylglycerol (DPPG) were obtained from Sigma Chemical Company, St. Louis, Missouri. Dimyristoyl phosphatidylglycerol (DMPG) was obtained from Calbiochem-Behring Corporation, La Jolla, California. Stock solutions (1 mM) were prepared in benzene. The exact concentrations were determined by phosphorus analysis according to Bartlett (13). A measured amount of lipid solution was carefully deposited at the air–water interface with a microsyringe to form a monolayer.

Measurement of monolayer-compression isotherms. Monolayer-compression isotherms were obtained by driving a mobile Teflon barrier at a rate of 1.71 cm/min across a single-compartment Teflon trough (16.1 × 27.1 cm). Surface pressure was measured by the Wilhelmy method using a rough-surfaced platinum plate attached to a Cahn RTL electrobalance. The compression isotherm was recorded on a chart recorder and digitized data were collected in an interfaced 8080-based microprocesser prior to transfer to a time-shared PDP 11/70 computer system where the data were analyzed. The temperature of the apparatus was maintained at 25°C and the subphase solutions were kept in a water bath at 25°C prior to placement in the trough. The trough was washed, after each experiment with protein, by scrubbing with Triton X-100 solution and rinsing thoroughly with water.

Monolayers were spread over a buffer containing 0.1 M NaCl and 5 mM Tris-chloride, pH 7. Water for the buffer was purified by two-stage reverse osmosis, mixed-bed deionization, charcoal filtration, and distillation in an all-glass still. Protein was added to the buffer to the desired concentration before placement in the trough. Compression isotherms were run alternatively with and without protein in the subphase. At least four sets of controls (without protein) and experiments (with protein) were obtained and averaged for each different protein concentration.

RESULTS

Penetration of Melittin into DDPC and DDPG

Penetration of melittin into phospholipid monolayers is reflected by an increase in surface area at constant surface pressure. Compression isotherms of phospholipid in the absence and presence of various concentrations of melittin in the subphase were analyzed at constant surface pressure. Differences in molecular areas with and without melittin (ΔA) were calculated at each melittin concentration. Plots of ΔA versus melittin concentration with DDPC and DDPG at 30 mN/m are shown in Fig. 1. Penetration into DDPG followed a normal saturation curve. Penetration into DDPC seemed to be biphasic. A normal saturation curve up to 0.2 μg/ml was followed by a steady increase at higher mel-

[3] Abbreviations used: DDPC, didecanoyl phosphatidylcholine; DMPC, dimyristoyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; DDPG, didecanoyl phosphatidylglycerol; DMPG, dimyristoyl phosphatidylglycerol; DPPG, dipalmitoyl phosphatidylglycerol.

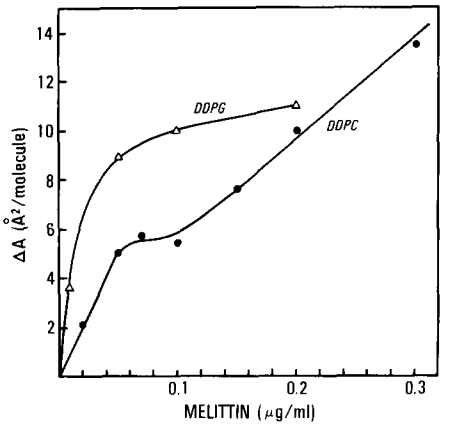

FIG. 1. Penetration of melittin into phospholipid monolayers. Surface pressure = 30 mN/m; T = 25°C.

ittin concentrations. Data for melittin penetration into DDPG and into DDPC below 0.2 μg/ml were fitted by regression analysis to double-reciprocal plots. Values for maximal area change (ΔA_{max}) and concentration of melittin giving 1/2 ΔA_{max} (K) were determined and are given in Table I.

Changes in molecular area as a function of surface pressure at constant melittin concentration (0.1 μg/ml) with DDPC and DDPG are shown in Fig. 2. Extrapolation to zero area change gives a surface pressure above which no penetration would occur. This is defined as the cutoff pressure. Cutoff pressures of about 45 and 60 mN/m were obtained for DDPC and DDPG, respectively. Experiments could only be done up to 40 mN/m due to film collapse above this pressure. The extrapolated cutoff pressures, thus, may be somewhat hypothetical, but do indicate the strength of interaction with phospholipid.

TABLE I

CONSTANT-PRESSURE PENETRATION OF MELITTIN

Phospholipid	ΔA_{max} (Å²/molecule)[a]	K (μg/ml)[a]
DDPC[b]	10.4 ± 2.0	0.065 ± 0.028
DDPG	12.7 ± 0.4	0.0245 ± 0.0027

[a] Computer fits to a Langmuir isotherm, $\Delta A = \Delta A_{max}[M]/(K + [M])$; ±SD.

[b] Fit to data below 0.2 μg/ml.

Penetration of Melittin into DMPC, DPPC, DMPG, and DPPG. Effect of a Phase Transition

Compression isotherms for DPPC and DPPG with and without melittin are shown in Fig. 3. Phase transitions occur at about 11 and 15 mN/m for DPPC and DPPG, respectively, at 25°C. DMPC and DMPG show no phase transitions under these conditions. Area changes as a function of surface pressure for melittin penetration are shown in Fig. 4 for DMPC and DPPC and Fig. 5 for DMPG and DPPG.

Penetration of Glucagon into DMPC, DPPC, DMPG, and DPPG

Penetration of glucagon into DMPC and DPPC as reflected by area change as a function of surface pressure is shown in Fig. 6. Penetration of glucagon into DMPG and DPPG is similarly shown in Fig. 7.

DISCUSSION

Melittin can exist in solution both as a monomer and as a tetramer. In this study it is important to know which form of melittin is present in the subphase. The monomer ⇌ tetramer equilibrium depends on the concentration of melittin, pH, and the ionic strength and nature of ions present. Increasing melittin concentration, pH, and ionic strength favor the formation of tet-

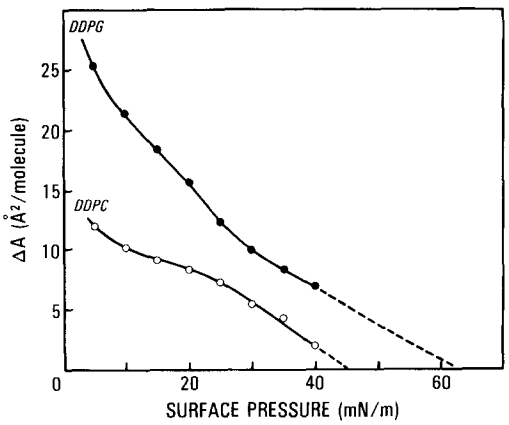

FIG. 2. Penetration of melittin into phospholipid monolayers. Melittin concentration = 0.1 μg/ml; T = 25°C.

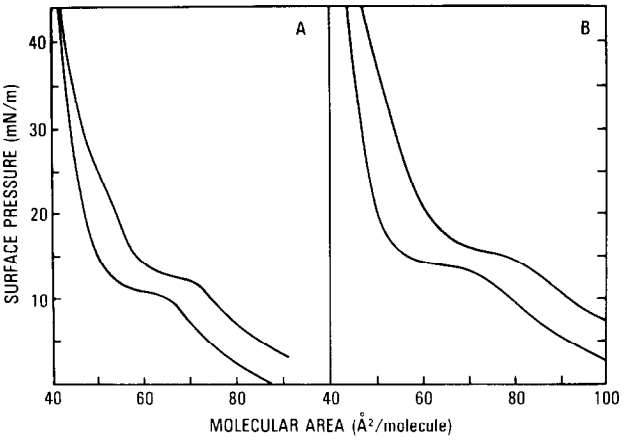

FIG. 3. Compression isotherms. (A) DPPC alone (lower curve), DPPC with 0.05 μg/ml melittin (upper curve). (B) DPPG alone (lower curve), DPPG with 0.075 μg/ml melittin (upper curve). T = 25°C.

ramers. Faucon *et al.* (14) showed that at 5.4 μg/ml (1.9 μM) melittin, pH 7.5, tetramers are only observed at NaCl concentrations above 1 M. In this study, melittin, even at the highest concentration used (0.3 μg/ml (0.1 μM), pH 7.0, in 0.1 M NaCl), is monomeric and well below the point where self-association to tetramers occurs. Coddington *et al.* (15) claimed to show differences in interactions of monomeric and tetrameric melittin with lipid monolayers. Under conditions where they assumed melittin to be tetrameric (0.25 μM melittin, pH 7.5, 1 mM NaCl), melittin is still largely monomeric according to the data of Faucon *et al.* (14). They presented no evidence to show that melittin was indeed tetrameric under those conditions. Talbot *et al.* (16) showed that only tetrameric melittin can bind to monomeric lipid, while both monomeric and tetrameric melittin can bind to aggregated lipid (above the critical micelle concentration) and the mixed micelles formed at saturation appear to be independent of the initial state of association of melittin.

Penetration of melittin into phospholipid monolayers is reflected by an increase in

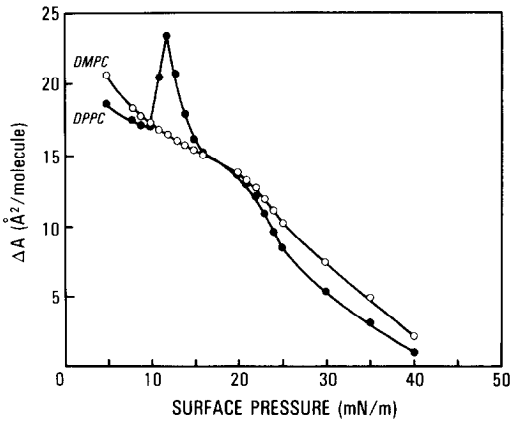

FIG. 4. Penetration of melittin into phosphatidylcholine monolayers. Melittin concentration = 0.1 μg/ml; T = 25°C.

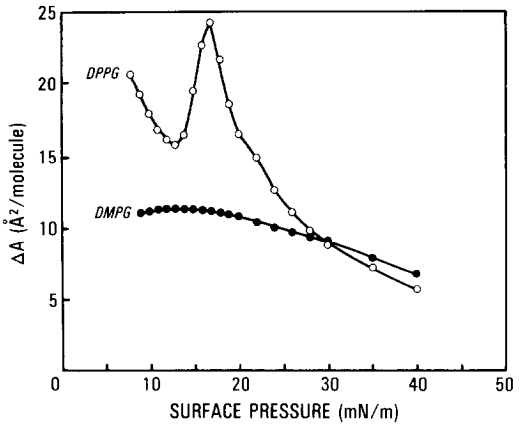

FIG. 5. Penetration of melittin into phosphatidylglycerol monolayers. Melittin concentration = 0.1 μg/ml; T = 25°C.

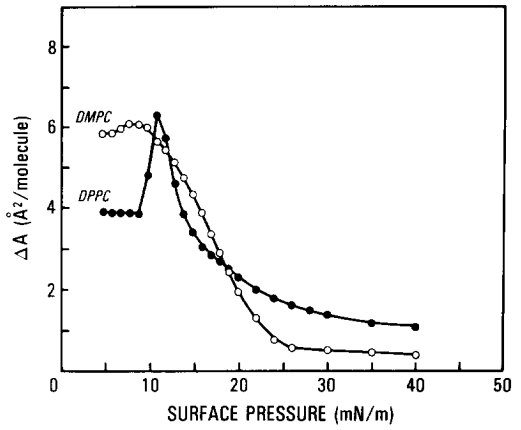

FIG. 6. Penetration of glucagon into phosphatidylcholine monolayers. Glucagon concentration = 0.1 μg/ml; T = 25°C.

surface area (ΔA) at constant surface pressure. This increase in area is due primarily to an increased number of melittin molecules in the interface rather than an increase in the molecular area of the lipid molecules. With anionic phospholipids there may be a decrease in the molecular area of the lipid molecules as the cationic protein causes a decrease in electrostatic repulsion between the anionic groups of the phospholipids (4). In this case, the increase in surface area may be less than a quantitative measure of the amount of melittin penetrated.

Penetration of melittin into DDPG, reflected by area change as a function of subphase concentration, showed normal saturation behavior (Fig. 1). Penetration into DDPC showed normal saturation up to 0.2 μg/ml, followed by a steady increase at higher concentrations. The latter increase was more pronounced at lower surface pressures and may be due to infinite expansion as melittin penetrates into interfacial regions already rich in melittin. This would be similar to melittin absorption at an air–water interface (3). This phenomenon of infinite expansion was discussed by Barnes (17). The preference of melittin for penetration into negatively charged monolayers of DDPG is indicated by a smaller K value (Table I) and a higher cutoff pressure (Fig. 2). Bougis *et al.* (4) observed a similar cutoff pressure (40 mN/m) for melittin penetration into dilauryl phosphatidylcholine by measuring surface pressure increase at constant area, and a higher cutoff pressure for penetration into anionic dilauryl phosphatidylserine.

The effect of a phase transition on melittin and glucagon penetration was studied with DMPC, DPPC, DMPG, and DPPG. DMPC and DMPG show no phase transitions upon compression at 25°C, while DPPC and DPPG show phase transitions at about 11 and 15 mN/m, respectively. With both DPPC and DPPG there is a dramatic increase in melittin penetration, as reflected by area change, within the region of the phase transition. Glucagon also showed increased penetration into DPPC and DPPG monolayers in the region of the phase transition. At the phase-transition temperature Epand (8) found that glucagon interacts with DMPC vesicles to form disk-like particles. The rate of glucagon interaction was found to be greatest at this temperature (18). The increased penetration of protein into phospholipid interfaces at the phase transition was observed in monolayers with β-casein by Phillips *et al.* (19). They ascribed this to an increased lateral compressibility. This explanation was also proposed by Marcelja and Wolfe (20) from theoretical calculations of lipid bilayer systems.

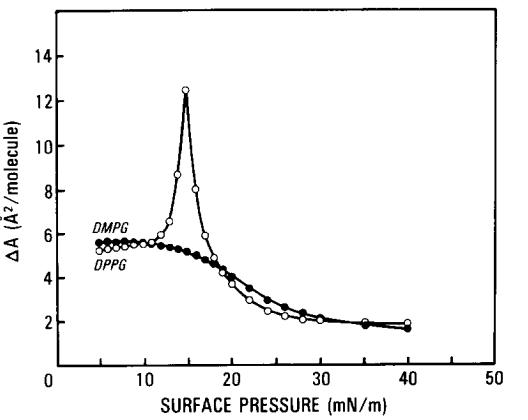

FIG. 7. Penetration of glucagon into phosphatidylglycerol monolayers. Glucagon concentration = 0.1 μg/ml; T = 25°C.

The interaction of glucagon with DMPC vesicles was reported by Epand *et al.* (9) to occur more strongly in the gel phase. This is unusual since most substances are more readily incorporated into the liquid-crystalline phase. Our results show that glucagon has a cutoff pressure for penetration into DMPC monolayers of about 25 mN/m. Since the state of compression believed to exist in bilayer membranes is equivalent to a monolayer at about 30 mN/m (21, 22), one would expect to see little interaction of glucagon with DMPC vesicles in the liquid-crystalline phase. Significant interaction would then only occur in the region of the phase transition, as shown in this study, due to increased lateral compressibility, and in the gel phase, due to defects in lipid packing (23) which exist in this state.

Our studies give further examples of increased protein penetration of phospholipid interfaces in the region of the phase transition. We also present a convenient method for the study of lipid-protein interaction at constant surface pressure by measurement of monolayer-compression isotherms in the presence and absence of protein in the subphase.

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