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MONOLAYER AND ^{13}C NUCLEAR MAGNETIC RESONANCE STUDIES ON THE INTERACTION BETWEEN MELITTIN AND CHLOROPLAST LIPIDS

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The interaction of the polypeptide melittin with chloroplast membrane lipids has been studied by monolayer and ^{13}C-NMR techniques. Under conditions favouring the existence of a melittin monomer, the interaction of the polypeptide with monolayers of chloroplast lipids is largely confined to negatively charged lipids. Conditions favouring the formation of the melittin tetramer result in a marked interaction of the polypeptide with monolayers of uncharged lipids. ^{13}C-NMR measurements of the rate of motion of fatty acyl carbon atoms in multibilayers of chloroplast membrane lipids demonstrate that melittin penetrates deeply into the bilayer irrespective of the proportion of negatively charged lipids. It is concluded that interaction of melittin which chloroplast membrane lipids is the primary factor in its capacity to inhibit membrane-associated reactions in chloroplasts.

The polypeptide, melittin, is a major component of bee venom but is devoid of enzymic activity [1]. Its primary structure (I) comprises 26 amino acids of which the 20 N-terminal amino acids are predominantly non-polar while the six C-terminal amino acids are polar and four are positively charged [2].

Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-

Ala-Leu-Ile-Ser-Try-Ile-Lys-Arg-Lys-Arg-Gln-Gln$(NH)_2$ (I)

Physicochemical studies have revealed that melittin can exist in both monomeric and tetrameric forms, the equilibrium state depending on pH and ionic strength [3–5], and that both forms may interact with lipids [3,6]. Melittin is very active biologically, being capable of potentiating the activity of both soluble and membrane-bound phospholipases A [7–9], causing hemolysis of erythrocytes [1,10], liberating enzymes from membranous structures such as lysosomal granules [11] and uncoupling mitochondrial oxidative phosphorylation [12].

We have recently demonstrated that melittin is capable of inhibiting a number of photochemical reactions in higher plant chloroplasts including photosynthetic electron transport, photophosphorylation, the light-induced absorbance change at 518 nm and the photooxidation of cytochrome *f* [13]. These effects occur without fragmentation of the membrane and the inhibition of both photosynthetic electron transport and photooxidation of cytochrome *f* can be relieved by addition of carrier

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Abbreviations: DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; SL, sulphoquinovosyldiacylglycerol; PC, phosphatidylcholine; ^{13}C-NMR, carbon-13 nuclear magnetic resonance; Hepes, 4(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

amounts of plastocyanin. Chloroplast membrane lipids differ from those of most other biological membranes in consisting predominantly of uncharged glycolipids, and this communication reports a monolayer and ^{13}C-NMR study of the interaction of melittin with those lipids.

Materials and Methods

Highly purified chloroplast lipids were isolated and purified as previously described [14]. Dilinolenylphosphatidylcholine (Serdary) and cardiolipin (General Biochemicals) were further purified by the same techniques. Chloroplast total polar lipid for monolayer experiments was isolated from pea chloroplasts [15] and contained, in mol%, MGDG, 53; DGDG, 33; SL, 10; PG, 5. Monolayer experiments were performed at 22°C in a Teflon tank with a surface area of 33.75 cm^2, similar to that described by Jackson et al. [16]. Monolayers were prepared at an initial pressure of 10 mN/m unless otherwise indicated.

The lipid samples used for ^{13}C-NMR measurements were prepared by two techniques. The first technique involved the mixing of highly purified chloroplast lipids [14] in the desired proportions and these samples are referred to as reconstituted lipids. In the second technique, pigments of neutral lipids are removed from a total extract of leaf or chloroplast lipids by passage through SepPak HPLC silica gel cartridges [17] and these are referred to as polar lipids. Multibilayer lipid dispersions for ^{13}C-NMR measurements were prepared by dispersing a lipid sample, freed of solvent under high vacuum, in small amounts of ^2H_2O and mixing on a vortex mixer. The final lipid concentration in the sample was 150 mg/ml. ^{13}C-NMR spectra and T_1 measurements were performed as previously described [18,19] using a Varian CFT-20 spectrometer and fast inversion recovery techniques.

Melittin (Sigma) was used without further purification, since monolayer experiments using material purified to remove any residual phospholipase activity or on melittin (Serva) containing low levels of *N*-formylmelittin, gave identical results.

Results

The interaction of melittin with monolayers of chloroplast lipids made on purified water (pH 6.0) is shown in Fig. 1. The concentration of melittin in the subphase was saturating, i.e., further increases did not increase the magnitude of the pressure rise. Virtually no pressure increases (i.e., less than 2.5 mN/m) occur in monolayers of MGDG or DGDG, which are uncharged, or in those of dilinolenylphosphatidylcholine, which has no net charge. In contrast, substantial pressure increases, of about 25 mN/m, occur in monolayers of the negatively charged chloroplast lipids SL and PG. The initial rate of increase in pressure of monolayers containing chloroplast total polar lipid is perhaps greater than that of either negatively charged lipid, but the final pressure increase is only about 10 mN/m. However, some marked differences are evident when the subphase consists of 10 mM Hepes (pH 7.5)/10 mM NaCl (Fig. 2). The increase both in ionic strength and in pH will tend to increase the content of the tetrameric form of melittin in the subphase. The rate of increase and final pressure obtained in monolayers of MGDG, DGDG and PC are substantially greater than those occurring on a water subphase, while both the rate and extent of the pressure changes in

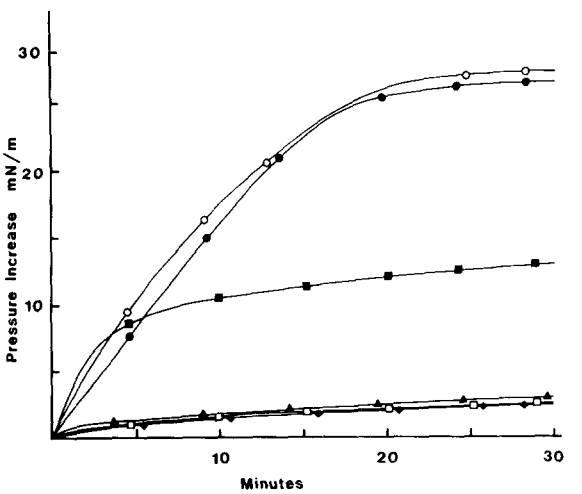

Fig. 1. Interaction of melittin with monolayers of chloroplast lipids on a water subphase (pH 6.0). Subphase concentration of melittin, 0.25 μM. ●——●, PG; ○——○, SL; ▲——▲, MGDG; ◆——◆, DGDG; □——□, di 18:3 PC; ■——■, chloroplast total polar lipid.

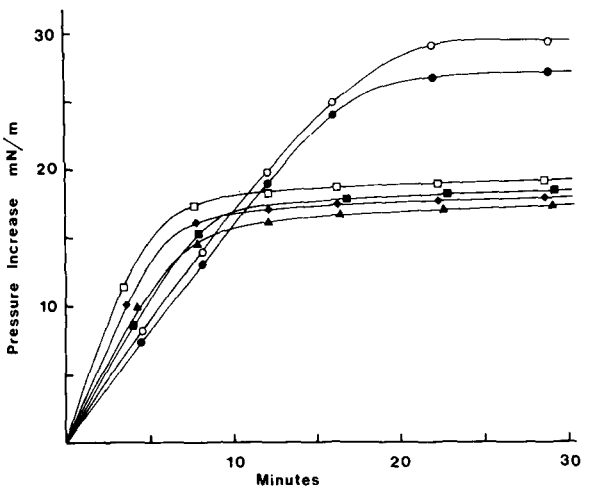

Fig. 2. Interaction of melittin with monolayers of chloroplast lipids on a subphase of 10 mM Hepes (pH 7.5)/10 mM NaCl/1 mM EDTA. Subphase concentration of melittin, 0.25 μM. Symbols as in Fig. 1.

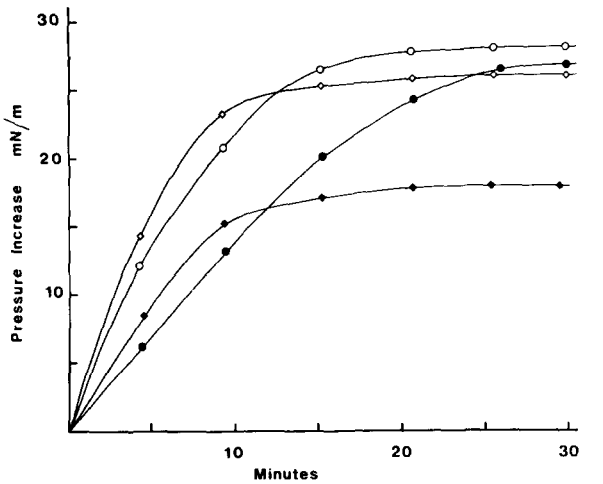

Fig. 3. Effect of ionic strength on interaction of melittin with monolayers of DGDG and PG. All subphases were buffered with 10 mM Hepes (pH 7.5)/1 mM EDTA. Closed symbols, 10 mM NaCl in subphase. Open symbols, 1 M NaCl in subphase. ◇——◇, DGDG; ○——○, PG. Subphase concentration of melittin, 0.25 μM.

monolayers of PG and SL are relatively unchanged (cf. Fig. 1). A greater pressure increase also occurs in monolayers of chloroplast total polar lipid, but the initial rate of pressure increase is similar irrespective of whether water or Hepes/NaCl is the subphase.

The results derived from Figs. 1 and 2 would suggest that increasing the monomer: tetramer equilibrium of melittin in favour of the tetramer would increase the interaction of the polypeptide with uncharged lipids such as MGDG and DGDG. This conclusion is further strengthened by the results shown in Fig. 3, where the subphase contains either 10 mM NaCl or 1 M NaCl. Increasing the subphase concentration of NaCl from 10 mM to 1 M caused marked increases in the rate and extent of penetration of melittin into monolayers of DGDG (Fig. 3) and the other uncharged chloroplast lipid, MGDG, and the zwitterionic lipid PC (tracings for MGDG and PC not shown). In contrast, while the rate of penetration of melittin into monolayers of negatively charged lipids such as PG was increased in 1 M NaCl, the final pressure increase varied only slightly (Fig. 3) and similar results were observed for the other negatively charged lipids, SL and cardiolipin.

Although the data presented in Figs. 1–3 have been obtained at an initial monolayer pressure of 10 mN/m, similar although smaller pressure increases have been obtained with monolayers at higher initial pressures. For example, at an initial pressure of 25 mN/m, which approximates that of biological membranes, melittin causes a 2.5 mN/m increase in PG monolayers.

It has been shown previously [10] that melittin itself is capable of forming a stable film on aqueous solutions, and the effect of pH and ionic strength on this property is shown in Fig. 4. At subphase concentrations of 0.25 μM, melittin shows no surface activity on water (pH 6.0) and only slight surface activity on 10 mM NaCl (pH 6.0). However, on 1 mM NaCl (pH 6.0), melittin forms a stable monolayer with a collapse pressure of about 27 mN/m within 30 min after injection into the subphase. The effect of increasing pH is shown by the observation that on 10 mM Hepes/10 mM NaCl (pH 7.5), a monolayer with a final pressure of about 19 mN/m is formed (Fig. 4), which final pressure is similar to that formed on a monolayer of 100 mM NaCl (pH 6.0) (tracing not shown). At 1 M NaCl concentrations in the subphase the effect of pH is not significant (Fig. 4) because the ionic strength of a 1 M NaCl solution is sufficient to allow the tetramer form of melittin to predominate.

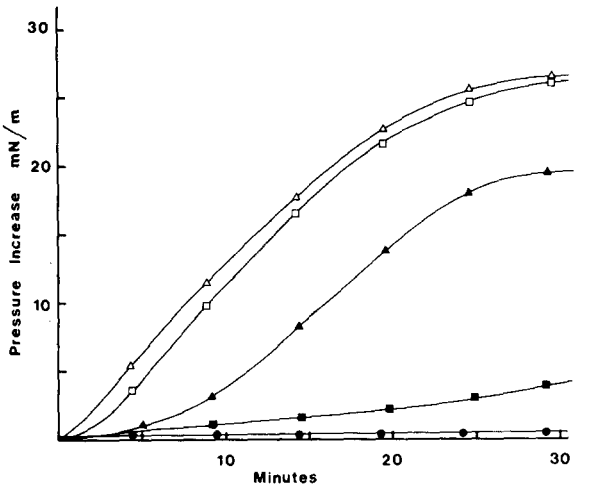

Fig. 4. Surface activity of melittin in absence of a lipid monolayer. ●———●, water subphase; ■———■, 10 mM NaCl subphase (pH 6.0); □———□, 1 M NaCl subphase (pH 6.0); ▲———▲, 10 mM Hepes (pH 7.5)/10 mM NaCl/1 mM EDTA subphase; △———△, 10 mM Hepes (pH 7.5)/1 M NaCl/1 mM EDTA subphase. Subphase concentration of melittin, 0.25 μM.

The interaction of melittin with multibilayers prepared from chloroplast polar lipids has now been studied by measuring the ^{13}C-NMR spin-lattice relaxation times (T_1) of the carbon atoms of the acyl chains of the lipid molecules [18,19]. The T_1 value of a carbon atom correlates directly with the rate of motion of the carbon atom such that the greater the rate the larger the T_1 value [18]. Multibilayers of two different lipid compositions were employed. The first, termed reconstituted multibilayers, contained highly purified chloroplast lipids, MGDG (45%), DGDG (45%) and SL (10%). The second, termed polar lipid multibilayers, were prepared from leaf polar lipids isolated on SepPak cartridges and contained MGDG (43%), DGDG (26%), SL (17%), PG (5%), PC (5%) together with small amounts of other phosphoacylglycerols.

A typical ^{13}C-NMR spectrum of a chloroplast lipid dispersion containing melittin is shown in Fig. 5. Only resonances corresponding to the carbon atoms of the lipid acyl chain are distinguishable and all of these are derived from α-linolenic acid, which is the major fatty acid of MGDG and DGDG and constitutes a significant proportion of the fatty acids of SL and PG [14].

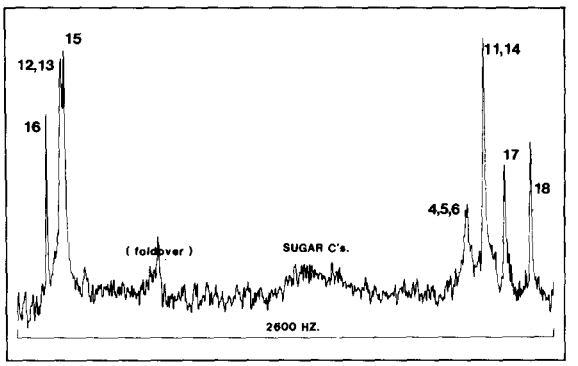

Fig. 5. ^{13}C-NMR spectrum of melittin in chloroplast total lipid (1 : 160) multibilayers in ^2H_2O with the acyl carbon assignments indicated.

The effect of increasing amounts of melittin on the T_1 of the acyl carbon atoms of lipids in reconstituted multilayers is shown in Table I. At molar ratios of melittin : lipid as low as 1 : 160, significant decreases in the T_1 of the acyl carbon atoms

TABLE I

EFFECT OF MELITTIN ON LONGITUDINAL RELAXATION TIMES (T_1) OF FATTY ACYL CARBON ATOMS IN RECONSTITUTED AND POLAR LIPID MULTIBILAYERS

All measurements were made at 30°C and T_1 values (s) are correct to ± 10%.

Acyl carbon	Multibilayers		
Melittin/lipid:	0 : 1	1 : 160	1 : 65
Reconstituted multibilayers			
4, 5, 6	0.27	0.26	0.16
11, 14	1.0	0.9	0.6
12, 13	1.0	0.9	0.7
15	2.4	1.4	1.3
16	2.7	1.4	1.7
17	3.5	2.5	1.9
18	3.7	3.0	3.1
Polar lipid multibilayers			
4, 5, 6	0.35	0.31	0.28
11, 14	0.8	0.7	0.7
12, 13	0.8	0.7	0.7
15	1.6	1.5	1.3
16	2.1	1.8	2.1
17	3.2	3.1	2.7
18	3.5	3.3	2.7

at all positions along the chain can be detected, indicating that the polypeptide penetrates deeply into the bilayer and restricts the rate of motion under these conditions. At higher melittin : lipid ratios (1 : 65) this effect is even more apparent.

Measurements of the effect of melittin on the T_1 of fatty acyl carbon atoms of polar lipid multibilayers (Table I) revealed a similar restriction effect. The major difference between the two lipid dispersions is in their content of negatively charged lipids, the polar lipid multibilayers containing 22% of such lipids, whereas the reconstituted multibilayers contain only 10%. Comparison of the data for the control samples indicates that the acyl carbon atoms of the polar lipid multibilayers have, in general, shorter T_1 values than those of reconstituted multibilayers except near the head-group where the T_1 of the acyl carbons 4, 5 and 6 are longer in the polar lipid multibilayers. However, despite the fact that melittin is positively charged, the differing contents of negatively charged lipids in the two preparations does not lead to significant differences in the changes in T_1 caused by melittin, confirming the monolayer data that electrostatic interactions between the peptide and the lipids are of little significance.

Discussion

The results presented here demonstrate clearly that melittin can interact with both monolayers and bilayers of predominantly uncharged lipids and that both the rate and extent of the interaction are dependent upon the degree of aggregation of the melittin. Under conditions which favour the formation of the melittin tetramer, i.e., high ionic strength and higher pH [3–5], the ability of melittin to penetrate monolayers of uncharged lipids such as MGDG and DGDG is greatly enhanced (Figs. 2 and 3). Although these conditions also favour the formation of a melittin monolayer in the absence of lipid, the rate of pressure increase in the presence of lipid monolayers is far greater than in their absence, indicating that a true interaction is occurring between lipid and polypeptide, even though under some conditions the final pressure increase in the presence of a lipid monolayer may only be equivalent to that of the final pressure of a melittin monolayer in the absence of lipid. In contrast, the final pressure increase obtained when melittin is injected under a monolayer of negatively charged lipids is only slightly affected by the pH and ionic strength, indicating that the monomeric form of the polypeptide is capable of readily penetrating such monolayers. It is difficult to assess the role of electrostatic forces between the negatively charged lipids and the positively charged polypeptide in this interaction. Conditions which decrease electrostatic interactions between proteins such as cytochrome *c* and lipid monolayers, i.e., increase in ionic strength in the subphase [20,21], lead in the present case to formation of the melittin tetramer with a consequent increase in the rate of penetration into the lipid monolayer, although the final pressure increase remains about the same. It should be noted, however, that release of anions or glucose from synthetic liposomes is induced to the same extent by melittin, irrespective of whether the liposomes possessed a net positive or net negative charge [10]. Although it appears certain that the tetrameric form of melittin penetrates monolayers of uncharged lipids more rapidly than does the monomeric form, the monolayer data do not provide any information as to whether melittin exists in the monomeric or tetrameric state after penetrating the monolayer. ^{13}C-NMR results suggest that the content of negatively charged lipids in the bilayer has little effect on the nature of the interaction between the polypeptide and the lipid. Previous studies of melittin-micelle [6,22] and melittin-bilayer interactions [23,24] have suggested that the hydrophobic region of the polypeptide penetrates the hydrophobic interior of the bilayer, but little attention has been given to the charge on the lipid head-group. Measurements of the interaction between melittin and phosphatidylcholine, a zwitterionic phosphoglyceride [25–27], have suggested that the polypeptide binds to the lipid in an aggregated form, most probably as a tetramer, and that about 5–7 lipid molecules form a boundary layer around the peptide. The monolayer experiments reported here would confirm that the tetrameric form of melittin interacts more rapidly with phosphatidylcholine, MGDG and DGDG, than does the monomer form. However, it must be emphasized that the ^{13}C-NMR measurements made in this study were obtained from lipid dis-

persion in ^2H_2O in the absence of buffers and ions, so there is little possibility of tetrameric melittin being present in the aqueous solution. Thus, melittin is capable of penetrating into a lipid bilayer under conditions where only a monomeric form is present, supporting the conclusions of Brown and Wüthrich [22,28] that melittin takes up a specific conformation in bilayer systems with the N-terminal 1–19 amino acid sequence situated within the lipid bilayer.

We conclude, therefore, that the biological activity of melittin is due largely to its ability to penetrate membrane lipid bilayers and modify the motional properties (fluidity) of the lipid molecules. A similar reasoning has been used to explain the effect of melittin on microsomal adenylate cyclase activity [29]. Concomitantly, it is likely that channels permeable to ions are formed, since melittin is capable of releasing ions and small molecules from phosphatidylcholine liposomes [10] and has recently been shown to form channels in PC bilayers [30]. Although such channels are more permeable to anions than to cations [30], they may be responsible for the inability of the chloroplast membrane to establish a light-dependent proton gradient after treatment with melittin [13,31]. The release of plastocyanin from melittin-treated chloroplasts (Ref. 13 an unpublished results) is probably by a mechanism analogous to that causing the leakage of hemoglobin and carbonic anhydrase from erythrocytes [32], and the selective release of enzymes from leucocytes or lysosomal granules isolated from them [11,33]. Plastocyanin is a water-soluble protein, thought to be located in a hydrophilic cleft on the inner side of the chloroplast membrane bilayer [34], and its release from melittin-treated chloroplasts would confirm our previous suggestion [15] that the lipid bilayer plays a major role in maintaining plastocyanin at its site in the chloroplast membrane. The mechanism of protein release from melittin-treated organelles remains unknown and the suggestion [32] that melittin can induce transient openings in membranes large enough to allow the release of proteins remains a speculation.

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