Interaction of melittin with endotoxic lipid A

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Several amphipathic and cationic substances are known to bind lipid A, the toxic component of bacterial lipopolysaccharides. In this report, we have characterized, by fluorescence methods, the interaction of melittin, an amphipathic and basic 26-residue polypeptide isolated from bee venom, with lipid A. The stoichiometry of the complex appears to be two molecules of melittin to one of lipid A with a dissociation constant of 2.5×10^{-6} M. The binding of melittin not only modifies the endotoxic properties of lipid A in a number of biological assays, but also results in abrogation of the hemolytic activity of melittin. A model of the complex is proposed based on the known structures of lipid A and melittin, and the observed stoichiometry of binding.

Introduction

Endotoxin or lipopolysaccharide (LPS), an integral part of the outer membrane of gram-negative bacteria [1–3], elicits diverse biological activities, such as pyrogenicity, lethal toxicity, activation of complement and induction of cytokines, in susceptible hosts [4,5]. Structurally, LPS is made up of three components: (i) a hydrophobic, lipid-rich portion termed lipid A, (ii) a core oligosaccharide and (iii) an outer polysaccharide composed of repeating hetero-oligosaccharide subunits [6,7].

Several substances are known to bind LPS and lipid A. The negatively charged phosphate groups on lipid A, and anionic sites on the polysaccharide regions of LPS facilitate their binding to cationic substances like protamine, polylysine [8], myelin basic protein [9], macrophage cationic protein [10], and cationic dyes [11,12]. The nonpolar acyl chains of lipid A also enable binding to hydrophobic domains of proteins such as lysozymes [13,14]. The interactions of many of these substances with LPS or lipid A manifest in altered endotoxic properties [9,13,15]. In particular, polymyxin B, a cationic cyclic decapeptide with a terminal 8-carbon fatty acid tail, binds to lipid A [16] and abrogates almost the entire spectrum of activities of the latter [17–21]. Both the cationic and hydrophobic components of polymyxin are thought to participate in the formation of the nontoxic complex [16,22].

It was therefore of interest to examine if amphipathic, basic polypeptides of diverse sequences would bind lipid A, and if the binding would manifest in altered endotoxicity of lipid A. In this manuscript, we report the binding of lipid A to melittin, a 26-amino acid polypeptide constituent of bee venom. Melittin is a structurally well characterized basic and amphipathic polypeptide, possessing a cluster of positively charged residues near the C-terminal [23]. Orientational segregation of polar and non-polar side chains gives rise to distinct hydrophilic and hydrophobic domains within the molecule [24,25]. The binding of melittin to lipid A was characterized by fluorimetry, employing the lone tryptophan residue of melittin as an intrinsic fluorescent probe. The activity profile of the complex was characterized using a panel of biological assays.

Materials and Methods

Lipid A prepared from *Salmonella minnesota* Re 595 LPS, melittin from *Apis mellifera* venom, dimyristoyl phosphatidylcholine (DMPC) and dipalmitoyl phosphatidylcholine (DPPC) were purchased from Sigma Chemical Co. (St. Louis, MO). The phospholipids were verified to be homogeneous by thin-layer chromatography using three different solvent systems. Melittin was further purified by gel filtration on a Sephadex G-50 column, eluted with 0.1 M acetic acid.

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The peak fractions (monitored by absorbance at 280 nm) were pooled, the pH adjusted to 7.0 with ammonium hydroxide, and lyophilized. Melittin thus prepared was shown to be homogeneous by reverse phase HPLC on a C-18 column (retention time: 8 min) using 87% methanol:water with 0.5% trifluoroacetic acid for isocratic elution at a flow rate of 0.8 ml/min. The concentration of melittin in solution was calculated by assuming a molar extinction coefficient value of 5570 M^{-1} at 280 nm [26].

Fluorimetry

A stock suspension of lipid A at a concentration of 1 mg/ml was prepared by sonication in water containing 3.5 mM triethylamine. 2 μl aliquots of the lipid A suspension were successively added to 1 ml of water containing 7.15 μM melittin, and was sonicated for 30 s after each addition before fluorescence measurements. As controls, suspensions of DMPC and DPPC (1 mg/ml in water), each containing the same concentration of triethylamine as the lipid A suspension were used. Lipid A:melittin molar ratios were calculated assuming molecular masses of 2800 for melittin, and 1800 for lipid A [27]. The pH of the melittin sample did not change appreciably due to the addition of solubilized lipid A.

Fluorescence experiments were carried out on a Perkin-Elmer MPF-44A fluorimeter at 28°C. All spectra are uncorrected. 10 mm path length matched quartz cuvettes were used. Band pass values for both monochromators were 5 nm and 2 nm for recording emission spectra and emission maxima, respectively. Excitation was at 280 nm. Steady-state polarization values were obtained with a bandpass of 10 nm and were computed using the following equation [28]:

$$P = \frac{I_\parallel - I_\perp G}{I_\parallel + I_\perp G}$$

where $I_\parallel$ and $I_\perp$ are the intensities of the emitted light with polarization planes parallel and perpendicular to the exciting beam, respectively. G is the correction factor for instrument polarization, given by the ratio of vertical and horizontal polarized emission components when the excitation light is polarized in the horizontal direction [29].

Dissociation constants of melittin for lipid A were obtained from Scatchard-type plots [30], plotting delta F, the fluorescence quenching with each addition of lipid A (corrected for quenching induced by triethylamine present in the lipid A suspension) against delta F/lipid A concentration.

Biologic assays

Limulus amoebocyte lysate gelation. Limulus amoebocyte lysate (LAL) sensitive to 0.05–0.2 ng LPS, endo-toxin reference (from *Shigella flexneri*) and endotoxin-free water were purchased from Sigma. Glassware were rendered endotoxin-free by heating in an oven at 250°C for 3 h. Polystyrene pipettes and test tubes used were sterile and endotoxin free.

The test substances were serially diluted ten-fold from 1 μg/ml to 10 pg/ml in endotoxin-free water and dispersed by prolonged, vigorous vortexing. The reaction mixtures containing LAL (0.1 ml) and test substance (0.1 ml) were incubated in a water bath at 37°C and examined for gelation after 1 h as described elsewhere [31]. The positive control contained the reference endotoxin at 2 μg/ml, and endotoxin-free water was used as negative control. A solid gel permitting complete inversion of the tube without disruption of the gel was taken to be a positive endpoint.

Pyrogenicity in rabbits

Domestic rabbits weighing 2–2.5 kg each were injected intravenously with indicated doses of test substances in 5 ml pyrogen-free saline per kg of body weight. Rectal temperatures were recorded every 30 min for 4 h after administration. An increase of temperature of more than 0.6°C was considered to be a positive febrile response [32].

Lethality in chick embryos

The method of Finkelstein [33] was used. Briefly, 11-day-old white Leghorn embryos were injected intravenously with 0.2 ml sterile saline containing the test substances, and lethality was assessed after 48 h.

Stimulation of murine splenocytes

Splenocytes (5×10^5 cells) of BALB/c mice (male, 8–10 weeks old) were cultured with graded doses of test materials in 0.2 ml RPMI medium supplemented with 10% fetal calf serum for 48 h [32]. Concanavalin A (Sigma) was used as a positive control to verify the viability and reactivity of cells. The cells were pulsed with 0.5 μCi of [^{3}H]thymidine during the final 24 h, harvested, and thymidine incorporation was quantitated by conventional methods using a toluene based scintillant.

Hemolysis assay

Fresh human erythrocytes (group O, Rh positive) were washed twice in 0.9% saline and resuspended to yield a 2% suspension. To 1 ml of the suspension, graded doses of test materials were added and incubated at 37°C for 1 h. The samples were centrifuged at 2000 RPM on a bench-top centrifuge, and the optical density of the supernatant was read at 578 nm. Hemolysis was quantitated as a percentage of the total haemoglobin releasable from erythrocytes after osmotic lysis in distilled water.

Results

Fluorescence experiments

The addition of lipid A at a constant melittin concentration resulted in a concentration dependent blue shift in the emission maxima of melittin fluorescence (Fig. 1), which plateaus off at 333 nm, corresponding to a lipid A:melittin molar ratio of 1:2. The blue shift indicates that the tryptophan fluorophore of melittin senses a nonpolar environment in the presence of lipid A.

This is accompanied by a marked increase in steady state emission polarization (Fig. 2), signifying hindered rotational mobility [34] of the tryptophan residue, suggesting that lipid A forms a complex with melittin. The inflection points of the emission wavelength and polarization curves suggest a stoichiometry of two molecules of melittin to one of lipid A.

With neutral phospholipids DMPC and DPPC, the blue shift (Fig. 1) and change in polarization (Fig. 2) are much smaller in magnitude. At the temperature that the fluorescence experiments were carried out, DMPC ($T_c = 24°C$) would be expected to be in the liquid crystalline state, while DPPC and lipid A with their higher phase transition temperatures, would be in the gel phase. To examine if thermotropic transitions of lipid A modified its propensity to bind melittin, polarization measurements on a 1:2 molar ratio mixture of lipid A and melittin were acquired from 10°C to 60°C in increments of 5°C, by means of a thermostatted cuvette holder. A sharp increase in polarization was noted at about 40°C (data not shown), which correlates well with the known phase transition temperature of heptacyl lipid A from *S. minnesota* [35]. From these
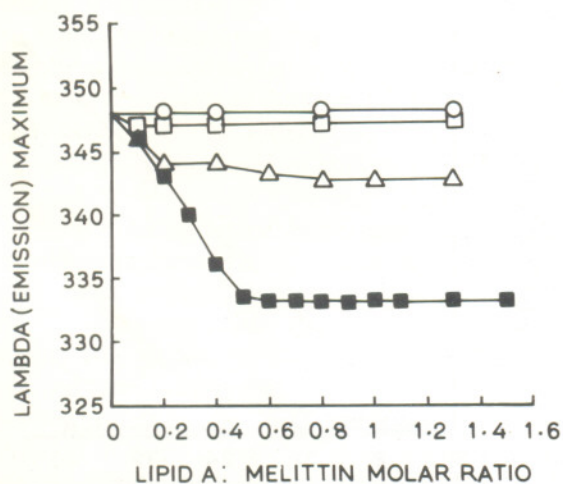
results, it would appear that the phase state of the lipid as well as its charge may influence its binding to melittin.

Concomitant with blue shifts, an enhancement of fluorescence intensity is usually observed [36]. In these experiments, however, quenching was noted (Fig. 3) when lipid A was added. Since triethylamine, an obligatory solubilizer of lipid A, by itself, was found to significantly quench melittin fluorescence, control experiments were performed with DMPC and DPPC

Fig. 2. Effect of addition of lipid A on the steady state emission polarization of melittin. Melittin concentration: 7.15 μM. Excitation: 280 nm. Each data point (■) represents the mean and S.D. of four de terminations. Also represented are controls: water (○), DPPC (□) and DMPC (△), containing identical concentrations of triethylamine as the lipid A suspension.

Fig. 1. Effect of addition of lipid A on the wavelength of maximal emission of melittin. Melittin concentration: 7.15 μM. Excitation: 280 nm. 2-μl aliquots of lipid A (1 mg/ml) in water containing 3.5 μM triethylamine (■) were successively added. As controls, water (○), DPPC (□) and DMPC (△), all containing 3.5 mM triethylamine were used.

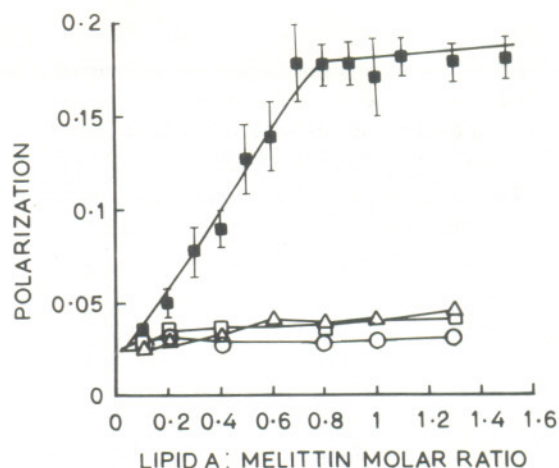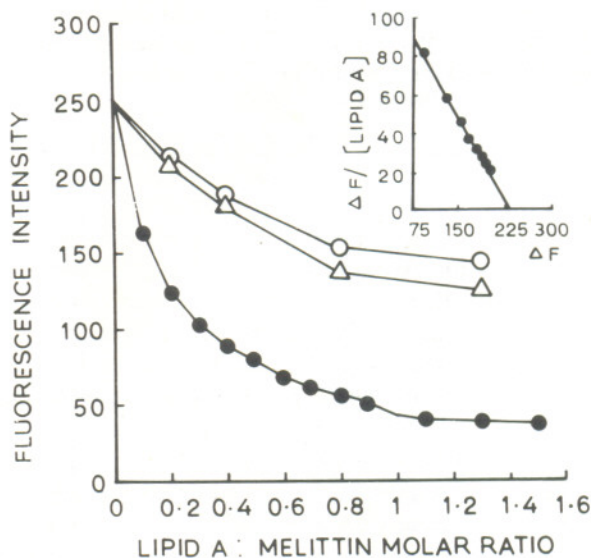Fig. 3. Quenching of melittin fluorescence by lipid A (●). Also represented as controls are DMPC (△) and DPPC (○). The triethylamine content of all suspensions were identical. Inset: Scatchard-type plots of fluorescence titration of lipid A with melittin. Melittin: 3 μM; Lipid A concentration range: 0.3–8.8 μM. Fluorescence intensity values are corrected for quenching by triethylamine present in the lipid A aliquots.

TABLE I

Pyrogenicity of lipid A, melittin and 1:2 molar ratio mixture of lipid A and melittin

Compound	Dose (μg/kg)	Avg. of max. increase of temp. (C^0)	No. positive/ No. tested
Lipid A	10	1.12	2/2
	2	0.47	1/2
	1	0.24	0/2
Melittin	10	0.08	0/2
	2	0.10	0/2
	1	0.11	0/2
Melittin- Lipid A *	10	0.17	0/2
	2	0.13	0/2
	1	0.10	0/2

* Dose indicated denotes concentration of lipid A in the complex.

suspensions containing identical triethylamine concentrations as that of the lipid A suspension. Quenching was observed with the neutral phospholipids as well (Fig. 3), but was of lesser magnitude, indicating that the observed quenching above the control values was attributable to lipid A.

A plot of F versus F/lipid A concentration resulted in a linear set of data points (Fig. 3, inset) from which an apparent K_D of 2.5×10^{-6} M was calculated.

Biological assays

Concentrations of 100 pg/ml and higher, of lipid A produce LAL gelation. Melittin does not show any activity in the range of 1 pg to 1000 ng/ml. A 1:2

TABLE II

Lethal toxicity of lipid A, melittin and 1:2 molar ratio mixture of lipid A and melittin in 11-day-old chick embryos

Compound	Dose		No. of embryos killed/ No. tested	Lethality
Lipid A	10	μg	5/5	100%
	1	μg	5/5	100%
	100	ng	4/5	80%
	10	ng	2/4	50%
	1	ng	1/3	33%
	0.1	ng	1/4	25%
Melittin	10	μg	5/5	100%
	1	μg	5/5	100%
	100	ng	5/5	100%
	10	ng	3/5	60%
	1	ng	0/5	0%
	0.1	ng	0/4	0%
Melittin- Lipid A *	10	μg	3/5	60%
	1	μg	2/5	40%
	100	ng	2/5	40%
	10	ng	1/4	25%
	1	ng	0/4	0%
	0.1	ng	0/4	0%

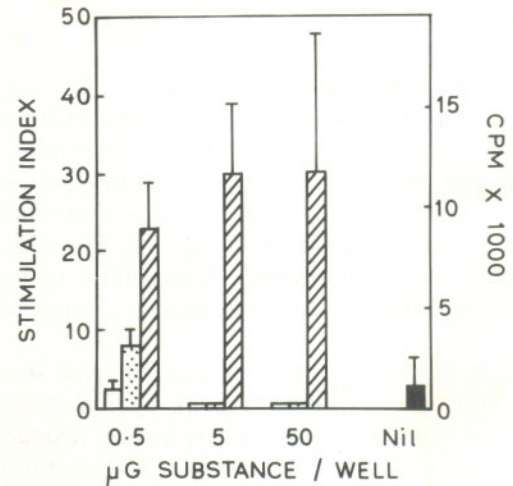* Dose indicated denotes concentration of lipid A in the complex.

Fig. 4. Effect of Lipid A (hatched bars), melittin (blank bars) and lipid A-melittin at 1:2 molar ratio (dotted bars) on [^{3}H]thymidine uptake of murine splenocytes. Stimulation indices denote percentages of the maximal uptake elicited by an optimal concentration of concanavalin A. For the lipid A-melittin complex, doses indicated correspond to concentrations of lipid A in the complex. Each data point represents the mean and S.D. of quadruplicate samples of a single representative experiment.

molar ratio mixture of lipid A and melittin also produces no gelation at these concentrations.

Lipid A evokes a dose dependent fever response (Table I). Melittin alone is nonpyrogenic, and in the presence of lipid A, attenuates the pyrogenicity of the latter.

Lipid A exerts a dose dependent lethality in chick embryos in the range of 0.1 ng to 10 μg (Table II). Melittin, by itself, was also lethal above concentrations of 100 ng. The mortality induced by lipid A-melittin, however, is less than that induced by either substance.
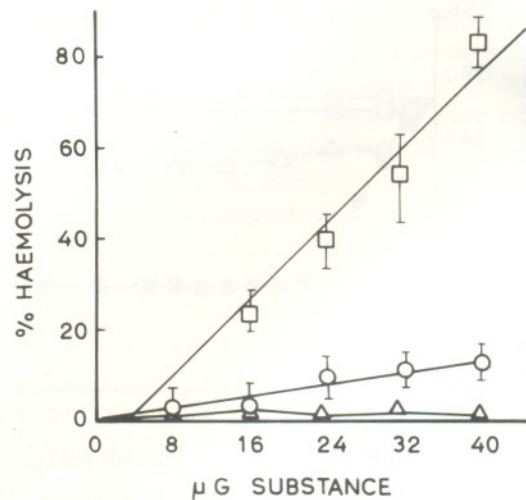

Fig. 5. The haemolytic activity of melittin ($\square$), lipid A-melittin complex at 1:2 molar ratio ($\bigcirc$) and lipid A ($\triangle$). Each data point is the mean and S.D. of three experiments. For the complex, doses indicated correspond to lipid A concentrations present in the complex.

Lethality in control experiments with pyrogen-free saline was about 10%.

The mitogenic activity of the test substances in murine spleen cells expressed relative to that of con-canavalin A (stimulation index) indicates that Lipid A has a marked stimulatory effect, as expected (Fig. 4). At 0.5 μg/ml concentrations, lipid A-melittin (0.5 μg lipid A, 1.0 μg melittin) is significantly less active than lipid A, while melittin alone is nonstimulatory, compared to control. At higher concentrations, melittin as well as the complex are inhibitory.

Melittin, being highly membranophilic, lyses erythrocytes [37]. Hemolysis experiments were therefore carried out to verify if the complexation recipro-cally inactivates both substances. The activity of melit-tin is markedly diminished when lipid A is added to it (Fig. 5). With excess of the latter, hemolysis is com-pletely inhibited; the addition of triethylamine alone to melittin has no effect (data not shown).

Discussion

The objectives of this study were to (i) determine if melittin binds to lipid A, given the physicochemical characteristics of both substances, and (ii) to verify if the interaction resulted in altered biological properties. The data presented in this paper confirms that melittin indeed binds lipid A, giving rise to stable complexes which behave differently from either parent substance in biological assays.

The stoichiometry of the complex of two molecules of melittin to one of lipid A is based on blue-shifts of melittin fluorescence induced by lipid A. Slightly dif-fering molar ratios of 1:0.7 were obtained from polar-ization data. It must be stressed that these values were obtained with natural lipid A; the intrinsic heterogene-ity of lipid A extracted from LPS with respect to acylation and phosphorylation of the glucosamine moi-eties is well recognized [38]. Therefore, the apparent stoichiometry of 2:1 reflects an averaged value of individual stoichiometries of differing species of lipid A.

The unexpected quenching of melittin fluorescence cannot be attributed to the presence of triethylamine alone, as is evident from control experiments per-formed with equivalent concentrations of trieth-ylamine. Thus, it seemed likely that the spatial proxim-ity of the terminal phosphates of lipid A to the trypto-phan fluorophore could be contributory. The addition of lipid A to melittin resulted in an increase in turbid-ity of the sample, as has been observed with polymyxin [16]. This may have resulted in increased light scatter-ing, further attenuating fluorescence intensity.

Melittin is an amphipathic molecule characterised by an overall hydrophobic N-terminal, and a hy-drophilic C-terminus [23]. In aqueous solutions of low ionic strength, melittin exists in an unfolded, random coil conformation, while under conditions of higher ionic concentrations, a bent alpha helical form is as-sumed [38]. The helix shows orientational segregation of hydrophobic and polar sidechains with respect to the 'inner' and 'outer' surfaces of the circumference [24,25]. The fluorescence experiments, carried out with melittin in distilled water, would have precluded the molecule from assuming the highly ordered, helical form. However, lipid bound melittin has been shown to be similar to the tetrameric form in solution [39,40]. It is possible, therefore, that a conformational change is associated with its binding to lipid A. We were unable to verify this by circular dichroism experiments because of the turbidity of the sample.

The complexation of lipid A to melittin results not only in attenuated toxicity of lipid A in several assay systems, but also in reduced hemolytic activity of melit-tin, suggesting that the complex is stable under physio-logical conditions. Noteworthy is the complete nonre-activity of the complex in the LAL gelation assay, even at concentrations of 1000 ng of lipid A. Because the batch of lipid A used was a mixture of the diphospho-ryl- and the nontoxic monophosphoryl species, the po-tency in biological assays was found to be less as compared to studies reported in the literature. For instance, the effect of 10 μg of lipid A on the fever response in rabbits is comparable to that produced by 1 μg of Re 595 lipid A in an earlier investigation [41]. However, the difference in the biologic activities be-tween free lipid A and the complex is unequivocal.

Based on the structures of lipid A and melittin, and the observed binding stoichiometry, a model of the complex could be proposed: two molecules of melittin are bound to a molecule of lipid A in a coaxial manner, such that the C-terminal regions of the polypeptide, bearing a cluster of positively charged groups are prox-imal to the phosphates on the disaccharide backbone of lipid A, while the apolar surfaces of melittin overlie the acyl chains. Thus, steric shielding of portions of lipid A may result in diminution or loss of its proper-ties.

Further experiments are being planned to examine the relative importance of electrostatic and hydropho-bic interactions in the formation of polypeptide-lipid A/LPS complexes using derivatives of melittin and synthetic peptides. The identification of molecules which neutralize the biological effects of lipid A may help in rationally developing therapeutically useful compounds.

Acknowledgements

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