Effect of permethylation on the haemolytic activity of melittin

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The cytolytic activity of the bee venom toxin, melittin, is abolished on permethylation of the ammonium groups into quaternary trimethylammonium groups. The loss of activity in permethylated melittin may result partly from the absence of the hydrogen bonding potential and partly from steric effects involving the bulky trimethylammonium groups. Displacing the trimethylammonium groups away from the backbone to relieve steric effects (by acylating melittin with glycine or 5-aminopentanoic acid followed by permethylation) restored moderate activity at 5-fold increase in concentration.

INTRODUCTION

Melittin is the major polypeptide isolated from bee venom and is noted primarily for its marked cytolytic activity (Habermann & Jentsch, 1967). The amino acid sequence, GIGAVLKVLTTGLPALISWIKRKRQQNH₂, is largely hydrophobic at the *N*-terminal region (1–20) and hydrophilic at the *C*-terminal region (21–26). There are five basic residues, four of which are present in the *C*-terminal region (21–24). The primary sequence of melittin is thus asymmetric with respect to the charge distribution. Melittin interacts with both biological and synthetic lipid membranes and has been extensively studied as a model for peptide–lipid interactions (see Bernheimer & Rudy, 1986; Dempsey, 1990, for reviews). In methanol or in membranes, melittin has been shown to adopt a monomeric helical structure, whereas in aqueous solutions its conformation and state of association are dependent on peptide concentration, pH and ionic strength (Talbot *et al.*, 1979; Knoppel *et al.*, 1979; Lauterwein *et al.*, 1980; Brown *et al.*, 1980; Bello *et al.*, 1982; Tatham *et al.*, 1983).

The lytic property of melittin has been studied in relation to the importance of the positive charges and the largely hydrophobic segment using melittin-like model peptides and melittin analogues (Schroeder *et al.*, 1971; DeGrado *et al.*, 1981). Melittin analogues that lack a few residues at either of the terminals are inactive (Schroeder *et al.*, 1971). A synthetic peptide possessing an amphiphilic *N*-terminal similar to melittin and retaining the *C*-terminal basic region was shown to be cytolytic (DeGrado *et al.*, 1981). Introduction of negative charges through succinoylation of the *N*-terminal amino group and the ϵ -amino groups of lysine led to the loss of activity (Habermann & Kowallek, 1970). These studies showed that a specific cationic region as well as an amphipathic helical segment are required for haemolytic activity. Recently, Blondelle & Houghten (1991a,b) have studied the importance of individual residues in the cytolytic activity of melittin. A series of position-specific omission analogues of melittin was synthesized and their relative haemolytic activity determined. Analogues lacking residues at the *C*-terminal were found to be slightly more active than melittin. In the second study, 26 analogues of melittin in which individual residues were replaced by Leu were synthesized and their haemolytic activity

compared with that of melittin. These studies indicated that the *C*-terminal region of melittin does not require all of its positive charges, but need only be sufficiently cationic to bind to the membrane and cause lysis (Blondelle & Houghten, 1991a,b).

The interaction of melittin with erythrocytes, initially, is probably a result of contact interaction between the positively charged *C*-terminal peptide and the negatively charged lipid phosphate head groups (Kini & Evans, 1989). Conversion of the ammonium groups of melittin to guanidinium groups resulted in a 3.5-fold increase in lytic activity compared to melittin (Habermann & Kowallek, 1970). This increase in activity is probably as a result of higher hydrogen-bonding capability of the guanidinium groups. In addition to the greater number of potential hydrogen-bonding hydrogens in the guanidinium group, there is also the possibility of forming bonds to two of the oxygens of a phosphate group. Also, it has been reported that the amino group of lysine-21 has a pK_a of 6.5 (Quay & Tronson, 1983). Conversion to guanidinium would render this residue cationic at the pH of lysis experiments, thereby providing a higher positive charge.

Dempsey *et al.* (1987) observed that melittin dimethylated on each of the four amino groups (α -amino group of glycine and ϵ -amino groups of lysines-7, -21 and -23) shows only 30% haemolytic activity compared to melittin. The loss of about two-thirds of lytic activity on replacement of two-thirds of hydrogens of ammonium groups in melittin suggests that hydrogen-bonding to phosphate groups of lipids may be important in the interaction of melittin with membranes. To test further this idea we permethylated (all the hydrogen atoms of the ammonium groups are replaced by methyl groups to give quaternary trimethylammonium groups) the ammonium groups of melittin (the α -amino group of glycine and the ϵ -amino groups of lysines-7, -21 and -23), so that these groups have no hydrogen-bonding capability. Permethylation of the ammonium groups, apart from retaining the positive charges (which are now pH-independent) and eliminating the hydrogen-bonding, may also introduce some steric effect as a result of the bulky trimethylammonium groups. We also prepared derivatives of melittin in which the amino groups were acylated with glycine (MLT-2) or 5-aminopentanoic acid (MLT-5) to move the ammonium groups farther from the main chain, and permethylated these derivatives (MLT-2Me and MLT-5Me, respectively) to determine the steric effects.

Abbreviations used: MLT, melittin; MLT-2, glycyl-melittin; MLT-5, 5-aminopentanoyl-melittin; MLT-Me, permethylated melittin; MLT-2Me, permethylated glycyl-melittin; MLT-5Me, permethylated 5-aminopentanoyl-melittin; poly[Lys(Me)₃], poly(*N*^ε,*N*^ε,*N*^ε-trimethyl-L-lysine); Boc, *t*-butoxycarbonyl; BSS, Hank's balanced salt solution.

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EXPERIMENTAL

Materials

Melittin (Sigma) was twice purified by chromatography using Sephadex G-50 (1.5 cm × 100 cm column, 20 mM-acetic acid). MLT-2 and MLT-5 were prepared by acylating the α - and ϵ -amino groups of the glycyl and lysyl residues of melittin with the succinimide esters of Boc-glycine or Boc-5-aminopentanoic acid followed by deprotection of Boc using *p*-toluenesulphonic acid/triphenylphosphine. The succinimide esters were prepared from glycine or 5-aminopentanoic acid using *N*-hydroxysuccinimide with ethyl-di-isopropylcarbodi-imide as the coupling agent (Bodanszky, 1988). The synthetic peptides were purified by Sephadex G-15 column chromatography (100 mM-ammonium acetate). The integrity of the tryptophan residue was checked by u.v. spectroscopy. The amino acid composition of the melittin and melittin derivatives was in accord with the expected values. Permethylation of MLT, MLT-2 and MLT-5 was carried out using dimethyl sulphate at about pH 9.5, following a reported procedure (Granados & Bello, 1979). The derivatives were desalted using Sephadex G-15 in 100 mM ammonium acetate. Amino acid analysis of the permethylated melittin showed the expected loss in the number of glycyl and lysyl residues, showing complete methylation. The permethylated derivatives, MLT-2Me and MLT-5Me, also showed the expected loss in the number of additional glycyl or 5-aminopentanoyl residues, respectively. C.d. spectra in 100 % methanol were obtained using a Jasco-500 spectropolarimeter at 25 °C, using a silica cell of 2 mm path length. Concentrations of the peptide stock solutions were determined from the tryptophan absorption spectra and an ϵ_{280} value of 5600 M⁻¹·cm⁻¹ (Bello *et al.*, 1982).

Haemolysis

Haemolytic activity of the peptides was monitored by the release of haemoglobin from erythrocytes (Hider *et al.*, 1983). A typical assay is as follows: 1 ml of the packed erythrocytes was diluted to 50 ml in BSS buffer (125 mM-NaCl, 5 mM-KCl, 4 mM-CaCl₂, 2.5 mM-MgCl₂, 5 mM-Tris, pH 7.4). To 1 ml of the blood suspension 1 ml of the peptide was added at 25 °C. After 1 h, cells were centrifuged and the absorbance of the supernatant containing the released haemoglobin was measured at 576 nm. A 100 % haemolysis control was determined using erythrocytes suspended in BSS and 0.05 % Triton-X100.

RESULTS AND DISCUSSION

The primary structures of the melittin derivatives are shown in Fig. 1. Permethylation of the derivatives was confirmed by amino acid analysis. The haemolytic activities of MLT and its permethylated derivative MLT-Me are shown in Table 1. At 3.5 μ M

Fig. 1. The primary structure of melittin and its derivatives

The amino groups of lysine, to which X is attached, are implied.

Table 1. Haemolytic activity of the melittin derivatives at 25 °C

Peptide	Concn. (μ M)...	Lysis (%)		$10^{-3} \times -[\theta]_{222}$ (degrees·cm ² ·dmol ⁻¹) in 100 % methanol
		3.5	17.5	
MLT	100	100		23.6
MLT-2	94	100		17.2
MLT-5	56	100		21.3
MLT-Me	0	5		14.8
MLT-2Me	0	33		16.6
MLT-5Me	4	41		23.0

Fig. 2. C.d. spectra of melittin and derivatives in 100 % methanol

1, MLT-2; 2, MLT-5; 3, MLT. (b) 4, MLT-Me; 5, MLT-2Me; 6, MLT-5Me.

(10 $\mu\text{g/ml}$) concentration, MLT is 100 % active. But MLT-Me loses its haemolytic ability completely (essentially zero within a probable error of $\pm 10\%$). Even at 17.5 μM (50 $\mu\text{g/ml}$) its activity is only 5 %. This result supports the idea that either the modified amino groups of glycine and lysines in melittin must have hydrogen-bonding potential in addition to the presence of positive charges, or that the permethylated ammonium groups bind more weakly to anionic lipids for steric reasons. For example, in the permethylated melittin the centres of charge cannot approach each other as closely as in the case of melittin.

Methylated lysyl residues are found in several proteins (for example, histones, muscle proteins, cytochrome *c*) that are widely distributed in nature (Paik & Kim, 1971, 1990). The biological significance of this modification is unclear. In cytochrome *c*, Kim *et al.* (1980) found that enzymic trimethylation of Lys-72 reduced the isoelectric point from 10.0 to 9.5 and, from space-filling models, they proposed that methylation disrupts a hydrogen bond between Lys-72 and Asn-70. Park *et al.* (1988) have suggested that trimethylation of lysine residues in cytochrome *c* lends stability to a more 'open' or unfolded structure that can be imported into mitochondria more readily. Methylation also diffuses the permanent positive charge on the ϵ -amino group of a lysine residue, leading to a weakening in the electrostatic interaction. Such weakened electrostatic contribution has been suggested to be one of the reasons for the easy dissociation of the poly[Lys(Me₃)]·DNA complex compared to poly(Lys)·DNA in the presence of salts (Granados & Bello, 1980). Also, from the interaction of poly[Lys(Me₃)] with SDS, it has been suggested that poly[Lys(Me₃)] may have reduced ability to interact with lipids compared with poly(Lys) (Granados & Bello, 1979). Thus, it is possible that the trimethylammonium groups of the permethylated MLT-Me, MLT-2Me and MLT-5Me bind less efficiently with the head groups of the lipids compared with the ammonium groups of melittin.

Steric effects were explored by using MLT-2 and MLT-5, in which the positive charge on the ϵ -amino group of lysyl residues and the α -amino group of glycine is displaced an additional two and five carbon atoms away from the melittin backbone. These unmethylated derivatives are 94 % and 56 % active at 3.5 μM (10 $\mu\text{g/ml}$) concentration. Permethylation of these peptides also results in negligible activity at 3.5 μM concentration. But when the concentration of permethylated peptides is increased to 17.5 μM (50 $\mu\text{g/ml}$), MLT-2Me and MLT-5Me are 33 % and 41 % active compared to melittin. The results suggest that hydrogen-bonding is not indispensable for lysis, and that a steric effect also operates. The bulky trimethylammonium groups of MLT-2Me and MLT-5Me, when displaced outward and less hindered sterically, appear to be able to interact with the membrane.

Melittin has been shown to adopt a helical structure in membrane surfaces such as egg yolk phosphatidylcholine liposomes (Drake & Hider, 1979). The ability of these permethylated

melittin derivatives to adopt helical structures was determined by measuring the c.d. spectra in 100 % methanol (to simulate the effect of membranes) (Figs. 2a and 2b). The c.d. spectra show that all of the derivatives are helical in methanol, with MLT-Me the least helical. The helix-forming potential in methanol shows that these permethylated peptides probably behave similarly to melittin in membranes.

We suggest that the decrease of lytic activity in permethylated melittin arises partly from the absence of hydrogen-bonding potential and partly from the steric effects of the bulky trimethylammonium groups.

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