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REVIEW ARTICLE

Melittin: a Membrane-active Peptide with Diverse Functions

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Abstract Melittin is the principal toxic component in the venom of the European honey bee *Apis mellifera* and is a cationic, hemolytic peptide. It is a small linear peptide composed of 26 amino acid residues in which the amino-terminal region is predominantly hydrophobic whereas the carboxy-terminal region is hydrophilic due to the presence of a stretch of positively charged amino acids. This amphiphilic property of melittin has resulted in melittin being used as a suitable model peptide for monitoring lipid–protein interactions in membranes. In this review, the solution and membrane properties of melittin are highlighted, with an emphasis on melittin–membrane interaction using biophysical approaches. The recent applications of melittin in various cellular processes are discussed.

Keywords Melittin · Hemolysis · Tryptophan fluorescence · Lipid–protein interactions · Melittin orientation · Aggregation and pores

Abbreviations

DTPC	1,2–ditetradecyl-*sn*-glycero-3-phosphocholine
DMPC	1,2-dimyristoyl-*sn*-glycero-3-phosphocholine
DLPC	1,2-dilauroyl-*sn*-glycero-3-phosphocholine
DPhPC	1,2-diphytanoyl-*sn*-glycero-3-phosphocholine
DOPA	1,2-dioleoyl-*sn*-glycero-3-phosphoacid
DOPC	1,2-dioleoyl-*sn*-glycero-3-phosphocholine
PC	Phosphatidylcholine
PG	Phosphatidylglycerol
PEI	Poly(ethyleneimine)
POPG	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol
REES	Red edge excitation shift

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Melittin is the principal toxic component in the venom of the European honey bee *Apis mellifera* and is a cationic, hemolytic peptide (Habermann 1972; Dempsey 1990). It constitutes 50% of the dry weight of the bee venom. It was first identified as a 'direct lytic factor' since it induced hemolysis in the absence of added lecithin (Neumann et al. 1953; Habermann 1972). The active peptide melittin is released from its precursor, promelittin, during its biosynthesis in honey bee and later gets formylated (Habermann 1972). It is a small linear peptide composed of 26 amino acid residues (NH_2-GIGAVLKVLTTGLPALISWIKRKRQQ-$CONH_2$) in which the amino-terminal region (residues 1–20) is predominantly hydrophobic whereas the carboxy-terminal region (residues 21–26) is hydrophilic due to the presence of a stretch of positively charged amino acids. The amphiphilic property of this peptide makes it water-soluble and yet it spontaneously associates with natural and artificial membranes (Dufourcq et al. 1984; Bernheimer and Ruby 1986; Dempsey 1990; Sansom 1991; Saberwal and Nagaraj 1994). Such a sequence of amino acids, coupled with its amphiphilic nature, is characteristic of many membrane-bound peptides and putative transmembrane helices of membrane proteins (Dempsey 1990; Shai 2002). This has resulted in melittin being used as a suitable model peptide for monitoring lipid–protein interactions in membranes as well as for cytolytic peptides.

There are several reviews available in the literature which cover pharmacological aspects of many cytolytic and channel-forming antimicrobial peptides (which includes melittin) as well as their interaction with membranes (Habermann 1972; Bernheimer and Ruby 1986; Dempsey 1990; Sansom 1991; Saberwal and Nagaraj 1994; Vernon and Bell 1992; Fletcher and Jiang 1993; Cserhati and Szogyi 1994; Bechinger 1997, 1999, 2004; Sitaram and Nagaraj 1999; Kourie and Shorthouse 2000; Dathe and Wieprecht 1999). However, an excellent review by Dempsey (1990) is the only review solely dedicated to melittin. Numerous studies have contributed significantly to our current understanding on the molecular mechanisms of melittin-induced lysis and the general features of lipid–protein interactions as well as its diverse cellular functions since then. Because melittin reaches target membranes through the aqueous phase, the properties of melittin in aqueous solution are relevant to its effects on membranes. The emphasis of this review is to provide a comprehensive account of the solution properties of melittin followed by a discussion on the actions of melittin on membranes. In the process, the structural requirements for the action of melittin, its orientation, aggregation state, and current view of pore formation as well as its various cellular actions are discussed.

Structure and Conformation of Melittin in Solution

Melittin, like other membrane-binding peptides and proteins, is predominantly hydrophobic. Yet, the peptide has a net charge of +6 at physiological pH, four of which are at a stretch in the highly basic C-terminal region (Lys-Arg-Lys-Arg) and the remaining two in the N-terminal region, Lys-7 and the N-terminal group (Gly-1). There is an asymmetric distribution of polar and non-polar amino acids which makes melittin amphipathic when the peptide is aligned in an α-helical configuration as shown in Fig. 1 as a helical wheel diagram (Dathe and Wieprecht 1999). Though melittin has a high proportion of non-polar amino acids, it is highly soluble in water (>250 mg/ml) and moderately soluble in methanol (up to 20 mg/ml). At low concentration, melittin is monomeric and adopts essentially a random coil conformation in aqueous solution.

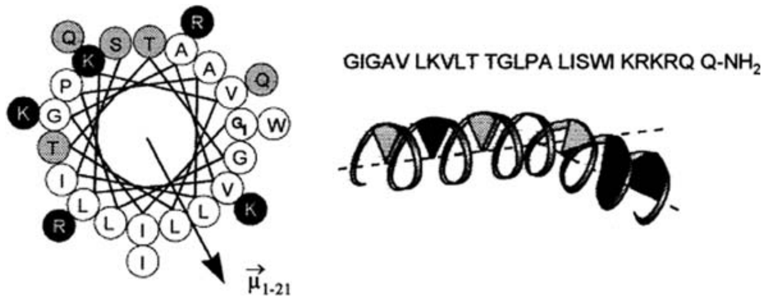

Fig. 1 Helical wheel projection and schematic representation of the amphipathic helix of melittin showing various structural features. The one letter code for amino acids is used. Hydrophobic residues are shown in white, polar residues in gray and cationic residues in black circles. The hydrophobic moment (μ) for the α-helical region is shown. Adapted and modified from Dathe and Wieprecht (1999)

Importantly, it adopts an α-helical conformation and aggregates into tetramers depending on several factors (Dempsey 1990) as described below (see 'Aggregation/self-association of melittin in solution').

High-resolution Structures of Melittin

The three-dimensional structure of the melittin tetramer in aqueous solution is known to atomic resolution as determined by X-ray crystallographic analysis at 2 Å resolution from melittin crystals grown from solutions of high ionic strength (Anderson et al. 1980; Terwilliger and Eisenberg 1982a, b; Terwilliger et al. 1982). The four melittin monomers in the tetramer are nearly identical in conformation. In the crystalline tetramer (see Fig. 2), each melittin chain is composed of two α-helical segments and the overall shape is that of a 'bent rod'. The peptide helix is bent due to the presence of proline at position 14 so that lines drawn through the helix axes of residues 1–10 and 16–26 intersect with an angle of about 120°. The large helix bend allows optimal packing of hydrophobic side chains within the melittin tetramer. Despite a large bend around Pro-14, the crystalline melittin monomer has an essentially helical conformation throughout. The degree of helicity of crystalline tetrameric melittin is greater than expected from the value determined by circular dichroism analysis for tetrameric melittin in solution which is around 50–60% (Knoppel et al. 1979; Bello et al. 1982). Due to the asymmetric distribution of polar and apolar side chains, the melittin chain has a hydrophobic amino terminal region, a central region with hydrophobic and hydrophilic faces, and an entirely hydrophilic (charged) C-terminal region.

In addition to the X-ray structure of tetrameric melittin in aqueous solution, high-resolution NMR structures of monomeric and tetrameric melittin in aqueous solutions have been determined (Lauterwein et al. 1980; Brown et al. 1980). Further, high-resolution NMR structures of monomeric melittin in methanol (Bazzo et al. 1988; Dempsey 1988, 1992) and dodecyl phosphatidylcholine micelles (Brown and Wüthrich 1981; Brown et al. 1982; Inagaki et al. 1989; Ikura et al. 1991) have also been determined. Melittin is monomeric and α-helical in methanol (Bazzo et al. 1988; Dempsey 1988, 1992) and the structure has been found to be similar to that found in melittin crystals. Surprisingly, the structure of the hinge region in the melittin chain is shown to be significantly different from that of the crystal structure, leading to a considerably smaller angle between the two helical segments. This indicates that the Pro-14 residue need not induce a large bend in the melittin structure and this should be

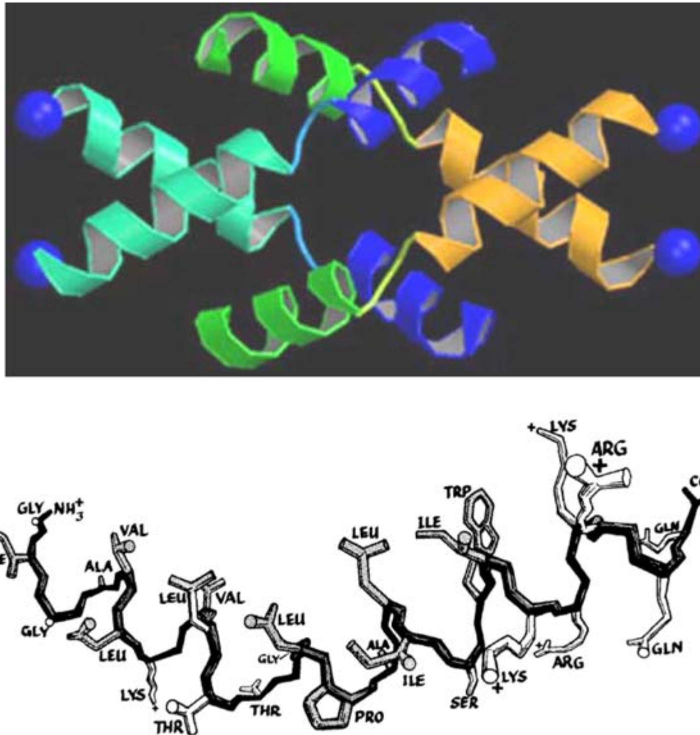

Fig. 2 X-ray crystal structure of melittin tetramer in solution at 2 Å resolution (top) (PDB code 2MLT). Each melittin monomer is composed of two α-helical segments and the overall shape is that of a 'bent rod'. Conformation of one melittin chain from the tetramer is shown below (Terwilliger and Eisenberg, 1982)

considered in models of the membrane-bound conformation in the absence of direct determination of perturbation induced by the proline residue. Interestingly, the NMR structure of melittin bound to micelles (Brown and Wüthrich 1981; Brown et al. 1982; Inagaki et al. 1989; Ikura et al. 1991) shares most of the features of the NMR structure obtained in methanol and aqueous solutions (Brown et al. 1980), and the peptide secondary structure is stable from pH 4 to pH 11 (Yuan et al. 1996). These high-resolution structures of melittin in the crystalline state, in solution of low dielectric constant and in micelles, have been considered as good models for the membrane-bound conformation (Dempsey, 1990). Interestingly, the secondary structure of membrane-bound melittin has also been shown to be predominantly α-helical (see 'Melittin–membrane interactions').

Aggregation/Self-association of Melittin in Solution

In aqueous solution, aggregation of monomeric melittin to a tetramer is promoted by high salt, high melittin concentration, and high pH. These factors strongly suppress the charge of melittin and promote the self-association of melittin monomers into tetramers since melittin has a high charge density and aggregation would be prevented by electrostatic repulsions. For example, Talbot et al. (1979) have shown by optical rotatory dispersion (ORD), gel filtration and fluorescence measurements that melittin is

monomeric and adopts a random coil conformation when the peptide concentration and ionic strength are low at physiological pH. This is in agreement with fluorescence experiments which show that melittin is predominantly monomeric at very low salt concentration (Quay and Condie 1983; Raghuraman and Chattopadhyay 2006a). In contrast, the aggregation of melittin is promoted by high concentration of the peptide and/or high ionic strength at neutral pH (Talbot et al. 1979; Faucon et al. 1979; Raghuraman and Chattopadhyay 2006a). The effect of increasing melittin concentration on tetramer formation has been supported by an increase in the helical content of the peptide (Bello et al. 1982). In addition, Knoppel et al. (1979) reported a change in the conformation of melittin to tetramer at high pH although the concentration of the peptide and the ionic strength are low. Further, Brown et al. (1980) observed aggregation of melittin to the tetrameric form at (i) elevated concentration at neutral pH, (ii) low pH in 1.5 M NaCl, (iii) high concentration in low pH, salt-free solution, and (iv) pH 9 in salt-free solution, by monitoring the ^{1}H NMR spectra and sedimentation behavior. An extensive study on the effect of pH and concentration on the conformation and aggregation of melittin (Bello et al. 1982) has shown that deprotonation of the α-amino group of melittin enhances the tendency to aggregate. These studies point out that the conformation of melittin in aqueous solution is a complex function of peptide concentration, ionic strength and pH. Further, dependence of melittin aggregation on temperature (Iwadate et al. 1998) and its thermodynamics determined by circular dichroism spectroscopy has also been reported (Wilcox and Eisenberg 1992). Interestingly, tetramer formation has been shown to occur when the net charge of melittin is modified by acetylation (net charge +2) or succinylation (net charge –2) and this conversion is promoted by an increase in peptide concentration (Bello et al. 1982).

The complexity of the aggregation behavior of melittin in solution with reference to electrostatic repulsion has been demonstrated by Ramalingam et al. (1992) using anionic melittin analogues. They changed the net charge of melittin to –6 by acetylation and showed that this anionic derivative forms a tetrameric helix at neutral pH without salt, and at relatively low concentrations under which native melittin is monomeric and adopts random coil conformation. This observation suggests that a high net charge is not sufficient to prevent the association and helix formation of melittin. Surprisingly, the anionic melittin analogues in which all five lysine and arginine residues are replaced with glutamate (net charge –4), and the acetyl and succinyl derivatives of this anionic melittin (net charges –5 and –6, respectively) were resistant to helix formation and required much higher salt concentration for helix formation than cationic melittin. Even divalent cations are found to be less effective in promoting helix formation in these derivatives when compared with melittin (Ramalingam et al. 1992).

Overall, the aggregation/self-association of melittin in aqueous solution is a complex phenomenon and strictly depends on the interplay between the peptide concentration and solution properties (ionic strength and pH), which in turn affect the hydrophobic, electrostatic, and helix–dipole interactions at the N-terminus of melittin. We have recently shown that the red edge excitation shift (REES) approach (Demchenko 1988; Chattopadhyay 2003; Raghuraman et al. 2005) could be used as a convenient tool to monitor the aggregation behavior of melittin in aqueous solution (Raghuraman and Chattopadhyay 2006a). REES represents a powerful approach which can be used to directly monitor the environment and dynamics around a fluorophore in a complex biological system (Demchenko 2002; Chattopadhyay 2003; Raghuraman et al. 2005). This effect is mostly observed with polar fluorophores in motionally restricted environments such as viscous solutions or condensed phases where the dipolar relaxation

time for the solvent shell around a fluorophore is comparable to or longer than its fluorescence lifetime. REES arises due to slow rates of solvent relaxation (reorientation) around an excited state fluorophore, which is dependent on the motional restriction imposed on the solvent molecules in the immediate vicinity of the fluorophore. Utilizing this approach, it becomes possible to probe the mobility parameters of the environment itself (represented by the relaxing solvent molecules) using the fluorophore merely as a reporter group. The unique feature about REES is that while all other fluorescence techniques yield information about the fluorophore itself, REES provides information about the relative rates of solvent (water in biological systems) relaxation dynamics, i.e., the dynamics of hydration, which is not possible to obtain by other techniques. Interestingly, the ultrafast hydration dynamics associated with the folding and self-association of melittin has been recently reported (Qiu et al. 2005).

Melittin–membrane Interactions

Several naturally occurring peptides have been used to characterize lipid–protein interactions in model membranes (Sitaram and Nagaraj 1999). Melittin is one of the most extensively used peptides to understand lipid–protein interactions at the molecular level (Dempsey 1990). The small size and easy availability of melittin makes it a suitable model peptide to monitor lipid–protein interactions using a variety of biophysical techniques. However, studies involving melittin–membrane interactions should be carefully carried out since bee venom also contains an endogenous phospholipase A_2, apart from melittin (Shipolini et al. 1971). Since melittin frequently retains phospholipase activity, probably as a result of binding of the peptide to the enzyme (Dempsey 1990; Banks et al. 1981), phospholipase A_2 contamination in melittin presents a major concern for artifacts in experiments involving melittin with phospholipid membranes unless the peptide is purified free of the enzyme. A number of reports on melittin-induced changes in thermal transition temperature in melittin–DMPC complexes have been shown to result from contaminating phospholipase A_2 activity (Dasseux et al. 1984; Dempsey and Watts 1987). Trace amounts of phospholipase A_2 are often present in melittin obtained from commercial sources. Fortunately, the effect of phospholipase A_2 is detectable only if a significant amount of Ca^{2+} is added to the sample. In fact, results from both commercially available melittin and pure synthetic melittin have been shown to be indistinguishable in the absence of added Ca^{2+} (Yang et al. 2001). Nevertheless, as a precautionary measure, it is advisable to use buffers with EDTA at concentrations of 5 mM in experiments involving melittin–membrane interactions to ensure the suppression of residual phospholipase A_2 activity (Dempsey 1990). Importantly, it has been shown that the phospholipase activity, assayed using radiolabeled phospholipids, could not be detected in lipid samples containing 5 mM EDTA in buffer even after keeping for a few days at room temperature (Ghosh et al. 1997).

Melittin as a Model Peptide for Studying Lipid–protein Interactions

As mentioned above, the amphipathic nature is characteristic of many membrane-bound peptides and putative transmembrane helices of membrane proteins (Dempsey 1990; Shai 1995). These include apolipoproteins and peptide hormones (DeGrado et al. 1982; Kaiser and Kezdy 1984; Morii et al. 1994), signal peptides (Garnier et al. 1980;

Golding and O'Shea 1995), the envelope glycoprotein gp41 from the human immunodeficiency virus (Eisenberg and Wesson 1990; Rabenstein and Shin 1995), the pore-forming peptide of pathogenic *Entamoeba histolytica* (Leippe et al. 1991, 1992) and the 25-residue presequence (p25) of subunit IV of yeast cytochrome oxidase (Clague and Cherry 1988). Interestingly, melittin exhibits sequence and structural similarities to a region of the tobacco mosaic virus coat protein (TMV CP), which is known to be critical for protein–protein and protein–RNA interactions (Marcos et al. 1995). Further, understanding of melittin–membrane interaction assumes significance due to the finding that melittin mimics the structure of N-terminal of HIV-1 virulence factor Nef1-25 (Barnham et al. 1997). This has resulted in melittin being used as a convenient and popular model for monitoring lipid–protein interactions in membranes. Melittin is intrinsically fluorescent due to the presence of a single tryptophan residue, Trp-19, which makes it a sensitive probe to study the interaction of melittin with membranes and membrane-mimetic systems (Lauterwein et al. 1979; Vogel 1981; Bernard et al. 1982; Georghiou et al. 1982; Yianni et al. 1986; Hermetter and Lakowicz 1986; Chandani and Balasubramanian 1986; Schulze et al. 1987; Batenburg et al. 1987; John and Jähnig 1988; Schwarz and Beschiaschvili 1989; Kaszycki and Wasylewski 1990; Sekharam et al. 1991; Nishiya and Chou 1991; Weaver et al. 1992; Watala and Gwozdzinski 1992; Bismuto et al. 1993; Teng and Scarlata 1993; Chattopadhyay and Rukmini 1993; Bradrick et al. 1995; van Veen et al. 1995; Benachir and Lafleur 1996; Hincha and Crowe 1996; Cajal and Jain 1997; Oren and Shai 1997; Ghosh et al. 1997; Wimley and White 2000; Raghuraman and Chattopadhyay 2003; 2004a, b, c; Raghuraman et al. 2006). This is particularly advantageous since there are no other aromatic amino acids in melittin and this makes interpretation of fluorescence data less complicated due to lack of interference and heterogeneity. Importantly, it has been shown that the sole tryptophan residue of melittin is crucial for its powerful hemolytic activity (see 'Actions of melittin on membranes'). The organization and dynamics of the tryptophan residue therefore become important for the function of the peptide.

Melittin targets the membrane from the aqueous phase and partitions into phosphatidylcholine (PC) membranes with a partition coefficient (K_p) of ~10^3–10^5 M^{-1} (Schwarz and Beschiaschvili 1989; Kuchinka and Seelig 1989; Beschiaschvili and Seelig 1990; Beschiaschvili and Baeuerle 1991; Mozsolits et al. 2001; Kriech and Conboy 2003). The association of melittin with PC membranes takes place in the order of milliseconds (Schwarz and Beschiaschvili 1989; Sekharam et al. 1991; Wolfe et al. 1998; Constantinescu and Lafleur 2004). The kinetics of the association of melittin with membranes is controversial since both monophasic (Schwarz and Beschiaschvili 1989; Sekharam et al. 1991; Constantinescu and Lafleur 2004) as well as biphasic (Constantinescu and Lafleur 2004; Wolfe et al. 1998) kinetics have been reported. Melittin adopts an α-helical conformation in membranes which is supported by a number of studies carried out in membrane-mimetic systems such as micelles and reverse micelles, and in liposomes using a variety of techniques (Brown and Wuthrich 1981; Brown et al. 1982; Chandani and Balasubramanian 1986; Vogel 1987; Inagaki et al. 1989; Ikura et al. 1991; Dempsey and Butler 1992; Weaver et al. 1992; Bismuto et al. 1993; Ghosh et al. 1997; Yang et al. 2001; Ladokhin and White 2001; Raghuraman and Chattopadhyay 2003; 2004a, b, c; 2006). Studies using CD and Raman spectroscopy indicate that the helix contains about 20 amino acids in model membranes (Vogel and Jähnig 1986; Vogel 1987), suggesting that the C-terminal region of melittin, which consists a stretch of charged residues, may adopt a non-helical conformation. Recently, NMR studies of membrane-bound melittin have also shown it to be α-helical with a kink in the middle (Naito et al. 2000; Lam et al.

2001). Further, it has been shown that the angle between the N- and C-terminal helical segments of melittin bound to DMPC bilayer is ~140° or ~160°, which is larger than the value of 120° determined by X-ray diffraction studies in aqueous solution (Naito et al. 2000). However, despite the availability of high-resolution crystal structure of tetrameric melittin in aqueous solution (Terwilliger and Eisenberg 1982; Terwilliger et al. 1982a, b), the structure of the membrane-bound form is not yet resolved by X-ray crystallography.

It is now well understood that certain specific membrane lipids modulate the dynamics and lytic activity of melittin. Melittin is known to interact selectively with negatively charged lipids (Beschiaschvili and Seelig 1990; Gromova et al. 1992; Kleinschmidt et al. 1997; Ghosh et al. 1997; Sheynis et al. 2003). The affinity of melittin for membranes composed of negatively charged lipids has been shown to be 100-fold greater than for zwitterionic lipids (Batenburg et al. 1988; Lee et al. 2001). Interestingly, the presence of negatively charged lipids in the membrane has been shown to inhibit membrane lysis by melittin and this inhibition is enhanced with increasing surface charge density (Benachir and Lafleur 1995; Monette and Lafleur 1995; Hincha and Crowe 1996; Ghosh et al. 1997; Pott et al. 2001). Results from studies utilizing the REES approach of the functionally important sole tryptophan residue of melittin showed that when bound to zwitterionic membranes, the microenvironment of the functionally active tryptophan of melittin is motionally restricted, consistent with the interfacial location of the tryptophan residue (Chattopadhyay and Rukmini 1993; Ghosh et al. 1997; Raghuraman and Chattopadhyay 2004c). Interestingly, further results employing REES indicate that the microenvironment of the tryptophan residue in melittin is modulated when bound to negatively charged membranes, and this could be related to the functional difference in the lytic activity of the peptide observed in these two cases (Ghosh et al. 1997). REES is therefore sensitive to change in dynamics of hydration around melittin tryptophan caused by varying electrostatic interactions. This has been recently confirmed using melittin bound to micelles of various charge types (Raghuraman and Chattopadhyay 2004a). This study shows that micellar surface charge can modulate the conformation and dynamics of melittin. These results assume significance in the overall context of the role of surface charge of membranes and membrane-mimetic media such as micelles in the organization and dynamics of membrane-active, amphiphilic peptides in general and melittin in particular.

Cholesterol is an essential component of eukaryotic membranes and plays a crucial role in membrane organization, dynamics, function and sorting (Yeagle 1985; Liscum and Underwood 1995; Simons and Ikonen 2000; Pucadyil and Chattopadhyay 2006). It has been proposed that the rigid ring system and perpendicular orientation with respect to the plane of the membrane make cholesterol an attractive target for many bacterial toxins and fungal antibiotics (de Kruijff 1990). In addition, it has been suggested that the tryptophans in the toxins could potentially form a stable complex with the rigid ring system of the cholesterol molecule. Several studies have shown that the presence of cholesterol in membranes inhibits the lytic activity of melittin in model membranes (Benachir et al. 1997; Raghuraman and Chattopadhyay, 2004c; Allende et al. 2005). Tight lipid packing and increased deformation energy induced by cholesterol (Needham, 1995) could account for such effects. The reduced lytic effect of melittin in the presence of cholesterol could also be due to reduced binding of the peptide to the bilayer. This is because the presence of increasing amounts of cholesterol strongly reduces the binding of melittin to the lipid vesicles i.e., 3-fold excess lipid is needed in order for melittin to be completely bound to DOPC membranes in the presence of

cholesterol (Benachir et al. 1997; Raghuraman and Chattopadhyay 2004c). The significance of the interaction of melittin with membrane cholesterol is based on the fact that the natural target for melittin is the erythrocyte membrane which contains high amounts of cholesterol (~45 mol%) (Yeagle 1985; Raghuraman and Chattopadhyay 2005). Importantly, cholesterol inhibits the lytic activity of melittin in erythrocytes (see below). In order to gain a better understanding of the organization and conformation of membrane-bound melittin in presence of cholesterol, penetration depths of the sole tryptophan residue of melittin in membranes were determined using the parallax method (Chattopadhyay and London 1987). Results from the parallax method have shown that the depth of penetration of the tryptophan residue for melittin bound to DOPC vesicles to be 10.6 Å from the center of the bilayer (Ghosh et al. 1997; Raghuraman and Chattopadhyay 2004c). Interestingly, the depth of penetration of the tryptophan residue of membrane-bound melittin is reduced (i.e., the tryptophan is localized at a relatively shallow depth) in membranes containing cholesterol (see Fig. 3 and Raghuraman and Chattopadhyay 2004c). Similar reduction in the depth of penetration has been reported for peptides such as temporin L (Zhao and Kinnunen 2002) and amphipathic class A peptide (Gorbenko et al. 2003). This is probably due to the increase in elastic modulus (and hence increase in bilayer deformation energy) by several folds in the presence of high amounts of cholesterol (Needham 1995).

The interaction of melittin with membrane cholesterol has recently been explored utilizing resonance energy transfer measurements using melittin tryptophan as the donor and dehydroergosterol (DHE) as acceptor (Raghuraman and Chattopadhyay 2004c). DHE is a naturally occurring fluorescent analogue of cholesterol which is found

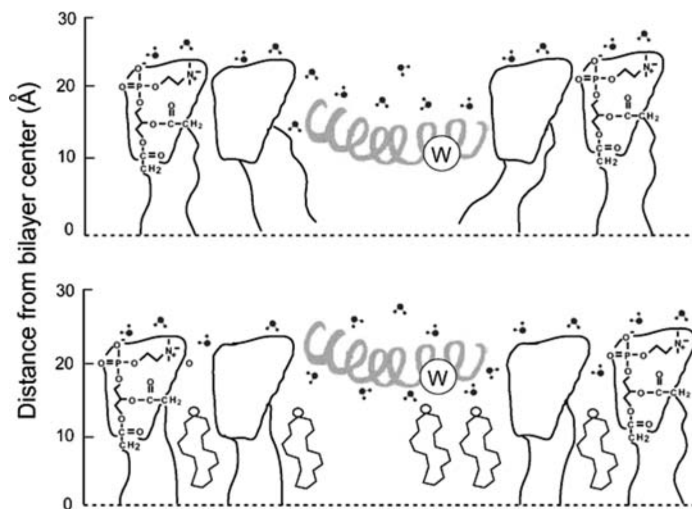

Fig. 3 A schematic representation of the membrane bilayer showing the orientation and location of membrane-bound melittin in the absence (upper panel) and presence (lower panel) of cholesterol. The small v-shaped structures represent membrane associated water molecules. The sole tryptophan residue (W) of melittin is localized in the interfacial region of membranes in both cases. However, melittin tryptophan is localized at a relatively shallow depth in membranes containing cholesterol. The specific interaction of melittin with membrane cholesterol is shown as a cluster of cholesterol molecules around the melittin tryptophan. The global bilayer effect of cholesterol is indicated by a change in headgroup packing and an increase in water penetration in the immediate vicinity of the tryptophan residue. The dotted line indicates the center of the bilayer (from Raghuraman and Chattopadhyay, 2004c)

in yeast and differs from cholesterol in having three additional double bonds and a methyl group. A number of reports have shown that DHE faithfully mimics natural cholesterol in biophysical, biochemical, and cell biological studies (Schroeder et al. 1991; Mukherjee et al. 1998). Results of energy transfer measurements were analyzed using the Fung and Stryer (1978) formalism for the energy transfer efficiency of randomly distributed donors and acceptors in membranes. Analysis of energy transfer results indicated that DHE is not randomly distributed in the membrane and is preferentially localized around the tryptophan residue of membrane-bound melittin, even at low sterol concentrations. Since DHE is a naturally occurring fluorescent cholesterol analogue, this result has been interpreted as specific interaction of melittin with membrane cholesterol (Raghuraman and Chattopadhyay 2004c).

In another study using fluorescence spectroscopic approaches, it has been shown that the melittin tryptophan is localized in a motionally restricted region in membranes when bound to membranes composed of various unsaturated lipids. Interestingly, the extent of motional restriction was found to increase drastically in membranes with increasing unsaturation, especially when the lipids contained more than two double bonds (Raghuraman and Chattopadhyay 2004b). In addition, increasing unsaturation in membranes was shown to cause a considerable change in the secondary structure of membrane-bound melittin. These results are potentially useful since lipid chain unsaturation has been shown to modulate the lytic power (Subbarao and MacDonald 1994) and bilayer micellization property (Monette and Lafleur 1996) of melittin in membranes. These effects are dependent on the degree of unsaturation of the lipid acyl chains and the fraction of unsaturated lipids present in the membrane.

Hydration plays a key role in cell structure and function and is crucial for lipid–protein interactions in membranes (Ho and Stubbs 1992). In addition, it has become increasingly evident that water molecules mediate lipid–protein interactions (Ho and Stubbs 1992; Essen et al. 1998; McAuley et al. 1999) and hence the function of membrane proteins (Sankararamakrishnan and Sansom, 1995; Okada et al. 2002; Kouyama et al. 2004). The effect of hydration on melittin–membrane interactions has been addressed using AOT (sodium bis(2-ethylhexyl)sulfosuccinate) reverse micelles as a membrane-mimetic system (Chandani and Balasubramanian 1986; Bismuto et al. 1993; Guz and Wasylewski, 1994; Ohman et al. 1996; Raghuraman and Chattopadhyay 2003). Reverse micelles offer the unique advantage of monitoring dynamics of embedded molecules with varying degrees of hydration which is difficult to achieve with complex systems such as membranes (Luisi and Magid 1986; Luisi et al. 1988). Interestingly, it has been shown that the magnitude of REES for melittin bound to reverse micelles is sensitive to the change in water content of the system (Raghuraman and Chattopadhyay 2003). In addition, increasing hydration significantly increases the propensity for α-helix formation in reverse micelles. Whether the observed effects of hydration on dynamics of melittin associated with the hemolytic activity of melittin poses an interesting question and needs to be explored.

Actions of Melittin on Membranes

Hemolytic Activity

The characteristic action of melittin is its hemolytic activity (Habermann 1972; DeGrado et al. 1981; DeGrado et al. 1982; Hider et al. 1983; Tosteson et al. 1985b; Dempsey 1990; Blondelle and Houghten 1991a, b; Blondelle et al. 1993; Saberwal and

Nagaraj 1994; Rudenko and Patelaros 1995; Rivett et al. 1996; Castano et al. 1999; Subbalakshmi et al. 1999; Raghuraman and Chattopadhyay 2005) since the target for the action of melittin is the erythrocyte membrane. At sub-micromolar concentrations and higher, melittin binds rapidly to erythrocytes (within seconds) and induces the release of hemoglobin into the extracellular medium. The apparent dissociation constant has been found in the range of 10^{-7}–10^{-8} M (Degrado et al. 1982; Tosteson et al. 1985b) depending on the conditions used for the assay. Interestingly, surface plasmon resonance experiments have shown that the apparent dissociation constant for melittin varies from 10^{-5} to 10^{-7} M depending on the surface charge density of model membranes (Lee et al. 2001). There are about 1.8×10^7 binding sites for melittin per erythrocyte (Degrado et al. 1982; Tosteson et al. 1985b), indicating that the primary site of interaction is the membrane lipid rather than specific receptors. It has been shown that melittin-induced hemolysis at room temperature occurs by a colloid osmotic mechanism (Tosteson et al. 1985b). This conclusion is based on the observation that the permeability of ions is rapid during the first few minutes of exposure to melittin and the release of hemoglobin is secondary to the formation of ion-permeable (hemoglobin-impermeable) lesions or 'pores'. This colloid osmotic mechanism is supported by the hemolytic action of truncated melittin analogues and the size of the lesions or 'pores' is estimated to be ~20 Å (Subbalakshmi et al. 1999).

Melittin-induced hemolysis follows reproducible, temperature-dependent biphasic kinetics (DeGrado et al. 1982; Hider et al. 1983) with characteristic fast and slow phases which dominate lysis at 4 and 37°C, respectively (DeGrado et al. 1982). This biphasic kinetics of melittin-induced permeabilization has been supported by experiments carried out using model membranes (Ghosh et al. 1997; El Jastimi and Lafleur, 1999; Gómara et al. 2003; Allende et al. 2005) as well as in nucleated mammalian cells (Su et al. 2001). Interestingly, the rates of the fast and slow kinetic phases are concentration-dependent and these rates have been interpreted in terms of the molecularity of the melittin species involved in each phase (DeGrado et al. 1982). At 4 °C in iso-osmotic sucrose (10 mM phosphate), the rate of the fast phase increases linearly with increasing peptide concentration while the rate of the slow phase increases as the square of the melittin concentration. The fast phase is interpreted as resulting from the perturbation of membrane structure and organization due to the rapid accumulation of melittin in the outer leaflet of the erythrocyte membrane and its decay into a slow phase is a result of the reorganization of peptide and membrane lipids to recover favorable packing geometry. It has been proposed that the internalization of the melittin dimer underlies the slow phase of hemoglobin release because of the second order dependence of the rate on peptide concentration (DeGrado et al. 1982). However, interpretation of the nature of molecularity of melittin from kinetic experiments produced variable and contrasting results depending on the concentration of peptide and temperature used. These variations could be due to the complex aggregation behavior of melittin in solution and possibly in membranes.

Importantly, it has been shown that the binding of melittin to erythrocytes as a monomer is necessary for its hemolytic activity (Hider et al. 1983; Dempsey 1990). This is based on the observation that monomeric melittin is fully active whereas the tetrameric melittin, as induced by high phosphate counter ion concentration, lacks such activity under identical conditions. This indicates that phosphate suppresses hemolysis to an extent which correlates with its effect on inducing aggregation of melittin to tetramers in solution. However, the cross-linked melittin oligomers using a series of bifunctional imido esters retained full hemolytic activity when compared to the native

melittin tetramer as induced by high phosphate concentrations (Hider et al. 1983; Knoppel et al. 1979). This is in agreement with the finding that the linear aggregates of melittin, obtained by cross-linking with dimethylsuberimidate, retain the lytic activity at high phosphate concentrations since cross-linking would suppress phosphate-induced self-association of melittin in solution (DeGrado et al. 1982). Whether the hemolysis induced by melittin requires reassociation of monomeric membrane-bound melittin to specific aggregates remains an interesting issue.

Although cell lysis by melittin has been extensively studied, the molecular mechanism of its hemolytic activity is still not well understood. In particular, the role of specific lipids on melittin-induced hemolysis is not yet clear. The natural target for melittin is the erythrocyte membrane which contains high amounts (~45 mol%) of cholesterol (Yeagle, 1985; Raghuraman and Chattopadhyay 2005). The modulatory role of cholesterol on the hemolytic activity of melittin has been explored recently (Raghuraman and Chattopadhyay 2005) by specifically depleting cholesterol from rat erythrocytes using methyl-β-cyclodextrin (MβCD), a well-characterized cholesterol-depleting agent (Ohtani et al. 1989; Christian et al. 1997; Steck et al. 2002; Pucadyil and Chattopadhyay 2004). Interestingly, depletion of cholesterol by ~55% without any appreciable loss of phospholipids was shown to increase the hemolytic activity of melittin by ~3-fold. This result clearly shows that the hemolytic ability of melittin is enhanced upon cholesterol depletion (Raghuraman and Chattopadhyay 2005). This could be due to increased penetration of melittin caused by a change in membrane packing in cholesterol-depleted erythrocytes since the presence of cholesterol is known to induce tight hydrocarbon chain packing in membranes (Yeagle 1985; Mitchell and Litman 1998). This is supported by the recent observation that the membrane penetration of melittin is decreased with increasing amounts of cholesterol in model membranes (Raghuraman and Chattopadhyay 2004c). This is further reinforced by previous results in which it was shown that bacterial lipopolysaccharides act similarly as cholesterol in providing protection to melittin-induced lysis due to tight packing of the lipid acyl chains in membranes (Allende and McIntosh 2003).

Voltage-gated Channel Formation

Melittin disrupts the barrier function of cell membranes (Dempsey 1990; Banemann et al. 1998; Epand and Vogel 1999) and has been shown to form channels in planar bilayers (Dempsey 1990; Tosteson and Tosteson 1981; Hanke et al. 1983; Gevod and Birdi 1984; Tosteson et al. 1985a; 1987; 1990; Pawlak et al. 1991; Stankowski et al. 1991; Tanaka et al. 1992). In the presence of a *trans*-negative membrane potential, melittin has been reported to induce increased permeability of ions in planar lipid membranes (Tosteson and Tosteson 1981). This observed change in conductance, under high ionic strength conditions, exhibits discrete multilevel conductances (Hanke et al. 1983; Tosteson et al. 1987). However, these are erratic and less well-defined currents. The voltage-dependent increase in conductance is consistent with the formation of channels in response to a voltage-dependent change in orientation of melittin in the membrane. The voltage-gated pores show selectivity for anions over cations, probably due to the accumulation of positive charges on the C-terminal region of melittin (Tosteson and Tosteson 1981; Pawlak et al. 1991). Interestingly, amino-acetylated melittin has also been shown to induce voltage-dependent conductance, indicating that the change in the orientation of melittin under the influence of transmembrane potential is not driven by the interaction of N-terminal or Lys-7 amino groups with the membrane potential

(Hanke et al. 1983). This is supported by the observation that synthetic melittins with a blocked N-terminal amino group or a Lys-7 to Asn-7 substitution show voltage-dependent ion permeability, and the apparent gating charge per monomer was found to be less (0.5–0.3) for these analogues than melittin which has an apparent gating charge of 1 (Tosteson et al. 1990). These results show that the positive charges in the amino terminal region of melittin play a major but not exclusive role in the voltage gating of melittin channels in bilayers.

The melittin-induced increase in ion permeability is characterized by a fourth power dependence on melittin concentration indicating that a tetrameric structure of melittin may be responsible for channel formation (Tosteson and Tosteson, 1981; Tosteson et al. 1990; Stankowski et al. 1991). However, the observation that melittin exhibits multiple conductance levels in POPC (Hanke et al. 1983) and asolectin (Tosteson et al. 1987) membranes indicates heterogeneity in the ‘pore’ structure. In addition, as mentioned above, structurally modified analogues of melittin show different concentration dependence on their ion permeability and reduced apparent gating charge per monomer (Tosteson et al. 1990). These studies therefore indicate that strictly tetrameric models for the voltage-dependent melittin channel formation are probably not justified (Dempsey 1990). In fact, it has been shown that the number of melittin monomers required to form molecular aggregates vary depending on the lipid composition and the physical conditions of the bilayer (see ‘Orientation of membrane-bound melittin’). Interestingly, the direct interaction of tricyclic tranquilizers with melittin has been proposed for the activation of melittin channels in PC membranes (Tanaka et al. 1992). Taken together, these observations provide a plausible explanation for the colloid osmotic mechanism of melittin-induced hemolysis (DeGrado et al. 1982; Hider et al. 1983; Tosteson et al. 1985b; Subbalakshmi et al. 1999).

Micellization and Fusion of Bilayers

Melittin-induced permeabilization of membranes is known to cause the breakdown of membranes into micelles at high peptide concentration. This is similar to the solubilization of membranes by detergents. Interestingly, micellization of melittin specifically occurs in membranes composed of pure saturated phosphatidylcholines and has an interesting dependence on the lipid phase transition temperature (liquid crystalline-to-gel state phase transition of the pure lipid, T_m) (Monette et al. 1993). Though the morphology of liquid crystalline bilayer remains unaltered in presence of low concentration of melittin (~5 mol%), the presence of intermediate amounts of melittin induces the liposome to fragment into small particles (micelles) upon lowering of temperature (below T_m) to form gel phase. This temperature dependence of bilayer to micelle formation by melittin and [Ala-14] melittin analogue in DMPC vesicles has been shown to be reversible using NMR spectroscopy and freeze-fracture electron microscopy (Dufourc et al. 1986; Dempsey and Watts 1987; Dempsey and Sternberg 1991). Freeze-fracture electron microscopy, light scattering and gel filtration experiments indicate that disk-shaped micelles with an approximate diameter of 235 Å are formed (Dufourcq et al. 1986). Interestingly, these discs are thermodynamically unstable and their size and formation show lipid acyl chain length dependence (Faucon et al. 1995). These studies conclude that melittin induces a reorganization of lipid assemblies which include vesicularization of multibilayers, fusion of small lipid vesicles, fragmentation into discs and micelles depending on the experimental conditions. Interestingly, the presence of cholesterol in membranes inhibits melittin-induced fusion

and formation of small discs (Monette et al. 1993; Pott and Dufourc 1995). In contrast, melittin exhibits bilayer-stabilizing effects when mixed with phosphatidylethanolamine membranes under conditions where the pure lipid arranges in a hexagonal phase (H_{II}) (Batenburg et al. 1988).

Like many basic amphipathic helical peptides (Svenaga et al. 1989), melittin exhibits fusogenic activity (Morgan et al. 1983; Dufourcq et al. 1986; Bradrick and Georghiou 1987; Bradrick et al. 1989). Even at very low concentration, melittin fuses a variety of phosphatidylcholine membranes when the temperature is cycled through the lipid phase transition temperature (Morgan et al. 1983; Pott and Dufourc 1995). In negatively charged lipids, fusion occurs under isothermal conditions (Morgan et al. 1983). It has recently been shown in DPPC and DLPC bilayers that α-helices of melittin molecules penetrate the hydrophobic core of the bilayer incompletely as a pseudo-transmembrane structure, which could induce fusion and bilayer disruption (Toraya et al. 2004).

Structural and Charge Requirements for the Activity of Melittin

It was initially believed that the action of melittin in membranes is mediated through channel formation. Structure–function studies of melittin indicate that replacement of Pro-14 with alanine (P14A melittin) removes the bend in the helical structure of melittin. Interestingly, it has been shown that P14A melittin is 2-fold more hemolytic than native peptide, but it forms less stable voltage-dependent channels, thereby indicating that channel formation may not dictate the hemolytic activity of melittin (Dempsey 1990; Dempsey et al. 1991). In another study, it has been shown that P14A melittin affects the self association, membrane binding and pore formation kinetics of melittin due to changes in structural and electrostatic properties (Rex 2000). The amphipathic helical segment of melittin between residues 1 and 20 appears to play only a structural role in its activity. The length of the amphipathic helical segment of melittin seems to be essential for cell lysis since analogues with shortened N-terminal sequences are very poor lytic agents (Gevod and Birdi 1984). Though membrane-active, the segment 1–20 of melittin does not possess any lytic activity. Interestingly, the cationic segment 21–26 present in the C-terminal region of melittin has been reported to be crucial for the lytic activity (Schroder et al. 1971). Removal of this cationic segment reduces the lytic activity although it is capable of binding to erythrocytes (Schroder et al. 1971). In addition, it is interesting to note that sequential removal of the C-terminal residues gradually reduces the lytic activity of melittin (Werkmeister et al. 1993) although Lys-23 and Arg-24 residues are shown to be important in binding of melittin to membranes (Otoda et al. 1992). However, these results are not consistent with other literature (see below).

In an elegant study, Blondelle and Houghten (1991a) analyzed the antibacterial and hemolytic activities of 24 individual omission analogues of melittin. The results indicate that deletion of Leu-6, Leu-9, Leu-13, Leu-16, Iso-17 and Trp-19 results in considerable decrease in hemolytic activity whereas apart from these residues, the deletion of Ala-4 and Lys-7 results in considerable reduction in antibacterial activity relative to native melittin. Analysis of these residues suggests that the residues making up the two separate helical regions (i.e., residues 1–9 and 13–20), except Pro-14 are important for the hemolytic action of melittin whereas the residues making up the C-terminal regions of melittin had no effect. However, using surface plasmon resonance and hybrid bilayer membrane systems, Mozsolits et al. (2001) demonstrated that the C-terminal region of melittin is essential for binding to both zwitterionic and anionic membranes. These

observations suggest that the structural requirements for the binding and the functional activity of melittin are entirely different. Based on the analysis of retention times during reverse-phase high performance liquid chromatography, it has been suggested that deletion of amino acids that caused lower hemolytic activity had lower amphiphilicity (decreased amphiphilicity was correlated with early retention times). This study, therefore, clearly indicated that amphiphilic structure is crucial for the hemolytic activity of melittin (Blondelle and Houghten, 1991a) which is in agreement with a recent report (Yan et al. 2003). Interestingly, the requirement of amphiphilic structure does not appear to be important for the antibacterial activity (Blondelle and Houghten 1991a). In contrast, hydrophobicity has been shown to be required for the antibacterial activity of melittin (Yan et al. 2003).

The requirement of amphiphilic helical structure for hemolytic activity (Blondelle and Houghten, 1991a; Rivett et al. 1996) but not for antimicrobial activity (Blondelle and Houghten, 1991a) is also borne out by studies using diastereomeric, retro and retro-enantiomers of melittin (Juvvadi et al. 1996; Oren and Shai 1997). Diastereomeric melittin, where Val-5, Val-8, Iso-17 and Lys-21 residues were replaced by D-isomers, showed a very low propensity for secondary structure and did not have any appreciable hemolytic activity but had significant antimicrobial activity (Oren and Shai 1997). This specificity is achieved due to the fact that melittin diastereomers bind to and destabilize only anionic membranes in contrast to native melittin, which binds strongly to both zwitterionic and anionic membranes. However, the partition coefficient, the depth of penetration into the membrane, and the membrane-permeabilizing activity of the diastereomers with negatively charged phospholipids are similar to that of native melittin. On the other hand, retro and retro-enantio analogues of melittin, which have high propensity for β-structure in aqueous medium but have high helical content in the presence of hexafluoroisopropanol, possess antimicrobial activity comparable to melittin but considerably lower hemolytic activity. The hemolytic activtity therefore appears to be sensitive to the direction of amide bonds and helix dipole moment (Juvvadi et al. 1996). Interestingly, using the retro and retro-enantio analogues of cecropin–melittin hybrids, it was concluded that chirality of the peptide was not a critical feature, and full antibacterial activity could be achieved with peptides containing either all L- or all D-amino acids in their respective right-handed or left-handed helical conformations (Merrifield et al. 1995). These studies emphasized the importance of amphipathicity and α-helicity not only to the cytolytic activity of melittin but for the activity of other cytolytic peptides. An additional attribute, common among native amphipathic helices including melittin, is peptide linearity. Experiments using the synthetic cyclic melittin analogue reveal that cyclization causes 4–30-fold decrease in melittin binding depending on the charge of the host membranes. Interestingly, the cyclic melittin analogue has increased antibacterial activity but decreased hemolytic activity (Unger et al. 2001). These results indicate that the linearity of the peptide is not essential for the disruption of the target phospholipid membrane, but rather provides the means to reach it.

Studies involving single amino acid omission analogues of melittin revealed the importance of the sole tryptophan of melittin (Trp-19) in its hemolytic activity (Blondelle and Houghten 1991a). This is further demonstrated by the remarkable decrease in activity observed upon photooxidation of the tryptophan (Habermann and Kowallek 1970), upon substitution of Trp-19 by leucine (Blondelle and Houghten 1991b) and the introduction of a second tryptophan residue in the melittin sequence (Blondelle et al. 1993). However, the position of Trp in the sequence does not seem to

be very critical for activity, as analogues of melittin containing a single Trp residue at positions 9, 11 or 17 also exhibited hemolytic activities with potencies in the order Trp-17 > Trp-19 = Trp-11 > Trp-9 (Weaver et al. 1989). Importantly, analogues of melittin containing a single Trp residue at positions 9, 11 or 17 do not form tetramers in solution at high peptide or salt concentrations unlike native melittin. The ability to form soluble tetrameric structures therefore appears to be unrelated to the hemolytic activity of melittin. This is in contrast to the previous observation in which it was shown that single amino acid substitution of Lys-7 prevented the self association of melittin and those substitutions, which prevented the inducible amphipathic folding ability, were found to result in a loss in hemolytic and antimicrobial activity (Pérez-Payá et al. 1995). It should be mentioned that there is no consensus regarding the role of Lys-7 in the lytic activity of melittin since some reports suggest that Lys-7 is crucial for the lytic activity of melittin (Gevod and Birdi 1984; Blondelle and Houghten, 1991a, b) which is not consistent with other indirect results (Werkmeister et al. 1993).

In addition to amphipathicity, α-helicity and amino acid specificity, certain charge constraints have also been shown to have an important role in the specificity of action of melittin, either hemolytic or antimicrobial (Werkmeister et al. 2002). As mentioned earlier, the net charge of melittin is +6 in solution (Dempsey 1990). It has been shown that the presence of at least two positive charges is essential for the activity of melittin (Werkmeister et al. 2002). Interestingly, melittin with extra positive charges introduced on the hydrophilic face of the helix possesses hemolytic activity greater than native melittin (Otoda et al. 1992). Further, a 15-residue synthetic peptide, corresponding to the C-terminal region of melittin, exhibits 5–7-fold less antimicrobial activity than melittin (Subbalakshmi et al. 1999). The hemolytic activity, on the other hand, is 300 times less than that of melittin. An analogue of this peptide in which two cationic residues have been transposed to the N-terminal region from the C-terminal region, has antibacterial activity comparable to that of melittin but has considerably lower hemolytic activity (Subbalakshmi et al. 1999). The biological activities of these peptides have been rationalized on the basis of their structure and aggregation properties. Interestingly, it has recently been shown that melittin is the first antimicrobial peptide to possess a leucine zipper motif, which is crucial for determining its hemolytic action, but not for antimicrobial activity (Asthana et al. 2004).

Energetics of Folding of Melittin in Membranes

The membrane interface has a potent ability to induce secondary structure in melittin (Ladokhin and White 1999) and this is also true for a wide range of other membrane-active peptides such as hormones, toxins and antimicrobial peptides (Kaiser and Kezdy 1983; 1984; Schwyzer 1992; Maloy and Kari 1995; White and Wimley 1998). In addition, it has been shown that membrane interfacial properties such as bilayer compressibility modulus modulate the binding of amphipathic peptides (Allende et al. 2003). Wimley and White (1996) have shown that the partitioning of peptides in membranes is dominated by the extremely unfavorable free energy cost of partitioning the peptide bonds (+1.2 kcal mol^{-1} per peptide bond for phosphocholine bilayers). They proposed that hydrogen bonding of peptide bonds reduces this high free energy cost and thereby promotes the formation of secondary structures of peptides/proteins in membranes.

In general, the folding of peptides is tightly coupled to their partitioning in membranes. Determination of the energetics of helix folding on membranes is therefore extremely difficult due to the experimental inaccessibility of the unfolded membrane-

bound form of peptides. Since melittin adopts unfolded (random coil conformation) and folded forms (α-helical) in solution and membranes, respectively, it has been used to test the hypothesis that hydrogen bonding reduces the high cost of partitioning the peptide bonds in melittin along with its diastereomeric analogue which has four D-amino acids (D_4,L-melittin). D_4,L-melittin serves as a model for the experimentally inaccessible unfolded bound form of native melittin since it has little secondary structure either in its free or bound form due to the presence of D-amino acids (Oren and Shai 1997). This is based on the assumption that membrane-bound D_4,L-melittin has only six residues for helix formation when compared to 18 residues in native melittin (Dempsey and Butler 1992). Thus, native melittin has 12 more residues in an α-helical conformation than D_4,L-melittin when membrane-bound. The free energy of partitioning of unfolded melittin from the aqueous phase to the bilayer interface of POPC vesicles as the folded form has been estimated to be –7.6 kcal mol^{-1} (Ladokhin and White 1999). The partitioning of native melittin into POPC membranes is 5.0 kcal mol^{-1} (~3-fold) more favorable than the partitioning of D_4,L-melittin which is –2.6 kcal mol^{-1}. These findings show that the free energy reduction per residue accompanying the folding of melittin in the membrane interface is about 0.4 kcal mol^{-1}, consistent with the hypothesis that hydrogen bonding reduces the high cost of partitioning of peptide bonds. This value has potential implications in estimating the energetic consequences of membrane-induced secondary structure formation of peptides/proteins. Ladokhin and White (1999) proposed that the cumulative effect of these relatively small per residue free energy reductions can be very large and assumes significance when tens or hundreds of residues are involved as in the assembly of the *β*-barrel transmembrane domain of α-hemolysin (Song et al. 1996) that buries ~100 residues in the membrane.

Orientation of Membrane-bound Melittin

The transmembrane potential has been proposed to affect the orientation of melittin in membranes (Tosteson and Tosteson 1981; Tosteson et al. 1985a). In an excellent study, Kempf et al. (1982) showed a voltage-dependent change in the orientation of melittin helix from a bilayer surface orientation to a transmembrane one under the influence of transmembrane negative potential. This process has been found to be reversible when the voltage is trans-positive. A recent study confirms the change in the orientation of membrane-bound melittin under the influence of transmembrane potential using a combination of high-performance liquid chromatography (HPLC) and liquid secondary ion mass spectrometry (Niu et al. 2000). All other studies concerning the orientation of membrane-bound melittin have been done in model systems in the absence of a transbilayer potential. In addition, it has been reported, based on energy calculations, that the lowest-energy orientation of melittin varies depending on the hydrocarbon thickness of the bilayer (Lazaridis 2003). Determination of the orientation of α-helical melittin bound to membranes using conventional CD spectroscopy is not possible since this technique cannot discriminate between the parallel and perpendicular orientations of the membrane-bound peptide. The direct determination of helix orientation has therefore been carried out using oriented bilayers utilizing IR spectroscopy and oriented circular dichroism (OCD) spectroscopy (see below).

The orientation of melittin in membranes is dependent on the lipid composition and the physical condition of the membrane. One of the key parameters that determine the orientation of membrane-bound peptides is their concentration. At low concentrations, melittin adopts parallel orientation with respect to the membrane surface whereas when

the concentration of peptide increases above a certain threshold value, an increasing fraction of peptide molecules change to the perpendicular orientation as explained by the two-state model for the action of antimicrobial peptides (Huang 2000). The orientation of α-helical segments of melittin in membranes is also found to be dependent on other important variables such as hydration, temperature and the phase state of the lipid (Vogel 1987; Frey and Tamm 1991; Toraya et al. 2004). The sensitivity of these variables changes with the peptide and lipid. For example, in gel phase DTPC or DMPC membranes at low hydration levels (6% w/w corresponding to 2–3 water molecules per lipid), the α-helical segments of melittin are oriented roughly perpendicular to the plane of the membrane (Vogel and Jähnig 1986). The perpendicular orientation of melittin is also observed in DLPC, DTPC and DMPC bilayers when the temperature is well above the phase transition temperature (Naito et al. 2000; Yang et al. 2001). In contrast, it has been shown that melittin is always oriented parallel to the DPhPC bilayers with little dependence on temperature and hydration (Yang et al. 2001). This is also true in case of melittin bound to DOPC membranes (Hristova et al. 2001). The parallel orientation in the membrane–water interface is such that the apolar residues face the hydrophobic core of the membrane and the polar residues face the bulk aqueous phase. This corresponds to the ‘‘wedge’’ model (Dawson et al. 1978; Terwilliger et al. 1982; Bernèche et al. 1998), and is consistent with experimental observations on the orientation of melittin in bilayers (Brauner et al. 1987; Maurer et al. 1991; Dempsey and Butler 1992; Bradshaw et al. 1994). Interestingly, in POPC bilayers, melittin can orient either parallel or perpendicular to the plane of the membrane depending on hydration (Ladokhin and White 2001), and peptide concentration (Yang et al. 2001).

In spite of a number of studies addressed to determine the orientation (parallel or perpendicular) of membrane-bound melittin with respect to the plane of the membrane bilayer, there is no consensus regarding the orientation of melittin in membranes (see Fig. 4). The orientation of melittin in membranes is further complicated by the observation that melittin does not adopt a fully transmembrane orientation in membranes, but a pseudo-transmembrane orientation (Wall et al. 1995; Bachar and Becker 2000; Toraya et al. 2004). These studies therefore point out the role of lipid composition and the physical factors that affect the membrane properties in the orientation of melittin in membranes. Recent results from our laboratory (Raghuraman

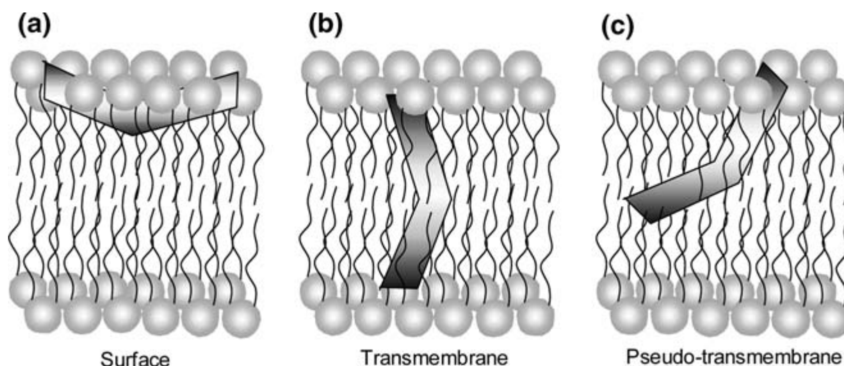

Fig. 4 A schematic representation of the membrane bilayer showing the various orientations and locations of membrane-bound melittin: (**A**) surface (**B**) transmembrane, and (**C**) psuedo-transmembrane orientations. The V-shaped bent rod represents the amphipathic, α-helical membrane-bound melittin

and Chattopadhyay 2006b) using the NBD-melittin analogues, where the fluorescent NBD group is attached to Gly-1 and Lys-7 residues, are in good agreement with the previous reports which show that membrane-bound melittin orients parallel to the membrane surface (Altenbach and Hubbell 1988; Dempsey and Butler 1992; Bradshaw et al. 1994; Bernèche et al. 1998; Yang et al. 2001; Hristova et al. 2001). It has recently been shown that in *E.coli* membranes, melittin adopts both forms of orientation in a wide range of melittin concentrations tested (Hung and Lee 2006).

Aggregation State of Melittin in Membranes

The aggregation state of melittin in membranes is an important issue since this property is presumed to be associated with the function of melittin. This can be appreciated by the fact that melittin forms voltage-gated channels (Tosteson and Tosteson 1981; Hider et al. 1983) which may require self association of melittin monomers to form pores in membranes. It is not known whether pore-forming melittin aggregates pre-exist in the membrane in the absence of an applied membrane potential. Further, extrapolating knowledge of the aggregation properties of melittin in solution to membranes is not justified since the membrane is a two-dimensional anisotropic fluid. This apparent lack of one dimension in membranes may increase the apparent local concentrations of melittin monomers and therefore favor self-association in membranes. In addition, it is well documented that lipids play a crucial role in the insertion and pore formation of melittin (see 'Orientation of membrane-bound melittin' and 'Melittin and pore formation'). Several studies have attempted to determine the aggregation state of membrane-bound melittin using modified (Vogel and Jähnig 1986), spin-labeled (Altenbach and Hubbell 1988) and *N*-methyl anthraniloyl-labeled melittins (Hermetter and Lakowicz 1986) utilizing various sensitive techniques such as fluorescence quenching, electron paramagnetic resonance and resonance energy transfer measurements. The results show that there is no consensus regarding the aggregation properties of membrane-bound melittin and led to contradictory conclusions which are summarized below.

Results from energy transfer measurements from Trp-19 to a melittin with a modified tryptophan, Vogel and Jähnig (1986) proposed a detailed model of tetrameric aggregation in membranes (at a lipid/melittin ratio above 1000:1), whereas utilizing energy transfer between Trp-19 and *N*-methyl anthraniloyl residue attached to Lys-21, Hermetter and Lakowicz (1986) concluded that the membrane-bound melittin is monomeric (at a lipid/melittin ratio above 50:1). This is further supported by energy transfer measurements involving chemically modified tryptophan that show a lack of association of melittin bound to membranes at low salt concentration (Schwarz and Beschiaschvili 1989). However, a related study in which the *N*-bromosuccinimide oxidized Trp-19 analogue of melittin has been used indicates the aggregation of melittin bound to fluid phase PC or PG membranes in the presence of NaCl (Talbot et al. 1987). In another study, it has been observed that the aggregation of melittin occurs only at special conditions such as high salt concentrations and lipid/melittin ratio below 200:1 (John and Jähnig 1991). In addition, based on the fluorescence quenching resolved spectra of melittin in lipid bilayers, it has been proposed that melittin bound to DMPC membranes exists in both monomeric as well as aggregated forms in presence of 2 M NaCl (Kaszycki and Wasylewski 1990). Using spin-labeled melittin analogues in DOPC and DOPC:DOPA (9:1) at lipid/protein ratios of about 200:1, Altenbach and Hubbell

(1988) not only confirmed that membrane-bound melittin is monomeric but also showed that melittin is bound parallel to the membrane surface. Interestingly, these results are consistent even in the presence of high salt concentration. The marked variation in these experimental results is most readily explained by differences in the aggregation properties of the differently labeled melittins and by the different conditions of ionic strength and membrane composition used. Overall, the results of experiments carried out to determine the aggregation state of membrane-bound melittin indicate that melittin is monomeric at peptide/lipid molar ratios below 1:100 in fluid phase membranes at low ionic strength (Dempsey 1990). Interestingly, using the dansylated analogue of melittin, it has been shown that melittin is monomeric even in erythrocyte ghost membranes (Pérez-Payá et al. 1997).

Kinetic studies suggest that the aggregation of melittin helices on the membrane surface act as a precursor of pore formation, with dimerization being the rate-limiting step in many (DeGrado et al. 1982; Schwarz and Beschiaschvili 1989; Schwarz et al. 1992) but not all (Rex and Schwarz, 1998) cases. Takei et al. (1999) provided direct evidence for the kinetic importance of melittin association in pore formation through studies of cysteine-substituted melittin analogues [(melittin K23C)$_2$ and (melittin K23Q, Q25C)$_2$] linked by disulfide bridges under low ionic strength conditions. The initial rate of melittin-induced pore formation increased with the square of the peptide concentration, whereas both disulfide-dimerized melittin analogues showed a first-order dependence of pore formation rates on peptide concentration indicating that peptide dimerization is the rate-limiting step for melittin-induced pore formation. Further, it has been shown that a dimeric form of melittin (Q25C) analogue causes larger structural perturbations in membranes when compared to native melittin at low concentrations under identical conditions (Hristova et al. 2001). Recently, the self association and membrane-binding behavior of mono- and tetra-5,5,5-trifluoroleucine-labeled melittin (which have enhanced hydrophobicity) have also been examined (Niemz and Tirrel 2001).

Melittin and Pore Formation

It is commonly believed that multimeric pore formation is the mode of action of many naturally produced peptides such as antimicrobial peptides and toxins (Parente et al. 1990; Schwarz et al. 1992; Wimley et al. 1994; Matsuzaki et al. 1994; Schwarz and Arbuzova 1995; Rapaport et al. 1996; Rex 1996). Under certain conditions, melittin molecules insert into the lipid bilayer and form multiple aggregated forms that are controlled by temperature, pH, ionic strength, lipid composition and lipid-to-peptide ratio. Neutron diffraction studies show that melittin-induced transmembrane pores are present only when the peptide is primarily oriented perpendicular to the membrane bilayer. No pores are detected when the peptide orients parallel to the bilayers. Hence, the perpendicular orientation of melittin with respect to the plane of a bilayer represents an important criterion for the pore formation of melittin. Several studies have been attempted to monitor the structure and function of such pores and their results show that melittin forms pores that have a rather wide distribution of sizes. For example, the sizes of the melittin pores which are characterized by the inner pore diameter, have been reported to be in the range of 10–60 Å (Rex, 1996), 13–24 Å (Matsuzaki et al. 1997) and 25–30 Å (Ladokhin et al. 1997) from vesicle leakage experiments. The diameter of these pores is expected to increase when the peptide concentration is increased. On the other hand, utilizing neutron diffraction, which

detects and measures the size of transmembrane pores, it has been shown that the inner pore diameter of melittin-induced pores is 44 Å (Yang et al. 2001). Interestingly, the pore size is shown to be independent of peptide concentration (peptide/lipid ratio ≥ 1/30) in this case. These results are consistent with the studies on osmotic protection of erythrocytes which show an estimated pore size of ~20–30 Å at high melittin concentrations (Katsu et al. 1988; Subbalakshmi et al. 1999).

The first model of peptide-induced pores (barrel-stave model) was proposed by Baumann and Mueller (1974) to account for the single channel conductance induced by alamethicin in black lipid membranes. In this model, alamethicin helices associate to form a bundle with a central lumen, like a barrel made of helical peptides as staves. Ever since its introduction, the barrel-stave model has been viewed as the prototype of peptide-induced transmembrane pores, which include the pore induced by melittin (Vogel and Jähnig 1986; Sansom 1991). Utilizing peptide orientation-sensitive techniques such as oriented circular dichroism and neutron diffraction, melittin pores have been shown to be consistent with the toroidal model (Yang et al. 2001) that was proposed to describe magainin-induced pores (Matsuzaki et al. 1996; Ludtke et al. 1996). The toroidal model differs from the barrel-stave model in that the peptides are always associated with the lipid headgroups even when they are perpendicularly inserted in the lipid bilayer. In forming such a pore, the lipid monolayer bends continuously from the top to the bottom in the fashion of a toroidal hole, so that the pore is lined by both the peptides and the lipid headgroups (see Fig. 5). In addition, molecular dynamics simulation study supports the toroidal pore model for melittin-induced pores (Lin and Baumgaertner 2000). Recently, Allende et al. (2005) have tested the model of melittin-induced toroidal pore formation by measuring the free energy of melittin transfer and melittin-induced vesicle leakage as a function of bilayer elastic properties. Interestingly, for bilayers with similar melittin binding, they found a linear relationship between melittin-induced vesicle leakage and elastic properties of bilayers

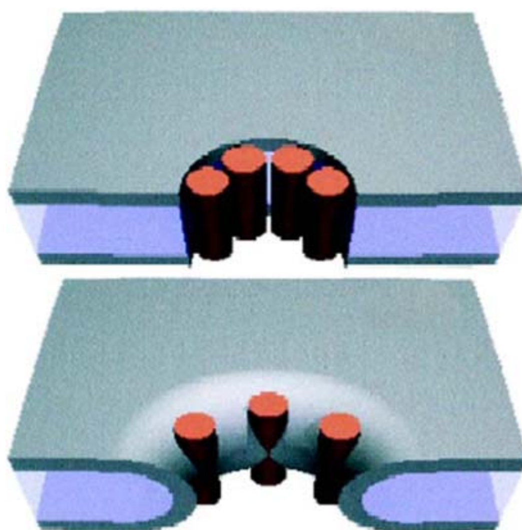

Fig. 5 A schematic representation of the barrel-stave model (top) and the toroidal model (bottom) for pore-forming peptides. The dark layers represent the headgroup regions of bilayers. Peptide monomers are represented by the cylinders (from Yang et al. 2001). See text for more detailed description

such as compressibility modulus and monolayer spontaneous curvature clearly indicating a role of bilayer elastic properties in membrane lysis. However, in contrast to what would have been expected for a barrel stave model, there is no correlation between melittin-induced leakage and hydrocarbon thickness of the bilayer. These results clearly demonstrate that melittin-induced pores are defects in the bilayer lined in part by lipid molecules and are supportive of the toroidal model for melittin-induced pore formation (Allende et al. 2005).

Lipid composition and phase separation appears to play a critical role in melittin-induced pore formation. For instance, it has been shown that sphingomyelin, one of the main lipids of erythrocyte plasma membrane, affects the ability of melittin to permeabilize lipid vesicles and stimulates melittin pore formation in electrically neutral bilayers (Gómara et al. 2003). Analysis of the leakage data according to a kinetic model of pore formation (Nir and Nieva 2000) shows a good fit for hexameric/octameric pores in sphingomyelin-containing neutral vesicles at low melittin concentration. This is attributed to the coexistence of gel and fluid phases induced by the presence of sphingomyelin in POPC bilayers. Interestingly, addition of cholesterol to this binary mixture reduces the efficiency of melittin pore formation since cholesterol induces the coexistence of fluid and liquid ordered (l_o) phases in sphingomyelin/POPC membranes. In another study, melittin-induced release of encapsulated fluorescent dextran markers from POPC vesicles is shown to be mediated by transmembrane pores (Ladokhin et al. 1997; Ladokhin and White 2001) whereas release from anionic POPG vesicles is found to be non-selective, i.e., 'detergent-like' (Ladokhin and White 2001). This is supported by surface plasmon resonance studies which show that melittin forms pores only in zwitterionic membranes and not in negatively charged membranes (Papo and Shai, 2003a). These studies clearly point out the importance of lipid composition and phase separation on the aggregation behavior and pore formation of melittin.

Cellular Activities of Melittin

Action of Melittin on Membrane Proteins

Apart from its ability to disrupt lipid bilayers, melittin affects the dynamics of membrane proteins. For instance, it has been shown that lytic concentrations of melittin dramatically reduce the rotational mobility of band 3 protein in human erythrocyte membranes (Dufton et al. 1984; Clague and Cherry 1988; Hui et al. 1990) and of bacteriorhodopsin in lipid vesicles (Hu et al. 1985). Further, melittin causes aggregation of membrane proteins including band 3 protein (Clague and Cherry 1988), bacteriorhodopsin (Hu et al. 1985) and Ca^{2+}-ATPase (Voss et al. 1991; Mahaney and Thomas 1991; Mahaney et al. 1992). In addition, melittin is a potent inhibitor of Ca^{2+}-ATPase (Voss et al. 1991; Mahaney and Thomas, 1991), H^+K^+-ATPase (Cuppoletti et al. 1989; Cuppoletti, 1990) and Na^+K^+-ATPase (Cuppoletti and Abbott, 1990). The experiments with bacteriorhodopsin suggest that immobilization due to aggregation of membrane proteins is a result of direct melittin–protein interactions rather than an indirect consequence of melittin–lipid interactions (Hu et al. 1985). This is supported by the observation that melittin effectively immobilizes membranes proteins in the plane of the lipid bilayer in erythrocyte membrane ghosts (Watala and Gwozdzinski 1992). Interestingly, the role of melittin–lipid interactions has also been shown to be responsible, along with more specific binding of melittin with membrane proteins, for

the inhibition of the Ca^{2+}-ATPase (Baker et al. 1995) and protein kinase C (Raynor et al. 1991).

Transfection Activity of Melittin and Nonviral Gene Delivery

Entry of exogenous DNA into the cytoplasm and subsequent transport into the nucleus are major cellular barriers for nonviral gene delivery vectors. Melittin, covalently attached to PEI, has been successfully used to enhance the transfection activity of PEI–DNA complexes in a broad range of cell lines including different tumor cell lines and also primary hepatocytes and human umbilical vein endothelial cells (Ogris et al. 2001). It has been shown that melittin not only enables efficient release of nonviral gene transfer particles into the cytoplasm due to its membrane lytic activity, as monitored by fluorescence microscopy and flow cytometry, but also enhances their transport into the nucleus. The latter effect of melittin has been attributed to the presence of the cationic cluster KRKR at the C-terminus of the peptide whose characteristics are similar to the classical nuclear localization sequence. These results suggest that naturally occurring melittin possesses a dual endosomolytic and nuclear-homing functionality that can form the basis of a powerful transfection agent and makes it an interesting candidate for the further development of systemic gene delivery in vivo. A very recent report supports the endosomolytic function of melittin conjugates (Lavignac et al. 2005).

In addition, melittin has also been used in peptide-mediated RNA delivery, a novel approach for the enhanced transfection of primary and post-mitotic cells (Bettinger et al. 2001). Endosomolytic activity was incorporated by conjugating PEI of 2 kDa with melittin and resulting PEI 2 kDa–melittin/RNA polyplexes mediated high transfection levels in certain cell lines. This suggests that melittin-modified low molecular weight polycations possess endosomolysis which enables efficient non-viral mRNA transfection of quiescent and post-mitotic cells.

Melittin and Cell Transformation

Oncogenes play an important role in the initiation and progression of the neoplastic phenotype. The *ras* oncogene is especially important with respect to human cancer (Bos 1989) since at least one-third of all human colorectal tumors analyzed express an activated *ras* oncogene (Bos et al. 1987; Forrester et al. 1987). Interestingly, it has been demonstrated that melittin specifically selects against cells in culture that express high levels of the *ras* oncogene (Sharma 1992). Melittin therefore exerts its anti-transformation effect(s) by specifically eliminating cells that express the oncoprotein. Further, acquisition of resistance to melittin is accompanied by a decrease in the number of copies of the *ras* genes, decrease in expression of the *ras* oncoprotein and a concomitant reversion of transformed cells to a normal morphology in a strict dose-dependent manner (Sharma 1992). Interestingly, it has been shown that the biochemical basis for melittin-mediated counterselection of *ras*-transformed cells is due to the ability of melittin to hyperactivate cellular phospholipase A_2 in *ras*-transformed cells by the mediation of enhanced influx of calcium ions (Sharma, 1993).

Melittin and Signal Transduction

It is known that cationic amphiphilic peptides such as mastoparan and melittin directly stimulate nucleotide exchange by heterotrimeric GTP-binding proteins

(G-proteins) in a manner similar to that of G-protein coupled receptors (Higashijima et al. 1988, 1990; Ross and Higashijima 1994). Further, it has been shown that melittin inhibits the activity of adenylyl cyclase in synaptic membranes. Both mastoparan and melittin are known to stimulate G_i or G_o activities in various cells, which may possibly be due to their common amphiphilic structure (Schwyzer, 1992). In addition, melittin has also been shown to inhibit G_s activity by reducing the affinity of both GTP (or GTP-γ-S) and GDP to G_s (Fukushima et al. 1998). It has therefore been proposed that G_i stimulation and G_s inhibition are involved in melittin-induced inhibition of adenylyl cyclase. Interestingly, melittin represents the first metabostatic peptide that inhibits the intrinsic activity of G protein (G_s) activity (Fukushima et al. 1998).

Leishmanicidal Activity of Melittin

Melittin induces membrane permeabilization and lyses prokaryotic as well as eukaryotic cells in a non-selective manner (Papo and Shai 2003b). This mode of action is responsible for its hemolytic, anti-microbial (Blondelle and Houghten 1991a; Bechinger 1997), anti-fungal (Lazarev et al. 2002), anti-tumor (Winder et al. 1998) and leishmanicidal (Diaz-Achirica et al. 1998) activities of melittin. The protozoan mammalian parasite *Leishmania* is the causative agent of leishmaniasis, which afflicts 12–14 million people worldwide (Herwaldt 1999). Though melittin has leishmanicidal activity, the cytolytic activity of melittin is considered an obstacle for its potential therapeutic use. Interestingly, it has been shown that cecropin A–melittin hybrid peptides show remarkable leishmanicidal activity with minimal cytolytic activity (Diaz-Achirica et al. 1998; Chicharro et al. 2001; Luque-Ortega et al. 2003). It has been shown that the action of cecropin–melittin hybrid peptides involves targeting of the plasma membrane of *Leishmania donavani* promastigotes, whereas the amastigote form is far more resistant to this peptide hybrid (Diaz-Achirica et al. 1998). Further, N-terminal fatty acylation has been shown to increase the leishmanicidal activity of the hybrid peptides (Chicarro et al. 2001). These hybrid peptides therefore show promise for the development of specific anti-*Leishmania* peptides.

Anti-viral Activity of Melittin

It has been shown that melittin reduces HIV-1 production in a dose-dependent mannner (Wachinger et al. 1992). The reduction in viral infectivity is proposed to be due to the affinity of melittin for the gag/pol precursor, thereby preventing the processing of gag/pol by the HIV protease. This intracellular action of melittin is supported by a recent study which shows that melittin decreases the levels of Gag antigen and HIV-1 mRNAs (Wachinger et al. 1998). Further, it has been indicated that melittin has a direct suppressive effect on the activity of the HIV long terminal repeat. Taken together, these studies point out that antimicrobial peptides such as melittin and cecropin are capable of inhibiting replication of HIV-1 by suppressing viral gene expression. In yet another study, melittin has also been shown to inhibit plant virus infection (Marcos et al. 1995). Synthetic analogues of melittin that have sequence and structural similarities to an essential domain of tobacco mosaic virus coat protein have been found to possess highly specific antiviral activity.

Conclusion and Future Perspectives

Melittin is probably the most extensively studied membrane-active lytic peptide for monitoring mechanisms of pore formation and lipid–protein interactions in membranes. Early biophysical studies utilizing fluorescence and NMR approaches have proved to be very useful in elucidating the salient structural features essential for its activity. The effect of melittin on cellular membranes is just beginning to be explored. The knowledge gained from previous biophysical studies should be potentially useful in understanding not only the mechanism of action of pore-forming peptides but also in elucidating the diverse cellular functions exhibited by melittin.

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