Differential Scanning Microcalorimetry Indicates That Human Defensin, HNP-2, Interacts Specifically with Biomembrane Mimetic Systems[†,‡]

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ABSTRACT: α-Defensins are antimicrobial peptides with 29−35 amino acid residues and cysteine-stabilized amphiphilic, triple-stranded β-sheet structures. We used high-precision differential scanning microcalorimetry to investigate the effects of a human neutrophil α-defensin, HNP-2, on the phase behavior of model membranes mimicking bacterial and erythrocyte cell membranes. In the presence of this positively charged peptide, the phase behavior of liposomes containing negatively charged phosphatidylglycerol was markedly altered even at a high lipid-to-peptide molar ratio of 500:1. Addition of HNP-2 to liposomes mimicking bacterial membranes (mixtures of dipalmitoylphosphatidylglycerol and -ethanolamine) resulted in phase separation owing to some domains being peptide-poor and others peptide-rich. The latter are characterized by an increase of the main transition temperature, most likely arising from electric shielding of the phospholipid headgroups by the peptide. On the other hand, HNP-2 did not affect the phase behavior of membranes mimicking erythrocyte membranes (equimolar mixtures of dipalmitoylphosphatidylcholine and sphingomyelin) as well as the pure single components. This is in contrast to melittin, which significantly affected the phase behavior of choline phospholipids in accordance with its unspecific lytic activity. These results support the hypothesis of preferential interaction of defensins with negatively charged membrane cell surfaces, a common feature of bacterial cell membranes, and demonstrate that HNP-2 discriminates between model membrane systems mimicking prokaryotic and eukaryotic cell membranes.

During the last decade, isolation and characterization of membranolytic peptides originating from various organisms has progressed greatly (Saberwal & Nagaraj, 1994). The biological activities of a large number of these peptides have been ratonalized in terms of the peptides having the ability to adopt amphiphilic α-helical structures and to associate with the lipid components of membranes (Kaiser & Kezdy, 1987). Therefore, numerous studies on model systems consisting of naturally occurring and chemically synthesized peptides were initiated to gain information on the parameters important in peptide−lipid interaction and to understand the structural basis for the different membrane actions of amphiphilic helices (Epand, 1993; Saberwal & Nagaraj, 1994; Shai, 1995).

α-Defensins are a family of arginine-rich, cationic peptides (Lehrer et al., 1991) whose members have been isolated from the neutrophils of humans, rabbits, rats, and guinea pigs; the pulmonary macrophages of rabbits; and the intestinal Paneth cells of humans (Jones et al., 1992, 1993) and mice (Eisenhauer et al., 1992; Quellette et al., 1994). These β-sheet peptides exhibit potent antimicrobial but relatively limited cytotoxic properties (Lehrer et al., 1991). A high-resolution crystal structure for human neutrophil peptide 3 (HNP-3)[1] revealed that the peptide adopted a triple-stranded β-sheet configuration stabilized by three intramolecular disulfide bonds, and that it formed basket-shaped dimers with a hydrophobic base and a polar top (Hill et al., 1991). This description was concordant with the solution structure of HNP-1, as defined by 2D-NMR (Zhang et al., 1992; Pardi et al., 1992).

A related family of antimicrobial peptides known as β-defensins has been isolated from avian and bovine leukocytes (Harwig et al., 1994a; Evans et al., 1994), bovine trachea (Diamond et al., 1993), and tongue (Schonwetter et al., 1995) and found in human plasma and genitourinary organs (Bensch et al., 1995). Although β-defensins contain 36−40 residues and have a different conserved cysteine motif, the peptide fold of bovine neutrophil β-defensin closely resembled that established for the α-defensins (Zimmermann et al., 1995). Both α- and β-defensins may be contemporary expressions of an ancestral system of innate immunity. The primary structures of several α-defensins and β-defensins are shown in Table 1.

Although few in number, biophysical studies on the interaction of defensins with model membrane systems have

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[1] Abbreviations: HNP, human neutrophil peptide; DPPC, 1,2-dipalmitoylphosphatidylcholine; DPPE, 1,2-dipalmitoylphosphatidylethanolamine; DPPG, 1,2-dipalmitoylphosphatidylglycerol; SM, egg sphingomyelin; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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Table 1: Amino Acid Sequences of α- and β-Defensins; Alignment Was Maximized for the Six Conserved Cysteine Residues by Addition of Gaps

	HUMAN α-DEFENSINS													
	HNP-1[a)]	A	C	Y - - - - -	C	RIPA	C	IAGERRY	G	T	C	IYQGRLWAF	CC	
	HNP-2[a)]		C	Y - - - - -	C	RIPA	C	IAGERRY	G	T	C	IYQGRLWAF	CC	
	HNP-3[a)]	D	C	Y - - - - -	C	RIPA	C	IAGERRY	G	T	C	IYQGRLWAF	CC	
	HNP-4[b)]	V	C	S - - - - -	C	RLVF	C	RRTELRV	G	N	C	LIGGVSFTY	CC	TRV
	HD-5[c)]	ARAT	C	Y - - - - -	C	RTGR	C	ATRESLS	G	V	C	EISGRLYRL	CC	R
	HD-6[c)]	TRAFT	C	H - - - - -	C	RR - S	C	YSTEYSY	G	T	C	TVMGINHRF	CC	L
	EPITHELIAL β-DEFENSINS													
Human	hβD-1[d)]	DHYN	C	VSSGGQ	C	LYSA	C	PIFTKIQ	G	T	C	YRGKAK - - -	CC	K
Bovine	TAP[e)]	NPVS	C	VRNKGI	C	VPIR	C	PGSMKQI	G	T	C	VGRAVK - - -	CC	RKK
	MYELOID β-DEFENSINS													
Bovine	BNBD12[f)]	GPLS	C	GRNGGV	C	IPIR	C	PVPNRQI	G	T	C	FGRPVK - - -	CC	RSW
Avian	Gal 1[g)]	GRKSD	C	FRKSGF	C	AFLK	C	PSLTLIS	G	K	C	SRFYL - - - -	CC	KRIW
cys. #			1		2		3				4		5 6	

[a] Selsted et al. (1985). [b] Wilde et al. (1989). [c] Jones & Bevins (1993). [d] Bensch et al. (1995). [e] Diamond et al. (1991). [f] Selsted et al. (1993). [g] Harwig et al. (1994).

provided important insights into the mechanism of action (Kagan et al., 1990; Fujii et al., 1993; Wimley et al., 1994; Lohner et al., 1995; White et al., 1995). Kagan et al. (1990) found that rabbit NP-1 and human defensin HNP-1 form voltage-dependent channels in planar bilayer membranes of neutral and negatively charged phospholipids. Modified (carboxamidomethylated) NP-1 had no significant effect on bilayer conductance, suggesting that an intact tertiary structure is required for channel formation. Circular dichroism and fluorescence spectroscopy of HNP-1 also indicated that the amphiphilic β-sheet structure is maintained when the peptide interacts with small unilamellar vesicles composed of a mixture of phosphatidylcholine and -serine (3:1 mol/mol) (Fujii et al., 1993). Very recently Wimley et al. (1994) demonstrated that HNP-2 caused vesicle leakage in an all-or-none manner when a minimum number of peptide dimers were bound to a model membrane containing negatively charged phospholipids. From the release of dextrans entrapped in large unilamellar vesicles of palmitoyloleoylphosphatidylglycerol, a pore diameter of about 2.5 nm was estimated, leading to a model of a pore consisting of a hexamer of HNP-2 dimers. Calcein release from unilamellar vesicles composed of zwitterionic and acidic phospholipids also indicated a preferential interaction of HNP-2 with negatively charged lipids (Lohner et al., 1995).

The human neutrophil peptide, HNP-2, containing 29 amino acid residues, possesses a much higher ability to kill bacteria than to lyse erythrocytes (Lehrer et al., 1993). It is well established that the composition of phospholipids, being the matrix of biomembranes, differs significantly between procaryotic and eucaryotic cell membranes. Whereas the outer leaflet of eucaryotic membranes almost exclusively consists of choline phospholipids, the procaryotic one has a high content of negatively charged phospholipids (Yorek, 1993). In order to gain insight on the impact of the membrane structure regarding the membranolytic activity of HNP-2, we used microcalorimetry to study the effects of this peptide on the thermotropic behavior of liposomes mimicking these cell membranes: the negatively charged dipalmitoylphosphatidylglycerol and dipalmitoylphosphatidylethanolamine for bacterial membranes, and the zwitterionic dipalmitoylphosphatidylcholine and sphingomyelin for erythrocyte membranes.

EXPERIMENTAL PROCEDURES

Lipids. 1,2-Dipalmitoylphosphatidylcholine (DPPC), -phosphatidylethanolamine (DPPE), and -phosphatidylglycerol (Na salt) (DPPG) as well as egg sphingomyelin (SM), where palmitate is the main side chain component, were purchased from Avanti Polar Lipids, Inc. (purity >99%), and used without further purification. Stock solutions of SM and DPPC were prepared in chloroform/methanol (2:1 v/v). DPPG and DPPE were dissolved in chloroform containing 1 vol % of methanol. In order to get complete dissolution of DPPG and DPPE, the solutions were titrated with double-distilled water till they were fully clear at room temperature. All stock solutions were stored at −18 °C. Before and after calorimetric experiments, the purity of the phospholipids was checked by thin-layer chromatography, which showed only a single spot using $CHCl_3/CH_3OH/NH_{3,conc}$ (75:25:6 v/v) as a solvent.

Peptides. Human neutrophil peptide, HNP-2, was isolated from human peripheral blood neutrophils and purified to homogeneity as described previously (Harwig et al., 1994b). The lyophilized samples were stored at 2 °C and rehydrated in 0.01% acetic acid at 1 mg of peptide/mL. Melittin, from bee venom, was purchased from Serva (Heidelberg, Germany). The peptide was used without further purification, after the integrity of the peptide regarding phospholipase A_2 contamination was assayed as described previously (Colotto et al., 1993).

Preparation of Liposomes. Appropriate amounts of the respective phospholipid stock solutions were dried under a stream of nitrogen and stored in vacuo overnight to remove the organic solvent totally. The exact lipid concentration (∼1 mg/mL) was determined according to the method of Bartlett (1959). The dried lipid films were then dispersed in double-distilled water containing 10 mM HEPES [*N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid] buffer, pH 7.4, and hydrated in the liquid-crystalline phase (SM, DPPC, and DPPG at 50 °C, DPPE at 70 °C) for several hours with intermittent, vigorous vortex mixing. This procedure resulted in multilamellar vesicles for the zwitterionic phospholipids. Liposome preparations containing DPPG appeared transparent in this buffer system. Negative-stain electron microscopy revealed unilamellarity of these liposomes with a diameter of about 80 nm. These experiments were kindly performed by Dr. S. Küpcü at the Zentrum für Ultrastrukturforschung, Universität für Bodenkultur, Vienna, Austria, with a Philips CM-100 transmission electron microscope at 80 kV using an 30 μm objective aperture. In an earlier study, formation of multilamellar vesicles was reported using the same method of vesicle preparation and a very similar buffer system (50 mM HEPES, pH 7.3), although either a lack of ordered layers or a small number of layers were suggested for these DPPG vesicles (Takahashi et al., 1992), as also described for both dimyristoyl-PG (DMPG) (Epand et al., 1992) and DPPG (Borochov et al., 1995) in the presence of salt. However, our finding is in good agreement with a recent negative-stain electron microscopy study on DMPG vesicles formed upon suspension in aqueous solution which demonstrated that multilamellarity strongly depends on sodium ion concentration (Kodama & Miyata, 1995). This is consistent with the general observation that charged lipids swell into large unilamellar vesicles in excess water (Hauser, 1984).

Unless otherwise stated, samples containing the peptide (lipid-to-peptide molar ratio of 100:1) were prepared by adding an aliquot of the HNP-2 stock solution to the preformed liposomes since this is how the peptide acts *in vivo*. After adding the peptide, the samples were incubated in the liquid-crystalline phase of the particular phospholipid for 15 min.

Differential Scanning Microcalorimetry. Calorimetric experiments were performed with a high-precision, adiabatic differential scanning calorimeter DASM-4 (Biopribor, Pushchino, Russia) designed by Privalov et al. (1975). The calorimeter was calibrated by the internal electrical power signal and was interfaced to a personal computer for automatic data collection. The cells were pressurized with nitrogen to about 2.5 atm to prevent bubbling on heating, and loss of solvent by evaporation. All heating scans were recorded at the same rate of 0.25 °C/min. Before starting each scan, the samples were equilibrated in the calorimetric cell for 15 min at 20 °C. After the first scan, the samples were cooled and rescanned in order to check the reproducibility of the thermograms. Calorimetric enthalpies were calculated by integrating the peak areas after base line adjustment and normalization to the scan-rate and the amount of phospholipid analyzed.

RESULTS AND DISCUSSION

The human neutrophil defensin peptide, HNP-2, displays broad antimicrobial activity against Gram-positive and Gram-negative bacteria as well as fungi (Lehrer et al., 1991) and is cytotoxic to eucaryotic cells under some specific conditions (Lichtenstein et al., 1986). In order to obtain a better understanding of their target cell specificity, we used differential scanning calorimetry to investigate the influence of the peptide on the phase transition properties of biomembrane mimetic systems, from which the nature of lipid−peptide interactions can be assessed (McElhaney 1982; Van Osdol et al., 1989).

The cell envelope of Gram-negative bacteria is a complex structure consisting of an inner membrane, a unique outer membrane layer, and an intervening layer of peptidoglycan in the periplasmic space (Lugtenberg & Van Alphen, 1983). Studies on the bactericidal activity of human defensins (Lehrer et al., 1989) and magainins from the skin secretion of the frog *Xenopus laevis* (Rana & Blazyk, 1991; Rana et al., 1991) demonstrated their ability to perturb the structure of the outer membrane. It is suggested that like the antibiotic polymyxin B (Hsuchen & Feingold, 1973), these cationic peptides bind to the lipopolysaccharide (LPS) layer located at the exterior of the outer membrane, disrupting its structure and thereby gaining access to the inner membrane. The inner (cytoplasmic) membrane is essentially a bilayer of lipids. A typical representative of Gram-negative bacteria is *Escherichia coli*. Although the major phospholipid subclass of its inner membrane is phosphatidylethanolamine (≈70% of total), a considerable amount of negatively charged phosphatidylglycerol ≈20% of total) as well as minor but significant amounts of cardiolipin (≈5%), a double negatively charged phospholipid, are also incorporated (Wilkinson, 1988).

Gram-positive bacteria have simpler lipid bilayer membranes than those found in Gram-negative organisms. Typically, these membranes possess very high phosphatidylglycerol contents (O'Leary & Wilkinson, 1988). For example, 40% of the total phospholipids of *Bacillus megaterium* or 60% of the total phospholipids of *Staphylococcus aureus* consist of PG.

Table 2: Pretransition and Main Transition Temperatures (T_{pre}, T_m) and Enthalpies (ΔH_{pre}, ΔH_m) of Liposomes in the Presence and Absence of the Human Neutrophil Peptide, HNP-2

	membrane mimetic system	HNP-2[b]	T_{pre} (°C)	ΔH_{pre} (kJ/mol)	T_m (°C)	ΔH_m (kJ/mol)
erythrocyte	DPPC	−	34.5	4.6	41.2	32.6
		+	34.4	4.6	41.2	33.0
	SM	−	−	−	37.4	27.6
		+	−	−	37.4	27.6
	DPPC/SM, 1:1	−	−	−	37.3	30.1
		+	−	−	37.4	30.9
bacterial	DPPG	−	33.8	3.3	40.2	34.3
		+[c]	34.2	3.3	40.7[d]	33.9
		+	−	−	41.3[d]	31.4
	DPPG/DPPE, 1:1[a]	−	−	−	54.4[d]	33.9
		+	−	−	52.0/55.4[d]	33.0
	DPPG/DPPE, 1:3[a]	−	−	−	61/61.4	33.4
		+	−	−	61.5/62.0[e]	33.9
	DPPE	−	−	−	63.7	36.0
		+	−	−	63.8	36.8

[a] Molar ratio. [b] Lipid-to-peptide molar ratio 100:1. [c] Lipid-to-peptide molar ratio 500:1. [d] Several shoulders. [e] Shoulder at ∼60.8 °C.

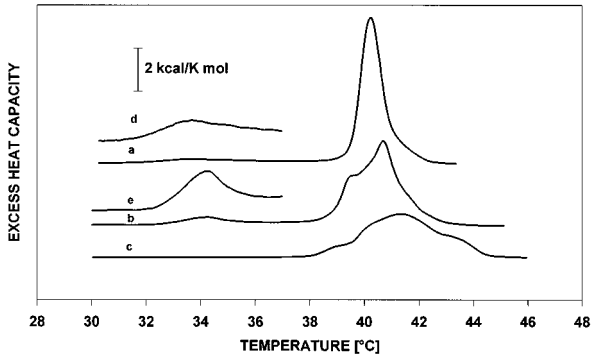

FIGURE 1: Heat capacity function of DPPG liposomes in 10 mM HEPES buffer, pH 7.4; scan-rate, 0.25 °C/min. Pure DPPG (a); DPPG in the presence of HNP-2 at a lipid-to-peptide molar ratio of 500:1 (b) and 100:1 (c). Pretransitions (d, e) were enlarged by a factor of 5.

Before studying phospholipid mixtures, which more closely resembled the composition of naturally occurring membranes, we recorded thermograms of single pure lipid model systems in order to simplify the nature and assessment of the effects of HNP-2 on their phase transitions. The heat capacity functions obtained for pure DPPG or DPPE liposomes and the calculated thermodynamic parameters (Table 2) are consistent with previously published data (Marsh, 1990; Caffrey, 1994). A very narrow, symmetrical transition was observed for DPPE at 63.7 °C indicative for a highly cooperative chain melting transition (data not shown). The respective transition of DPPG, centered at 40.2 °C, is broader as compared to DPPE. Furthermore, DPPG exhibits a pretransition at 33.8 °C (Figure 1a). The enthalpy associated with this transition is about 1 order of magnitude smaller than the enthalpy found for the main transition.

Similar heat capacity functions were obtained for DPPE liposomes in the presence or absence of HNP-2 (data not shown). A minor increase of the main transition temperature and enthalpy (Table 2) was found in the presence of the peptide. Also the cooperativity of this transition was slightly

increased as indicated by the slightly reduced width at half-height of the transition. These changes are characteristic for surface binding of the peptide to the lipid bilayer without penetration into the hydrophobic core of the lipid from these characteristics (McElhaney, 1982). However, the measured differences are rather small and will need further verification to determine their significance.

The thermograms of DPPG liposomes recorded in the presence of HNP-2 displayed (Figure 1) a complex shape and differed markedly from the one obtained for the pure lipid system. At a lipid-to-peptide molar ratio of 500:1, the peak maximum was shifted toward higher temperatures by about 0.5 °C (Figure 1b). A pronounced shoulder remained around 40 °C reflecting the fraction of lipid unaffected by the presence of the peptide. The thermograms were reproducible after the second rescan, from which additional shoulders at around 39 and 42 °C were discernible. The enthalpy calculated for the whole transition range is comparable to the value of pure DPPG (Table 2). As the enthalpy for the main transition is primarily due to the chain melting as a result of the disruption of the intra- and intermolecular van der Waals interaction and trans-gauche isomerization (Lewis et al., 1987), we can assume that the hydrocarbon chain packing is hardly affected at this lower lipid-to-peptide molar ratio.

The changes were enhanced at higher HNP-2 concentrations, i.e., a lipid-to-peptide molar ratio of 100:1. The temperature of the heat capacity maximum was further increased to 41.3 °C, though a strong shoulder at the position of pure DPPG (40.2 °C) was still observed. Additionally, the shoulders at the low (39 °C) and high (43 °C) temperature side were more clearly resolved (Figure 1c). An increase of the main transition temperature of DPPG was also reported in the presence of poly(L-lysine), which is frequently used as a model for cationic peptides (Takahashi et al., 1992). It was assumed that the addition of cationic peptides raises the phase transition temperature owing to nonspecific screening of the negative charges, as seen in the presence of monovalent ions (Cevc et al., 1980). The appearance of multiple peaks in the transition heat capacity function of the DPPG/HNP-2 systems with one of the peaks corresponding to the pure lipid may be interpreted by phase separation resulting in some domains being peptide-rich and others peptide-poor. The same result was obtained when dried DPPG was hydrated using a HEPES buffer solution containing HNP-2 to generate the liposomes in the first place, which ensures that the peptide has access to both leaflets of the bilayer. This indicates that the peptide does not only affect the outer leaflet of the bilayer which is supported by observations that HNP-2 caused vesicle leakage of liposomes containing negatively charged phospholipids (Wimley et al., 1994; Lohner et al., 1995).

The increase of the main transition temperature of DPPG might be also indicative for a preferrential stabilization of the gel phase when HNP-2 is present. However, many amphiphilic peptides do not interact with lipids in the gel state (Matsuzaki et al., 1993). Therefore, we also incubated DPPG liposomes with the human neutrophil peptide at room temperature, where the lipid is in such a phase. It was found that incubation of DPPG liposomes with HNP-2 below and above the main transition temperature gave qualitatively the same heat capacity functions (Figure 2). These data show the ability of human defensin to bind to DPPG liposomes in

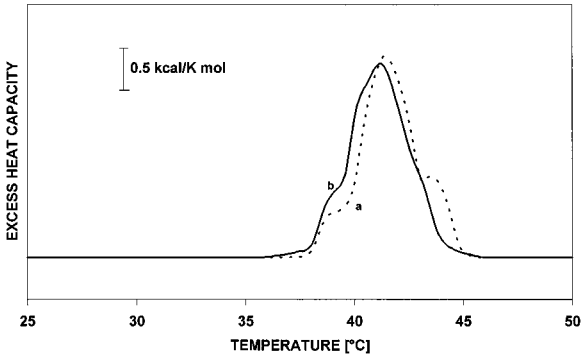

FIGURE 2: First heating scans of DPPG in the presence of HNP-2 (molar ratio 100:1) incubated at 25 °C (a) and 45 °C (b), respectively. Buffer system and scan-rate are the same as in Figure 1.

the gel state. Such an interaction was also reported for this lipid and tachyplesin I (Matsuzaki et al., 1993), a broad-spectrum antimicrobial peptide of 17 amino acid residues isolated from the horseshoe crab (Nakamura et al., 1988). Like human defensins, it forms a rigid antiparallel β-sheet structure owing to two disulfide bridges (Kawano et al., 1990). Referring to this study, we can also assume that this interaction is due to strong electrostatic attraction, which is energetically more favorable than the disordering of the more rigid hydrocarbon chain packing of the gel-phase lipids.

The pretransition, which is observed for some saturated phospholipids like DPPG and DPPC, is related to the untilting of the hydrocarbon side chains and usually is even more sensitive to the presence of foreign molecules than the main transition. Even minor amounts of additives like small organic molecules (Lohner, 1991) or the membrane-active peptides, melittin (Posch et al., 1983) and δ-lysin (Lohner et al., 1986), have strong effects (including abolition) on the pretransition, without strongly affecting the main transition of the lipid. In this study, we found that at a lipid-to-peptide molar ratio of 500:1 the pretransition temperature was increased by about 0.4 °C. The transition range itself was narrower, indicating a higher cooperativity of the transition as compared to pure DPPG liposomes, though the enthalpy was not affected (Figure 1d, e and Table 2). At higher HNP-2 concentrations, i.e., lipid to peptide molar ratio of 100:1, no pretransition was discernible from the heat capacity profile (Figure 1c). It can be inferred from these results that HNP-2 induces an untilting of the hydrocarbon side chains of the lipid bilayer. This would be consistent with the reduced main transition enthalpy which is found for these liposomes (Table 2), also indicating some interaction affecting the hydrocarbon chain packing. At present, it cannot be determined if changes in the headgroup structure, or insertion of the peptide into the hydrophobic core of the bilayer, cause the different hydrocarbon chain arrangement, or if both effects operate.

The latter mechanism may be supported by preliminary monolayer experiments performed in our laboratory (to be published elsewhere). These measurements show that HNP-2 partitions from the aqueous subphase into a DPPG monolayer at the air/water interface, suggesting that hydrophobic interactions may also play a role in the interaction between the cationic peptide and membranes. Further, these data indicate mixing at a molecular level and that the peptide may form distinct domains with the anionic lipid. This would be in agreement with the assumption that the initial step of

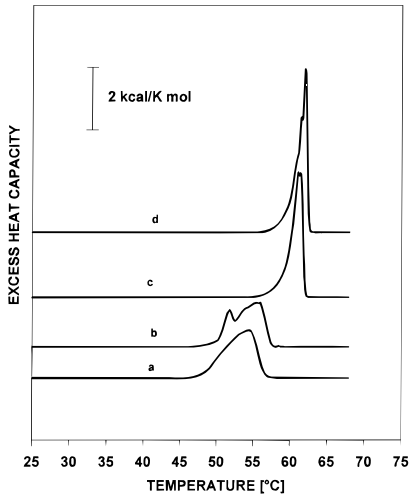

FIGURE 3: Heat capacity function of liposomes composed of DPPG and DPPE in 10 mM HEPES buffer, pH 7.4; scan-rate, 0.25 °C/min. Equimolar mixtures of both lipids without (a) and with HNP-2 (b). DPPG/DPPE liposomes (1:3 mol/mol) without (c) and with HNP-2 (d). Lipid-to-peptide molar ratio was 100:1 for both samples.

interaction between defensins and membranes involves electrostatic interactions (White et al., 1995). On the other hand, untilting of the hydrocarbon chains could be simply explained by neutralization of the headgroup charge by the cationic peptide, which will result in a reduced electrostatic headgroup repulsion and concomitantly in a smaller headgroup area as shown for pure uncharged DPPG at low pH (Watts et al., 1981).

Figure 3 shows the thermograms of two liposome preparations composed of mixtures of DPPG and DPPE, which can be considered to be representative for the phospholipid matrix of the plasma membranes of *E. Coli* (DPPG/DPPE, 1:3 mol/mol) and *Bacillus megaterium* (DPPG/DPPE 1:1 mol/mol). A broad, weakly cooperative chain-melting transition was found for the equimolar mixture of these lipids skewing toward low temperatures with a maximum heat capacity at 54.4 °C. Closer inspection of this thermogram reveals additional shoulders around 53.5 and 50.4 °C, respectively (Figure 3a). On the other hand, liposomes containing DPPG and DPPE at a molar ratio of 1:3 still exhibited a highly cooperative main transition at 61.4 °C similar to pure DPPE liposomes, with a significant tailing at the low-temperature side (Figure 3c).

Addition of HNP-2 altered significantly the phase transition characteristics of both lipid mixtures as shown in Figure 3 and summarized in Table 2. Besides the small peak at 61.4 °C, which is less resolved on recans, a transition at 62 °C was detected for liposomes mimicking the membrane of *E. coli*. The cooperativity of the transition was not affected by the peptide. In the presence of HNP-2, a high-melting component around 55.4 °C can be also observed for liposomes resembling the membrane of *Bacillus megaterium*. Furthermore, there was still a fraction detectable arising from the unperturbed DPPG/DPPE mixture. Additionally, a rather sharp transition was observed at about 52 °C. These results demonstrate that HNP-2 exerts the same effect on liposomes consisting of DPPG/DPPE mixtures as on pure DPPG liposomes, emphasizing the role of electrostatic interactions. This is consistent with the promotion of fusion of negatively charged phospholipid vesicles by HNP-1 (Fujii et al., 1993)

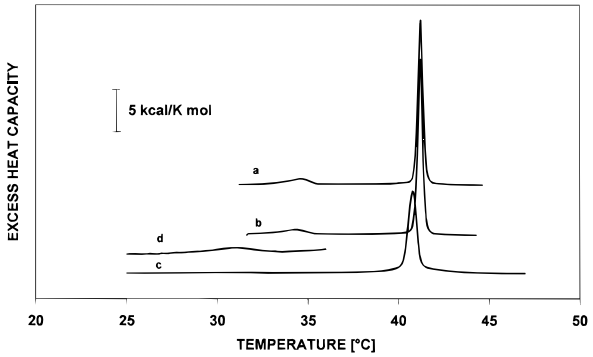

FIGURE 4: Thermograms of pure DPPC liposomes (a) and in the presence of HNP-2 (b) and melittin (c) at a lipid-to-peptide molar ratio of 100:1. The pretransition range for liposomes of DPPC/melittin (d) was enlarged by 1 order of magnitude. Scan-rate was 0.25 °C; samples were prepared in 10 mM HEPES buffer, pH 7.4.

and the permeabilization of large unilamellar vesicles containing anionic phospholipids by HNP-2 (Wimley et al., 1994).

It is well established that the plasma membrane of human erythrocytes displays an asymmetrical distribution of phospholipid subclasses between the inner and outer layer of the membrane (Opden Kamp, 1979). Thereby, the choline phospholipids, phosphatidylcholine and sphingomyelin, occur predominantly in the external leaflet at a nearly equimolar ratio (Yorek, 1993). This implies that in a first approach a model membrane consisting of phosphatidylcholine and sphingomyelin can be considered to mimic the phospholipid matrix of the human erythrocyte membrane.

Thermograms obtained for liposomes composed of the zwitterionic DPPC and SM are in agreement with earlier results (Marsh, 1990; Caffrey, 1994). Phase transition temperatures and enthalpies are summarized in Table 2. DPPC exhibits a pretransition at 34.5 °C and a very sharp main transition at 41.2 °C (Figure 4a). On the other hand, the gel to liquid-crystalline phase transition of SM, located at 37.4 °C, is broad and characterized by a tailing at the low-temperature side (data not shown) owing to the heterogeneous side chain composition in position 2 of this molecule (about 78% palmitate).

Incubation of DPPC multilamellar vesicles with HNP-2 at a lipid-to-peptide molar ratio of 100:1 did not lead to any significant changes in the phase transition properties, as compared to the pure phospholipid model system (Figure 4b, Table 2). The phase behavior of large unilamellar DPPC vesicles, formed by extrusion, was also not affected by the peptide (data not shown). This is in accordance with observations that no efflux on the fluorescence marker calcein was detected from small unilamellar vesicles composed of a mixed-chain PC in the presence of HNP-2 (Lohner et al., 1995). In contrast to phosphatidylcholine, sphingomyelin has a free OH group at the hydrophilic−hydrophobic interphase, which, because of the sphingosine backbone increasing the polarity of this region (Ali et al., 1991), might result in a different interaction between these choline phospholipids and HNP-2. However, superimposable heat capacity functions were obtained for SM liposomes in the absence and presence of the human neutrophil peptide (Table 2). Furthermore, the peptide did also not affect the thermotropic phase behavior of liposomes mimicking erythrocyte membranes, i.e., equimolar mixtures of DPPC and SM

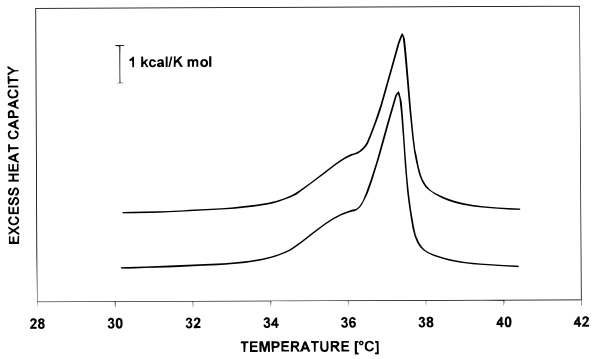

FIGURE 5: Heat capacity function of liposomes composed of an equimolar mixture of DPPC and SM (a) and with HNP-2 (b) at a lipid-to-peptide molar ratio of 100:1. Buffer system and scan-rate are the same as in Figure 4.

(Figure 5, Table 2). Again no calcein efflux was detected from small unilamellar vesicles of PC/SM mixtures (Lohner et al., 1995). These results demonstrate that HNP-2 does not bind to choline phospholipids under these experimental conditions, which is in accordance with the limited hemolytic activity of this peptide.

In contrast to these findings, melittin affected markedly the thermotropic behavior of DPPC liposomes at the same lipid-to-peptide molar ratio (Figure 4c). Melittin, the main component of bee venom (Habermann & Jentsch, 1967), has been studied in most detail with respect to its structure, conformation, and interaction with model and natural membranes (Dempsey, 1990; Saberwal & Nagaraj, 1994). Prior studies of the lytic activity of this amphiphilic, cationic peptide did not reveal any specificity toward a distinct target membrane. It is almost equally efficient in lysing erythrocytes (Sessa et al., 1969) or bacteria like *E. coli*, *B. subtilis*, and *S. aureus* (Boman et al., 1989). The interaction of melittin with phospholipids bearing different headgroups has been shown to have selective effects on the morphologies of these lipids (Batenburg & De Kruijff, 1988a), although it has a higher affinity to negatively charged phospholipids (Bhakoo et al., 1982; Batenburg & De Kruijff, 1988b). We find that in the presence of this cationic peptide, the pretransition of DPPC is significantly shifted to lower temperatures, hardly discernible from the base line (Figure 4d). The temperature of the main transition is less affected, but the transition is significantly broadened, indicating a loss of cooperativity in agreement with earlier reports (Posch et al., 1983; Colotto et al., 1993). These experiments reveal clearly the different interactions of melittin and defensins with phospholipids typical for eucaryotic cell membranes.

Conclusions. These results demonstrate that HNP-2 discriminates between model systems mimicking bacterial and erythrocyte cell membranes in a manner consistent with its biological activity. Moreover, they support the hypothesis of preferential interaction with negatively charged membrane cell surfaces (White et al., 1995). This is consistent with the specific release of calcein from DPPG-containing vesicles (Lohner et al., 1995), the negligible interaction between defensins and vesicles formed from DPPG and cholesterol (Fujii et al., 1993), and the binding of HNP-2 to vesicles composed of varying amounts of phosphatidylglycerol and -choline, indicating that peptide binding is initiated through electrostatic interactions (Wimley et al., 1994).

The utility of using different membrane mimetic systems to investigate antimicrobial and cytolytic peptides is illustrated by our findings that defensins and melittin had divergent effects on zwitterionic phospholipids, despite their similar actions on membranes composed of anionic phospholipids. Further systematic biophysical investigations can help to elucidate the interplay between such effector molecules and their target membranes, and identify factors responsible for the specificity and mechanism of their effects.

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