Interaction of 18-residue peptides derived from amphipathic helical segments of globular proteins with model membranes

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We investigated the interaction of six 18-residue peptides derived from amphipathic helical segments of globular proteins with model membranes. The net charge of the peptides at neutral pH varies from –1 to +6. Circular dichroism spectra indicate that peptides with a high net positive charge tend to fold into a helical conformation in the presence of negatively charged lipid vesicles. In helical conformation, their average hydrophobic moment and hydrophobicity would render them surface-active. The composition of amino acids on the polar face of the helix in the peptides is considerably different. The peptides show variations in their ability to permeabilise zwitterionic and anionic lipid vesicles. Whereas increased net positive charge favours greater permeabilisation, the distribution of charged residues in the polar face also plays a role in determining membrane activity. The distribution of amino acids in the polar face of the helix in the peptides that were investigated do not fall into the canonical classes described. Amphipathic helices, which are part of proteins, with a pattern of amino acid distribution different from those observed in class L, A and others, could help in providing newer insights into peptide–membrane interactions.

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1. Introduction

Amphipathic helices play crucial roles in modulating the interaction of proteins and peptides with membranes (Segrest *et al.* 1974; Kaiser and Kezdy 1984, 1987; Segrest *et al.* 1990; Epand *et al.* 1995; Huang *et al.* 1995; Phoenix *et al.* 1998; Johnson and Cornell 1999; Phoenix and Harris 2002; Cornell and Taneva 2006). Due to the distribution of polar and apolar residues on opposite faces of the helix, these peptides prefer aqueous–membrane interfaces. The average hydrophobic moment and average hydrophobicity values that would favour surface activity have been described by Eisenberg *et al.* (1982a, b). Based on the angle subtended by the polar face and distribution of anionic and cationic residues in the polar face, amphipathic helices have been classified into different categories (Segrest *et al.* 1990; Phoenix *et al.* 1998; Phoenix and Harris 2002). Helices that cause membrane lysis belong to class L and those that bind to lipids but are not lytic, such as those occurring in apolipoproteins, are classified as class A. Interest in amphipathic helices has further stemmed from the observation that a large number of linear host-defence antibacterial peptides form helices in membrane environments that are amphipathic in nature (Saberwal and Nagaraj 1994; Oren and Shai 1998; Matsuzaki 1998, 1999; Sitaram and Nagaraj 1999; Tossi *et al.* 2000; Giangaspero *et al.* 2001).

Modulation of the amphipathic character of helical peptides has been observed to result in altered haemolytic and/or antibacterial activities (Degrado *et al.* 1981; Agawa *et al.* 1991; Dathe *et al.* 1996; Kiyota *et al.* 1996; Wieprecht *et al.* 1997a, b; Uematsu and Matsuzaki 2000), underlining the importance of amphipathicity for interaction with

Keywords. Amphipathic helical peptides; average hydrophobicity; hydrophobic moment;; membrane-permeabilisation; model membranes

Abbreviations used: CD, circular dichroism; HPLC, high-performance liquid chromatography; LUV, large unilamellar vesicles; MRE, mean residue ellipticity; naf, normal accessibility factor; NBS, N-bromosuccinimide; PC, 1-palmitoyl-2-oleoyl-*sn*-glycerophosphocholine; PDB, Protein Data Bank; PG, 1-palmitoyl-2-oleoyl-*sn*-glycerophosphoglycerol; P/L, peptide:lipid; SUV, small unilamellar vesicles

membranes. In fact, model peptides composed of only hydrophobic and cationic residues, suitably engineered to result in amphipathicity that would favour membrane interaction, do exhibit membrane activity (Degrado *et al.* 1981; Agawa *et al.* 1991; Blondelle and Houghten 1992; Kiyota *et al.* 1996). However, naturally occurring host-defence peptides that interact with membranes have marked differences in their amino acid composition, lengths, amphipathicity and propensity for α-helical conformation (Saberwal and Nagaraj 1994; Oren and Shai 1998; Matsuzaki 1998, 1999; Sitaram and Nagaraj 1999; Tossi *et al.* 2000; Giangaspero *et al.* 2001). We have analysed protein structures in the Protein Databank (PDB) and delineated helices with a varying mean hydrophobic moment (<μH>) and hydrophobicities (<H>) (Sharadadevi *et al.* 2005). The analysis indicates that a large number of these helices would be surface-active. We have shown earlier that synthetic peptides corresponding to such helices with <μH> and <H>, which would favour 'surface activity', have antibacterial and/or haemolytic activities (Sharadadevi *et al.* 2005). The structures of these peptides, as observed in the X-ray structure of their parent proteins, are shown in figure 1. In the present study, we investigated how these peptides interact with model membranes. The bee-venom peptide melittin, whose model-membrane activity has been studied extensively (reviewed in Dempsey 1990; Raghuraman and Chattopadhyay 2007), and a potent analogue of the frog-skin antibacterial peptide magainin, MSI-78 (Maloy and Kari 1995), have been included for comparison.

2. Experimental procedures

2.1 *Peptide synthesis*

The following peptides were manually synthesised on Fmoc-amide handle crowns from Chiron, Australia, employing Fmoc chemistry (Atherton and Sheppard 1989) as described earlier (Sharadadevi *et al.* 2005): Pihf (Ac-RDAKELVELFFEEIRRAL-am), Pc3c (Ac-WYSEMKRNVQRLERAIEE-am), Pqc7 (Ac-LK-DVEEAQQKIINIIRRL-am), Pc9k (Ac-NRLARHFRDIAG-RVNQRL-am), Pill (Ac-KQLIRFLKRLDRNLWGLA-am), Pbuy (Ac-FRKLFRVYSNFLRGKLKL-am), and MSI-78. Melittin obtained from Sigma Chemical Co., USA, and purified by high-performance liquid chromatography (HPLC) was used for the experiments.

2.2 *Far UV circular dichroism measurements*

Spectra were recorded in a JASCO-J-815 spectropolarimeter using a quartz cell of 1 mm path length at 25°C. Data are represented as mean residue ellipticities (MRE). The lipids used for the preparation of lipid vesicles were 1-palmitoyl-2-oleoyl-*sn*-glycerophosphocholine (PC) and 1-palmitoyl-2-oleoyl-*sn*-glycerophosphoglycerol (PG). Small unilamellar vesicles (SUV) were prepared by sonicating a dispersion of the required lipid in 5 mM HEPES (pH 7.4) with 150 mM NaCl to clarity in a Branson sonifier.

2.3 *Calcein release*

The ability of the peptides to perturb membranes was determined by the release of the entrapped calcein at self-quench concentrations on a Hitachi 4500 spectrofluorimeter. The lipids used for the preparation of lipid vesicles were PC and PG. A lipid film composed of the required phospholipid was dispersed in 5 mM HEPES (pH 7.4) containing 70 mM calcein and passed through a Lipofast extruder (100 nm pore filter) (MacDonald *et al.* 1991) to obtain dye-encapsulated large unilamellar vesicles (LUV). The excess free dye was separated from the liposomes by gel filtration on a Sephadex G-75 column (elution buffer: 5 mM HEPES pH 7.4/150 mM NaCl, 5 mM EDTA). Release of the dye after addition of the peptide was continuously monitored by setting the excitation and emission wavelengths at 490 and 520 nm, respectively. Complete release of the dye was obtained by addition of Triton X-100 (0.1% v/v). The self-quenching property of the marker dye calcein was exploited in order to elucidate the induced release from the entrapped liposomes. To determine the mechanism of membrane permeabilisation, the dynamic self-quenching factor Q (Schwarz and Arbuzova 1995; Arbuzova and Schwarz 1999) was obtained as follows: after the calcein-containing LUV were mixed with the peptide, aliquots were drawn at different time points (different stages of efflux) and loaded on a Sephadex G-75 column for gel filtration. The vesicle fraction was separated from the leaked dye and the dynamic self-quenching factor Q was obtained by measuring fluorescence intensity before (F_b) and after (F_a) the addition of Triton X-100, as denoted by $Q = F_b/F_a$. To the remaining solution in the cuvette, Triton X-100 was added to lyse the vesicles completely. The apparent retention 'E' was calculated according to the following equation: $E=(F_t - F)/(F_t - F_o)$, where F and F_t denote the intensity of fluorescence before and after addition of the detergent, respectively. Fluorescence from intact vesicles is denoted by F_o.

2.4 *Diffusion potential dissipation assay*

The model membrane permeabilising ability was determined by monitoring the dissipation of the diffusion potential set up in lipid vesicles by valinomycin (Sims *et al.* 1974). K^+ (100 mM KCl) was entrapped in the lipid vesicles. During the experiment the vesicles were diluted in 5 mM HEPES buffer, pH 7.4, containing 100 mM NaCl, followed by serial

addition of the cyanine dye (diS-C_3-5), valinomycin and the peptides. The excitation and emission wavelengths were 620 and 670 nm, respectively. Percentage of fluorescence recovery F was calculated using the equation $F=(F_t - F_o)/(F_f - F_t)$ x 100, where F_t is the fluorescence after addition of the peptide, F_o after addition of valinomycin, and F_f is the total fluorescence.

2.5 *Tryptophan fluorescence*

Peptide–lipid interaction was studied by monitoring changes in the fluorescence properties of the Trp-containing peptides upon titration with lipid LUV. The emission spectra were recorded between 300 and 400 nm, with the excitation wavelength set at 280 nm, with 5 nm slit widths. All measurements were carried out on a Hitachi 4500 fluorescence spectrometer. LUV were obtained by passing through polycarbonate filters (pore size=100 nm) in a Lipofast extruder. In the quenching experiments, increasing amounts of the two quenchers, KI and acrylamide, were added to peptide–lipid solutions. The KI solution contained 1 mM $Na_2S_2O_3$ to prevent I^{3-} formation. The data were analysed according to the Stern–Volmer equation. Another quencher, N-bromosuccinimide (NBS), was also included in the study. NBS (8 *u*M) was added to the lipid-bound peptide and the spectra of the residual fluorescence were recorded after 10 min.

3. Results

Examination of the structures shown in figure 1 indicates that the polar face of the Pbuy and Pill helices is composed of cationic amino acids. In peptides Pc9k, Pqc7, Pc3c and Pihf, both cationic and anionic amino acids are present in the polar face. The sequence, mean hydrophobic moment, average hydrophobicity and net charge at neutral pH of

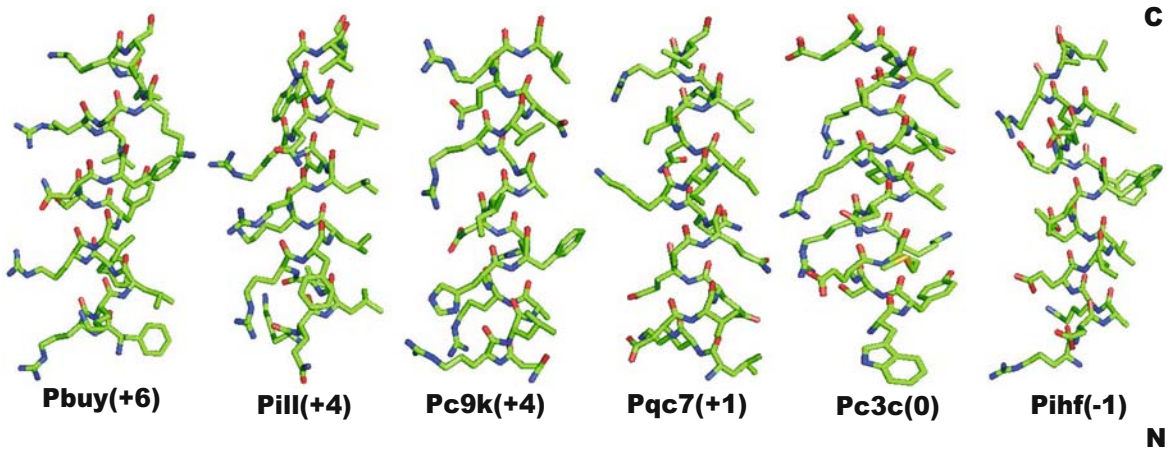

Figure 1. Structures of the 18-residue peptides as observed in their parent proteins. Their Protein Data Bank (PDB) IDs are 1buy, 1ill, 1c9k, 1qc7, 1c3c and 1ihf, respectively. The net charge at neutral pH is indicated in parentheses. C and N denote the carboxy- and amino-terminal ends. The N-terminal acetyl and C-terminal amide modifications are not included. Red, blue and green colours denote O, N and C atoms, respectively. Structures were generated using PyMOL (Delano W L [2002], Delano Scientific, San Carlos, CA, USA)

Table 1. Primary structure and physical properties of amphipathic peptides

Sequence of the peptides	<μH>	<H>	Net charge	Mean <H>$_{nonpolar}$
Ac-RDAKELVELFFEEIRRAL-am Pihf	0.72	–0.21	–1	0.51
Ac-WYSEMKRNVQRLERAIEE-am Pc3c	0.69	–0.45	0	0.26
Ac-LKDVEEAQQKIINIIRRL-am Pqc7	0.71	–0.2	+1	0.45
Ac-NRLARHFRDIAGRVNQRL-am Pc9k	0.71	–0.5	+4	0.49
Ac-KQLIRFLKRLDRNLWGLA-am Pill	0.75	–0.19	+4	0.39
Ac-FRKLFRVYSNFLRGKLKL-am Pbuy	0.69	–0.19	+6	0.32
GIGAVLKVLTTGLPALISWIK RKRQQ- am Melittin	0.55 [a]	–0.09	+6	
GIGKFLKKAKKFGKAFVKILKK-am MSI-78	0.75 [a]	–0.18	+9	

[a]<μH > are the highest mean values obtained for a window of 18 residues.

the six 18-residue peptides, along with that of MSI-78 and melittin, are shown in table 1. Zwitterionic PC vesicles and anionic PC/PG vesicles, mixed in a ratio of 1:1 were used, as membrane activity of melittin and MSI-78 show considerable variation against zwitterionic and anionic vesicles, which has been attributed to differences in their orientation in these lipid vesicles (Benachir and Lafleur 1995; Ladokhin and White 2001; Ramamoorthy *et al.* 2006).

3.1 *Circular dichroism*

The circular dichroism (CD) spectra of peptides were recorded in buffer and in the presence of increasing concentrations of lipid vesicles (figure 2). The structural propensities of the peptides vary to different extents. In buffer, Pill, Pbuy and Pc9k show spectra with a minimum at ~205 nm and a small shoulder at ~225 nm. These features indicate largely unordered conformation with only a small fraction populating helical conformation (Manning *et al.* 1988; Woody 1995; Venyaminov and Yang 1996). The shoulder at ~225 nm is more pronounced in Pqc7, indicating a greater fraction populating ordered conformation. Pihf shows a single minimum at ~217 nm, characteristic of a *β*-structure.

In the presence of lipids, Pill and Pbuy show minima at ~205 nm and ~225 nm, with a crossover at 200 nm, characteristic of helical conformation (Manning *et al.* 1988; Woody 1995; Venyaminov and Yang 1996). Pc9k shows similar features in the presence of PC/PG vesicles, but PC vesicles do not induce ordered conformation. The helical content does not increase for Pqc7 in the presence of PC and PC/PG lipid vesicles. Pihf shows two minima in PC/PG vesicles, suggesting transition from *β*-structure in aqueous medium to helical conformation. In PC vesicles, *β*-structure predominates. The CD data indicate that peptides with a high net positive charge tend to fold into helical conformation in the presence of negatively charged vesicles. In all the peptides, no further changes were observed in the CD spectra at peptide–lipid ratios >1:20 or 1:40. Pc3c, with a net charge of 0, did not yield good spectra in the presence of lipids, probably due to precipitation.

3.2 *Membrane permeabilisation*

In order to determine whether the amphipathic peptides can permeabilise model membranes, release of entrapped calcein was monitored at different peptide:lipid (P/L) ratios. The ability of the peptides to release calcein from PC LUV is shown in figure 3. Among the 18-residue peptides, Pill is most effective in causing release of calcein, followed by Pbuy, Pqc7 and Pc9k. Initially, leakage occurs rapidly, followed by a slower rate of release, except in the case of Pbuy, where the release is instantaneous and complete. The efficacy of Pill is comparable to melittin. In spite of its greater positive charge of +6 (similar to melittin), Pbuy is less effective. MSI-78, the magainin analogue, is the most potent among all the peptides in causing calcein release.

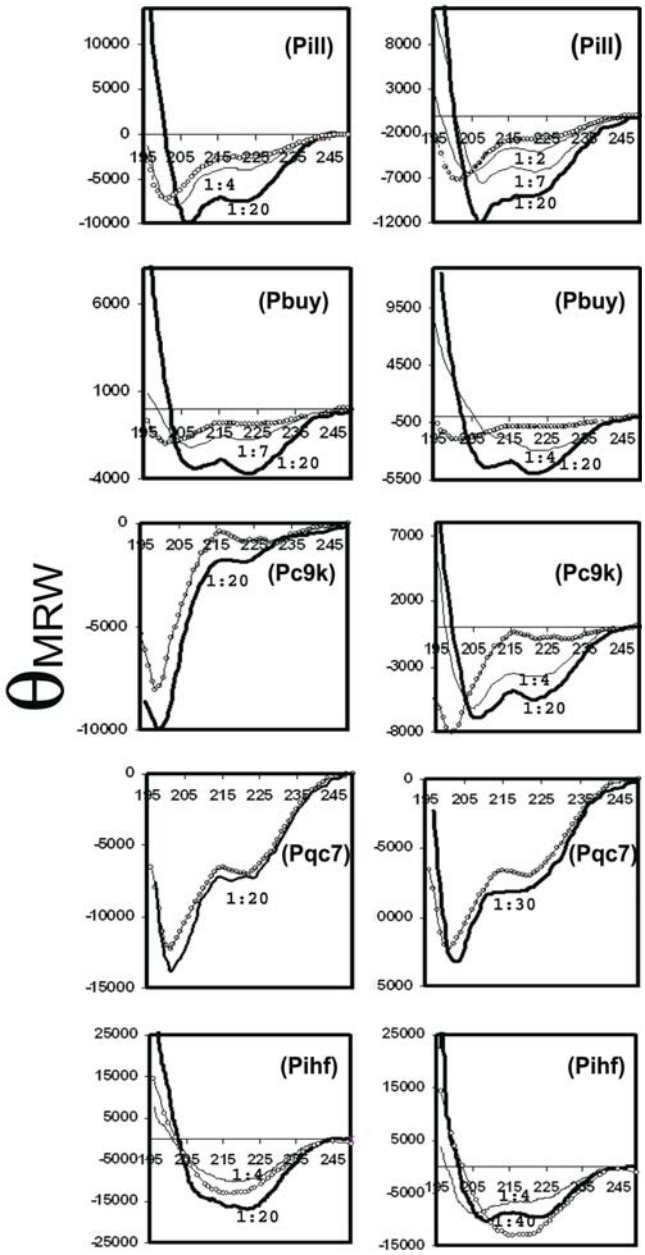

Figure 2. Far-UV circular dichroism (CD) spectra of the 18-residue peptides upon titration with lipid vesicles. Panels on the left are with PC small unilamellar vesicles (SUV) and panels on the right with PC/PG (1:1) SUV. Peptide concentration = 25 μM. (o---o) represents the spectrum of peptide in buffer. Lipid/peptide (L/P) ratios are indicated against each trace. PC, 1-palmitoyl-2-oleoyl-*sn*-glycerophosphocholine; PG, 1-palmitoyl-2-oleoyl-*sn*-glycerophosphoglycerol.

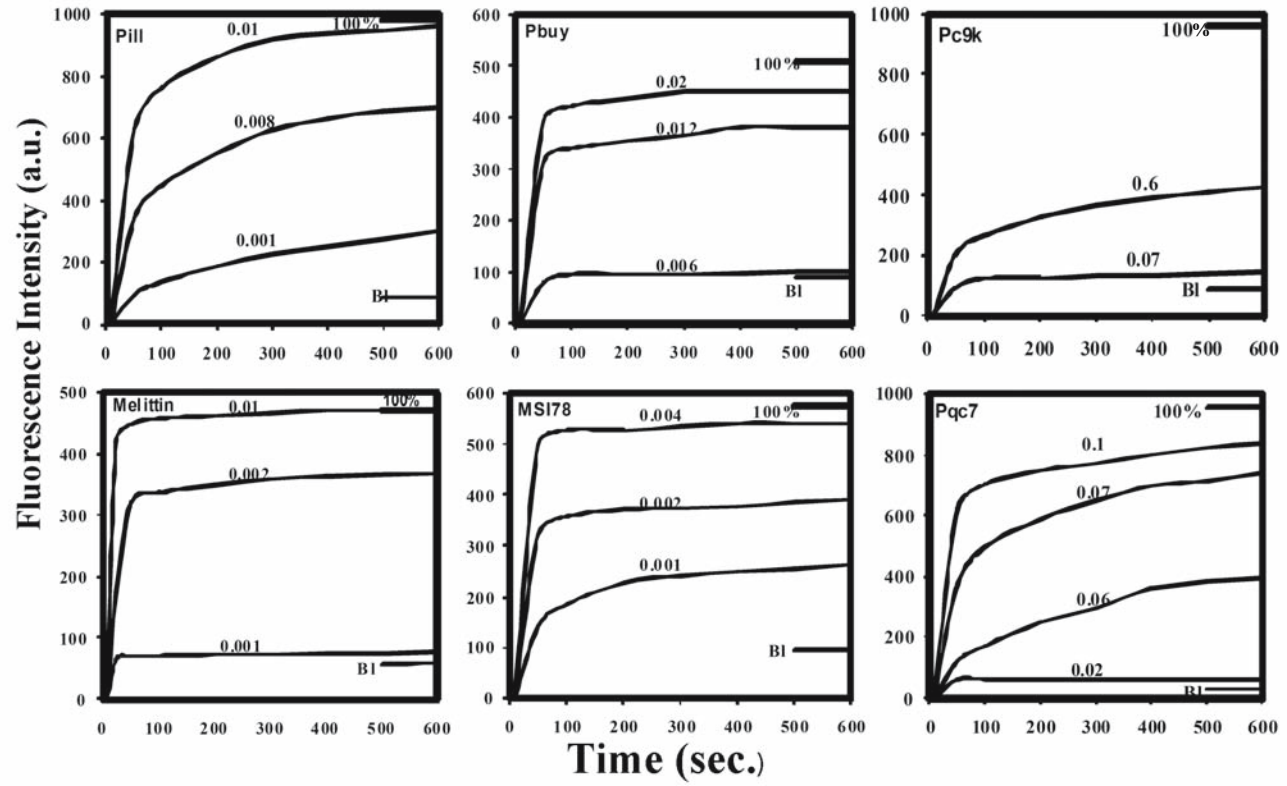

Figure 3. Release of calcein from PC large unilamellar vesicles (LUV) at different peptide–lipid ratios. Lipid = 30 μM. Release obtained with Triton X-100 indicated by dash on the right side Y-axis was taken as 100%. Peptide–lipid ratios are indicated above each trace.

All the peptides are less effective in causing the release of calcein from PC/PG (1:1) vesicles (figure 4). Ten-fold more peptide is necessary to cause a similar extent of dye release in PC/PG, compared with PC LUV. Even melittin and the highly cationic MSI-78 are less effective in causing calcein release as compared with PC vesicles. It has been shown that calcein release by melittin is inhibited in the presence of negatively charged lipids. This inhibition is presumed to result from peptide binding to the anionic charged surface (Benachir and Lafleur 1995; Ladokhin and White 2001), which prevents the formation of defects, thereby inhibiting calcein release. Interaction of MSI-78 with lipids is also dependent on the composition of the lipid (Ramamoorthy *et al.* 2006). Despite the requirement of a larger concentration of peptide to cause release of calcein, an initial rapid release is observed in the presence of Pbuy, Pill, MSI-78 and melittin. Peptides Pqc7 and Pc9k are not as effective in causing the release of calcein. Also, their activities against both PC and PC/PG vesicles are comparable, indicated by the similar range of P/L ratios to cause the efflux of calcein from both neutral and acidic lipids (figures 3 and 4). Peptides Pihf and Pc3c did not cause release of calcein even at a P/L ratio of 1:1.

In order to assess the mechanism of perturbation and calcein release, the rate of initial release (at t=1 min) was examined as a function of P/L ratios (figure 5). There are differences in the rates of calcein release by the peptides. Illustrated in the inset is the expanded portion of the X-axis at very low P/L ratios. It is clearly evident from the steep increase in the initial rates that Pill displays efficiency, comparable to MSI-78 and melittin, whereas the other three peptides exhibit lower potencies. The curves do not suggest cooperativity or formation of aggregates on the bilayer surface. This is further confirmed in the log–log plots in which slopes of ~1 were obtained (data not shown). Hence, the permeabilisation process does not appear to arise due to the formation of transmembrane channels.

The mechanism of calcein release was examined, as described in *Experimental procedures*. The data presented in table 2 indicate an 'all-or-none' mechanism of vesicle disruption by Pill and Pc9k. The data also suggest 'all-or-none' release for Pqc7 from PC/PG vesicles and some extent of graded release from PC vesicles. Pbuy exhibits 'all-or-none' release from both PC and PC/PG vesicles.

It is possible that the low activity of some of the peptides could arise as a result of less extensive membrane damage, and hence a lesser extent of calcein efflux. Therefore, the ability of peptides to dissipate the diffusion potential created by valinomycin was examined (figure 6). Peptides Pill, Pbuy, MSI-78 and melittin, although they caused the release of calcein from PC/PG vesicles at high P/L ratios (figure 5),

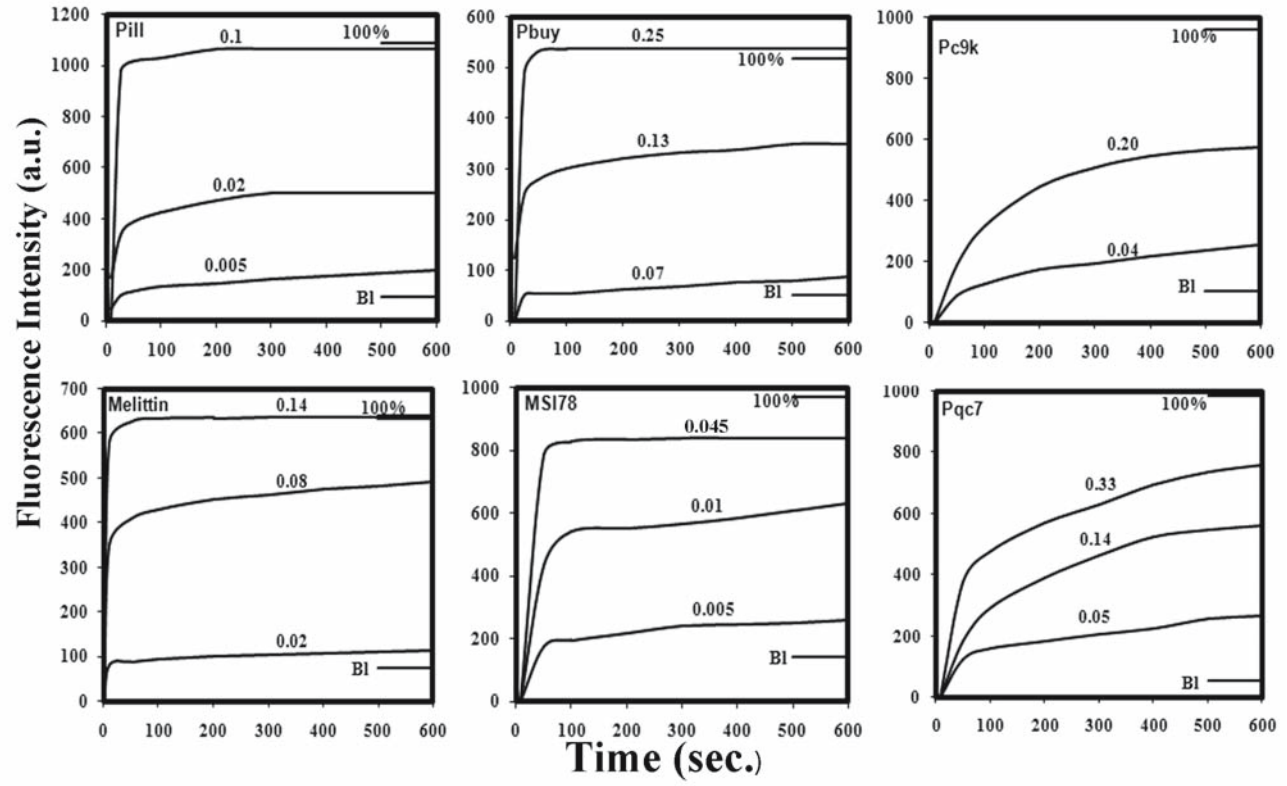

Figure 4. Release of calcein from PC/PG (1:1) large unilamellar vesicles (LUV) at different peptide–lipid ratios. Lipid = 30 μM. Release obtained with Triton X-100 indicated by a dash on the right side Y axis was taken as 100%. Peptide–lipid ratios are indicated above each trace. PC, 1-palmitoyl-2-oleoyl-*sn*-glycerophosphocholine; PG, 1-palmitoyl-2-oleoyl-*sn*-glycerophosphoglycerol.

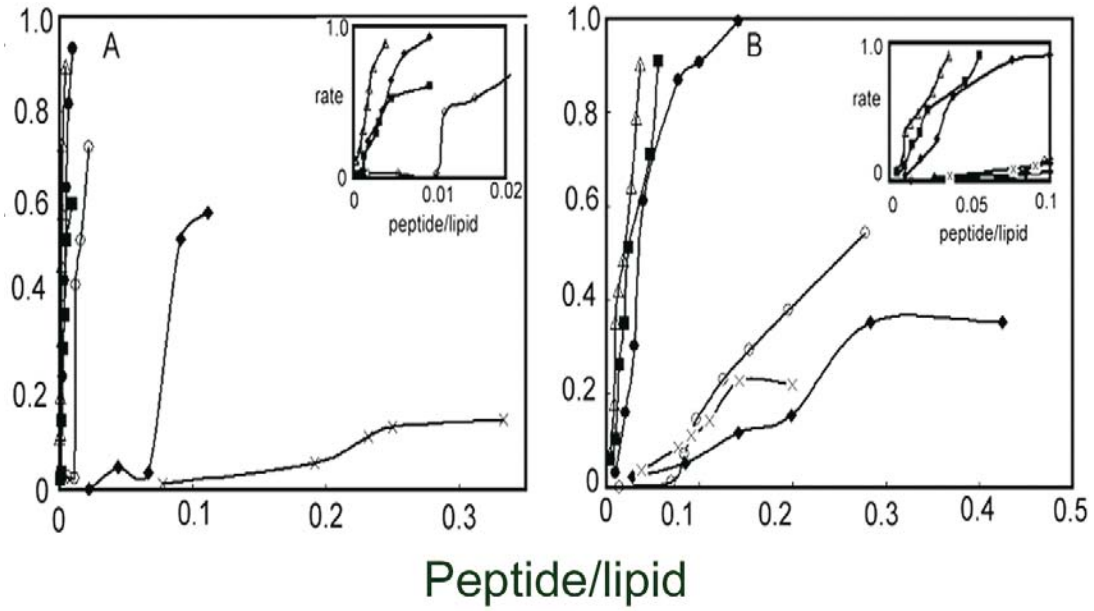

Figure 5. Plots of rate of initial calcein release as a function of peptide–lipid ratio. (**A**) PC and (**B**) PC/PG (1:1) . t = 1 min. *Key:* (Δ---Δ), MSI-78; (o—o), Pbuy; (■--■), Pill; (x-x), Pc9k; (♦---♦), Pqc7 and (●-----●) melittin. *Inset:* expanded portion of the X-axis. PC, 1-palmitoyl-2-oleoyl-*sn*-glycerophosphocholine; PG, 1-palmitoyl-2-oleoyl-*sn*-glycerophosphoglycerol.

could dissipate diffusion potential from PC/PG vesicles at considerably lower P/L ratios. Pbuy, in spite of possessing a higher charge, exhibited a lower ability to dissipate the diffusion potential compared with Pill. In fact, Pqc7 showed considerable activity at P/L ratios as low as 0.075 against PC/PG vesicles (figure 6B), unlike PC where no further dissipation of diffusion potential was observed above a P/L ratio of 0.02 (figure 6A). Pc9k was also moderately active in permeabilising vesicles, resulting in the efflux of calcein, as well as causing dissipation of the diffusion potential. Even at a P/L ratio of 0.1, only ~20% fluorescence recovery was observed (data not shown) in both PC and PC/PG vesicles, unlike the other four membrane-active peptides, *viz*. MSI-78, melittin, Pill and Pbuy.

Table 2. Modes of peptide-induced calcein leakage

Peptide	PC		PC/PG (1:1)	
	E[a]	Q[a]	E	Q
Pqc7	0.91	0.17	0.85	0.11
	0.42	0.23	0.55	0.11
	0.34	0.27	0.40	0.17
Pc9k	0.80	0.15	0.80	0.11
	0.59	0.20	0.61	0.11
Pill	0.96	0.08	0.85	0.12
	0.59	0.11	0.60	0.14
	0.32	0.10	0.43	0.20
Pbuy	0.70	0.12	0.90	0.26
	0.19	0.16	0.60	0.25

[a] E and Q are apparent retention and quenching factors, respectively, at different time points.

3.3 *Tryptophan fluorescence*

As the emission properties of Trp are sensitive to the surrounding environment, the fluorescence of Trp in two of the peptides that have Trp, viz., the very active Pill and the inactive Pc3c, were examined in the absence and presence of lipid vesicles. Variations in the emission spectrum of Trp in buffer and in the presence of lipid vesicles are presented in figure 7 A–D. Increasing concentrations of lipid vesicles were added to a fixed concentration of the peptide (4 μM). Both the peptides exhibit a λ_{max} ~355 nm in buffer, indicating exposure of Trp to an aqueous environment. Quenching of fluorescence intensity with no blue shift was observed when titrated with PC vesicles. In the presence of PC/PG vesicles, a blue shift in λ_{max}, with a concomitant increase in intensity were observed for Pill, whereas a decrease in fluorescence intensity was observed for Pc3c. The maximum blue shift of approximately 12 nm was observed for Pill, up to a P/L ratio of 1:30 (shown in figure 7). A further increase in P/L ratio did not cause any further changes in both λ_{max} and fluorescence intensity.

The localisation of Trp in the lipid bilayer in these peptides was examined by accessibility to quenchers, such as I^-, acrylamide and NBS. Quenching of fluorescence in Pc3c by KI was comparable both in the absence and in the presence of lipid vesicles, reflected by the superimposable lines (figure 7E). The Trp in Pill was shielded from I^- (figure 7F) and acrylamide (figure 7G) in both PC and PC/PG vesicles, with inaccessibility more pronounced in the latter. The normal accessibility factor (naf) values, which are a measure of the accessibility of the fluorophore in lipids with respect to free peptide in buffer, were found to be around 0.25–0.26 in the presence of PC, whereas it was in the range of 0.04–0.06 in anionic PC/PG. Such low values of naf indicate that the Trp is almost completely shielded from the

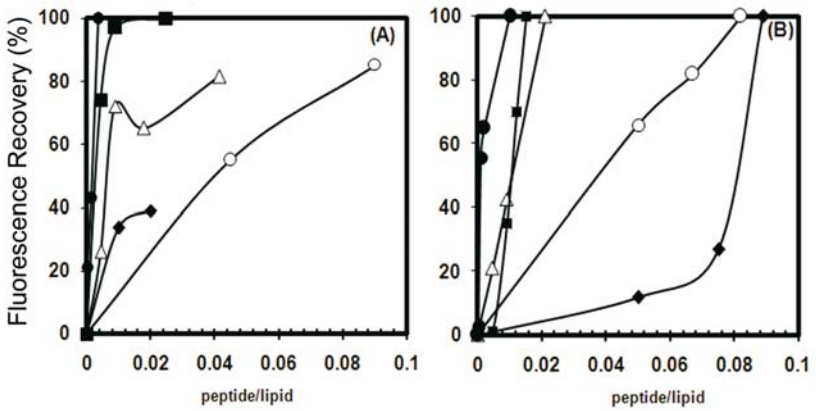

Figure 6. Dissipation of diffusion potential in lipid vesicles induced by peptides. Panel **A**: PC and panel **B** for PC/PG (1:1) LUV. Peptides were added to lipid vesicles in K^+-free buffer pre-equilibriated with the fluorescent dye diS-C3-(5) and valinomycin. Key: (Δ---Δ), MSI-78; (o—o), Pbuy; (■--■), Pill; (♦---♦), Pqc7 and (●-----●), melittin. PC, 1-palmitoyl-2-oleoyl-*sn*-glycerophosphocholine; PG, 1-palmitoyl-2-oleoyl-*sn*-glycerophosphoglycerol.

quenchers. As the quenching curves almost overlap in the presence and absence of lipid vesicles (figure 7E), in Pc3c the naf would be almost equal to 1, indicating considerable exposure to the quencher KI. It is also conceivable that since the Trp in Pc3c occurs at the extreme N-terminus, it is exposed to an aqueous environment, even though the rest of the peptide chain is associated with lipid vesicles. When the emission spectra of Pill, after quenching with acrylamide, were examined, a distinct blue shift of approximately 10 nm was observed, indicating a population of peptides, with Trp in a moderately hydrophobic environment (data not shown). The susceptibility of Trp to oxidation by NBS in the presence of PC and PC/PG vesicles at varying P/L ratios is shown in figure 8 A–D. In buffer, the fluorescence of Trp was quenched within 2−3 min on treatment with NBS (data not shown). In the presence of PC/PG vesicles, considerable fluorescence was retained even after treatment with NBS for 30 min, indicating that Trp is not accessible to NBS. Whereas a small decrease in fluorescence intensity was observed at a P/L ratio of 1:30 (figure 8A, trace 3) compared with buffer (figure 8A, trace 1), the value was higher when P/L=1:100 (figure 8B, trace 3) compared with buffer (figure 8B, trace 1). The blue shift obtained on addition of lipid vesicles (figure 8A, B, trace 2) was observed to remain unchanged in the emission spectrum of Trp that was not oxidised (figure 8A, B, trace 3), indicating a hydrophobic location of Trp. In the presence of PC vesicles, irrespective of the P/L ratio, there was further quenching upon addition of NBS to lipid-bound vesicles (figure 8C, D).

4. Discussion

The six peptides described in table 1 are helical segments derived from globular proteins (figure 1). These peptides show a propensity for helical conformation in the presence of zwitterionic and anionic lipids, albeit to different extents. They display varying degrees of membrane perturbation, as indicated by their ability to induce calcein release or cause dissipation of the diffusion potential. The helical wheel diagrams, which have been used extensively to explain the membrane activity of amphipathic peptides (Segrest *et al.* 1990; Epand *et al.* 1995; Phoenix and Harris 2002) shown in figure 9, indicate that the most active peptide Pill has a polar angle ≤180°, in which the boundary residues are both cationic, or one cationic and the other an uncharged polar residue. Three out of the five cationic residues are Arg. This pattern is different from the canonical class L amphipathic helix, which has a characteristic narrow, acute polar face comprising Lys or Arg residues (Segrest *et al.*

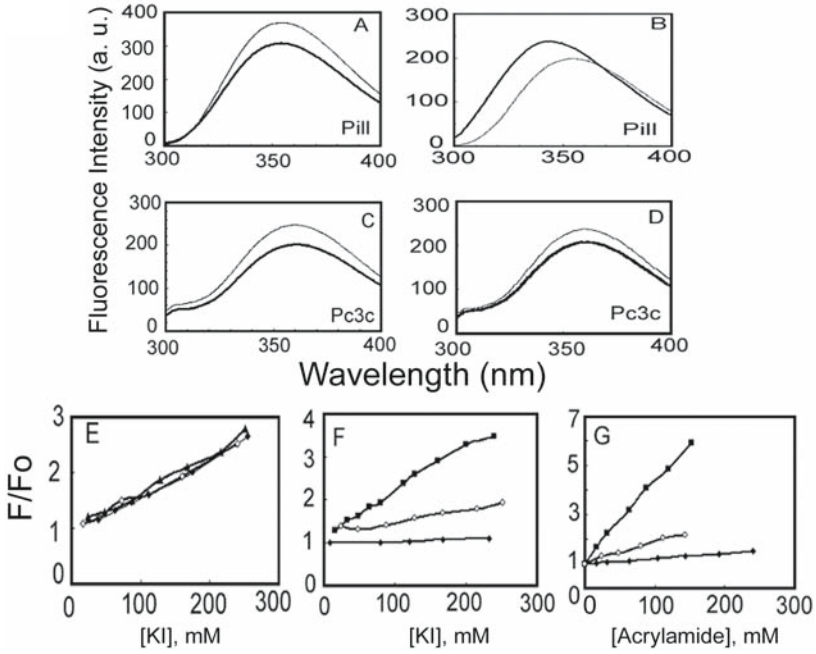

Figure 7. Tryptophan fluorescence spectra of peptides Pill and Pc3c. (**A, C**) PC large unilamellar vesicles (LUV); (**B, D**), PC/PG LUV. Increasing concentrations of lipid vesicles were added to a fixed concentration of peptide (4 μM). Data shown are for a peptide–lipid (P/L) ratio of 1:30 where maximal changes were observed. Thin and thick lines denote absence and presence of lipid vesicles, respectively. Stern–Volmer plots showing quenching of Trp fluorescence of Pc3c by KI (panel **E**), Pill by KI (panel **F**), and Pill by acrylamide (panel **G**). Initial concentration of peptide = 4 μM. Lipid = 400 μM. Key: (■--■) buffer, (o—o) PC LUV and (♦---♦) PC/PG (1:1) LUV. PC, 1-palmitoyl-2-oleoyl-*sn*-glycerophosphocholine; PG, 1-palmitoyl-2-oleoyl-*sn*-glycerophosphoglycerol.

1990; Epand *et al.* 1995). Yet, this observed pattern does impart considerable model membrane-perturbing ability comparable with melittin and MSI-78 (whose polar face is composed of only Lys residues, figure 9) against PC vesicles. Peptides Pbuy and Pc9k, despite having net positive charges of +6 and +4, respectively, are less active than Pill. In fact, Pbuy also has three Arg and two Lys residues in the polar face, similar to Pill. Nevertheless, the presence of a Lys also in the apolar face in Pbuy appears to attenuate the membrane perturbation compared with the presence of Asp in Pill, which may be facilitating the interactions with lipid vesicles. Also, the contiguous stretch of Leu residues that are stacked in the apolar face in Pill (figure 1) could be a feature that contributes to its higher activity. Peptide Pc9k with all the five Arg residues in the polar face is significantly less membrane-active compared with Pill and Pbuy. Lack of Lys residues, occurrence of an acidic Asp residue, and a large number of other uncharged polar residues on the polar face of Pc9k could reduce the charge density, thereby resulting in this attenuation of activity. The mean hydrophobicity per residue of the non-polar face in the peptides, using the consensus scale of Eisenberg *et al.* (1982b) is shown in table 1. Membrane-active peptides have a lower average hydrophobicity value compared with inactive peptides, with the exception of Pc3c. In Pihf, Pc3c and Pqc7, although there

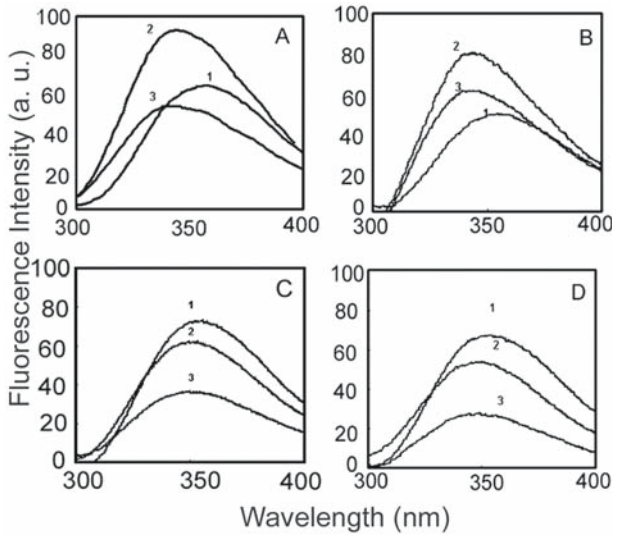

Figure 8. Quenching of Trp in Pill on treatment with NBS. (**A, B**) In the presence of PC/PG (1:1) LUV; (**C, D**) in the presence of PC large unilamellar vesicles (LUV). Traces 1, 2 and 3 denote fluorescence spectra of peptide in buffer, lipid-bound and lipid-bound after N-bromosuccinimide (NBS) modification. Concentration of peptide = 4 μM; NBS = 8 μM. Lipid concentration = 120 μM (**A** and **C**) and 400 μM (**B** and **D**) to yield peptide–lipid ratios of 1:30 and 1:100, respectively. PC, 1-palmitoyl-2-oleoyl-*sn*-glycerophosphocholine; PG, 1-palmitoyl-2-oleoyl-*sn*-glycerophosphoglycerol.

are several cationic residues in the polar face, there are also anionic residues and uncharged polar residues that appear to play a role in decreasing the membrane-perturbing activity.

All the peptides studied perturb negatively charged lipids to a lesser extent. A higher concentration of melittin was required to cause the release of entrapped markers from lipid vesicles containing anionic phospholipids (Benachir and Lafleur 1995; Ladokhin and White 2001). Benachir and Lafleur (1995) have argued that binding of melittin to the charged surface prevents the formation of defects that allow leakage. Ladokhin and White (2001) have attributed the lower activity to the inability of melittin to translocate PG-containing vesicles and adopt a transmembrane orientation. The low activity against PC/PG vesicles could arise because of binding to the bilayer surface, which prevents the formation of defects, as proposed for melittin (Benachir and Lafleur 1995; Ladokhin and White 2001).

More than one model has been proposed to rationalise permeabilisation of model membranes by amphipathic helical peptides. According to the toroidal pore model proposed for the interaction of magainin and analogues with model membranes, formation of a pore composed of a peptide–lipid supramolecular complex and translocation of peptide across the lipid bilayer is responsible for membrane permeabilisation (Matsuzaki 1999). In the 'carpet model', peptides bind to the membrane surface and cover it like a carpet. Membrane destabilisation occurs due to electrostatic interaction between the positively charged peptides and the negatively charged membrane surface, as well as hydrophobic interactions (Shai 1999). Certain amphipathic helical peptides corresponding to the helices of plasma apolipoprotein A-1 stabilise membranes (Segrest *et al.* 1990). The 'snorkel model' proposed for the insertion of this class of peptides (class A amphipathic peptides), in which the cationic residues occur at the boundary of the apolar/polar face and anionic residues towards the centre of the polar face, indicate that the alkyl side chains of Lys/Arg, which occur in the boundary of the apolar/polar face, are associated with the hydrophobic region of the lipid bilayer. The positively charged amino groups in contact with water extend to a location similar to the negatively charged carboxyl groups (Segrest *et al.* 1990; Epand *et al.* 1995). Our results, taken together from the different assays, suggest that these 18-residue peptides exert membrane activity in lipid vesicles by a process of membrane destabilisation, as described in the 'carpet model', rather than by the formation of pores due to peptide aggregates. It is unlikely that the lack of membrane-disrupting activity of some peptides is due to membrane stabilisation, as in the case of class A amphipathic peptides, as the arrangement of residues in the polar face is not similar to those of class A amphipathic peptides. The occurrence of anionic amino acid residues in the polar face could result

in a relatively weaker affinity for the membrane surface and consequently ineffective membrane destabilisation. A recent study on 17-residue model peptides highlights the importance of hydrophobic moment in modulating the partitioning of an amphipathic helix into the lipid bilayer interface (Fernandez-Vidal *et al.* 2007).

These authors concluded from their study that helical amphiphilicity is far more important than hydrophobicity for binding to membrane surfaces. The free energy of partitioning of melittin into PC vesicles was lower compared with its diastereomer, which had a lower propensity for helical conformation in PC vesicles (Ladokhin and White 1999). Hence, lower membrane activity of the two 18-residue peptides, viz., Pqc7 and and Pc9k, can be attributed to lower helical propensity in the presence of POPC vesicles, which would result in unfavourable free energy of partitioning.

Although our analysis is confined to only six peptides, it covers a broad range of net charge and composition of residues on the polar and apolar faces. Our results indicate that the amphipathic helical segments present in proteins could have a distribution of polar and apolar residues that is different from the canonical classes. The widely varying model membrane-perturbing activities of the six peptides indicate how variation of amino acids in the polar face region can modulate activity. Hence, amphipathic helices, which are a part of proteins, with a pattern of amino acid distribution different from those observed in class L, A and others, could help in providing newer insights into peptide–membrane interactions and their self-aggregating properties could also lead to the generation of novel organised assemblies.

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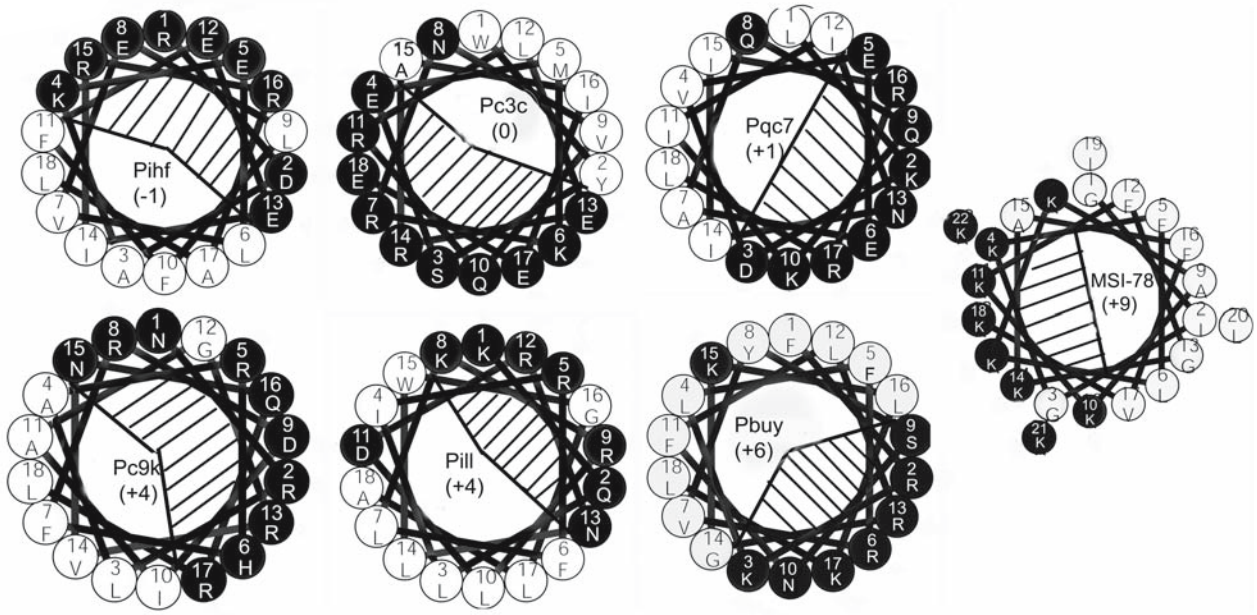

Figure 9. Helical wheel projections of the six 18-residue amphipathic helical peptides and MSI-78. The polar face is shown as a striped region. The polar residues are shaded dark.

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