Biochimica et Biophysica Acta 1778 (2008) 2041–2050

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Oxidized phospholipids as potential molecular targets for antimicrobial peptides

Juha-Pekka Mattila [1], Karen Sabatini [1], Paavo K.J. Kinnunen *

Helsinki Biophysics and Biomembrane Group, Institute of Biomedicine/Medical Biochemistry, P.O. Box 63 (Haartmaninkatu 8), FIN-00014 University of Helsinki, Helsinki, Finland

ARTICLE INFO

Article history:
Received 17 December 2007
Received in revised form 3 March 2008
Accepted 25 March 2008
Available online 8 April 2008

Keywords:
Monolayer penetration
Peptide–lipid interactions
Tryptophan fluorescence
Reactive oxygen species

ABSTRACT

The effects of oxidatively modified phospholipids on the association with model biomembranes of four antimicrobial peptides (AMPs), temporin B and L, indolicidin, and LL-37(F27W) were studied by Langmuir balance and fluorescence spectroscopy. In keeping with previous reports the negatively charged phospholipid phosphatidylglycerol (PG) enhanced the intercalation of all four peptides into lipid monolayers and liposomal bilayers under low ionic strength conditions. Interestingly, similar effect was observed for 1-palmitoyl-2-(9′-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine (PoxnoPC), a zwitterionic oxidized phospholipid bearing an aldehyde function at the end of its truncated *sn*-2 acyl chain. Instead, the structurally similar 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine (PazePC) containing a carboxylic moiety was less efficient in promoting the membrane association of these peptides. Physiological saline reduced the binding of the above peptides to membranes containing PG, whereas interactions with PoxnoPC were found to be insensitive to ionic strength. Notably, membrane intercalation of temporin L, the most surface active of the above peptides could be into PoxnoPC containing monolayers was strongly attenuated by methoxyamine, suggesting the importance of Schiff base formation between peptide amino groups and the lipid aldehyde function. PoxnoPC and similar aldehyde bearing oxidatively modified phospholipids could represent novel molecular targets for AMPs.

Introduction

Innate immune system of multicellular organisms relies as a first line of defense against opportunistic infections on inducible gene-encoded host-defense peptides [1–3], referred to as antimicrobial peptides. To date, hundreds of AMPs from various sources have been identified or synthesized de novo [4,5]. While they exhibit substantial diversity in their sequences, two recurrent structural characteristics are evident, viz. a net positive charge and amphipathicity owing to both hydrophobic and hydrophilic patches on their molecular surfaces [6]. These features promote the interactions of AMPs with pronounced selectivity towards membranes enriched in negatively charged phospholipids and lipopolysaccharides present in bacteria. Several models accounting for the disruption of membrane integrity following the binding of AMPs have been forwarded [7–9], each resulting in permeabilization and depolarization of the target cell or organism membrane. There is, however, evidence indicating that for certain AMPs membrane interactions in vivo are accompanied by antimicrobial activity due to association to intracellular targets or immunomodulation via stimulation in vertebrates of their adaptive immune response [10,11].

We have investigated the membrane association of several AMPs, including temporins B and L, indolicidin, and the F27W mutant of LL-37 [12–14]. Temporins were found in the skin of the European red frog *Rana temporaria* [15] and constitute a family of some of shortest (10–13 residues) linear AMPs known with a net positive charge and an amidated C-terminus. Temporin B (LLPIVGNLLKSLL–NH_2, charge=+2) and temporin L (FVQWFSKFLGRIL–NH_2, charge=+3), assume an amphipathic α-helical conformation in apolar solvent [14] and have been suggested to permeabilize target membranes by the barrel-stave mechanism [15]. They also form similarly to several other AMPs Congo red staining amyloid-type fibrils in the presence of acidic phospholipids [8,9].

Indolicidin is secreted by human macrophages and is a linear peptide consisting of 13 amino acids, amidated at the C-terminus and with an unusual amino acid composition with five Trp and three Pro residues, ILPWKWPWWPWRR–NH_2 (charge=+4). It does not adopt any classical secondary structure and does not display the characteristic amphiphatic nature of antimicrobial peptides [16]. Indolicidin adopts an extended conformation, with a wedge-type shape and with its Trp residues in a trough, flanked by the positively charged regions

Abbreviations: AMP, antimicrobial peptide; LUV, large unilamellar vesicle; oxPL, oxidized phospholipid; PazePC, 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine; PoxnoPC, 1-palmitoyl-2-(9′-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol; PC, phosphatidylcholine; PG, phosphatidylglycerol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol; RFI, relative fluorescence intensity; ROS, reactive oxygen species; K_{SV}, Stern–Volmer quenching constant; X_Y, mole fraction of compound Y; π, surface pressure; π_0, initial surface pressure; π_c, critical packing pressure; $\Delta\pi$, change of surface pressure

* Corresponding author. Fax: +358 191 25444.
E-mail address: paavo.kinnunen@helsinki.fi (P.K.J. Kinnunen).

[1] These authors contributed equally to this work.

0005-2736/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2008.03.020

of the peptide [16]. The peptide is thought to insert into the membrane with the hydrophobic core making contacts in the interfacial regions while the flanking parts would interact more prominently with the lipid headgroups. Indolicidin has been suggested to insert into the hydrophobic core of the membrane and form channel aggregates [16].

The 4.5 kDa cationic amphipathic peptide LL-37 (LLGDFFRKSKEKIG-KEFKRIVQRIKDFLRNLVPRTES) is found in humans and belongs to the class of α-helical AMPs. At physiological pH it contains 11 positive charges (due to 6 Lys and 5 Arg) and 5 negative charges (due to 3 Glu and 2 Asp) with a resulting net charge of +6. Expressed throughout the body, LL-37 has, in addition to its direct antimicrobial activity, been shown to be involved in reproduction [17], differentiation [18], and modulation of the innate immune system [19]. It has been shown to associate on the surface of the bilayer in the form of stable α-helices and subsequently permeate the membrane by a carpet-like mechanism [20,21]. In the present study we utilized a F27W mutant of LL-37 so as to allow the

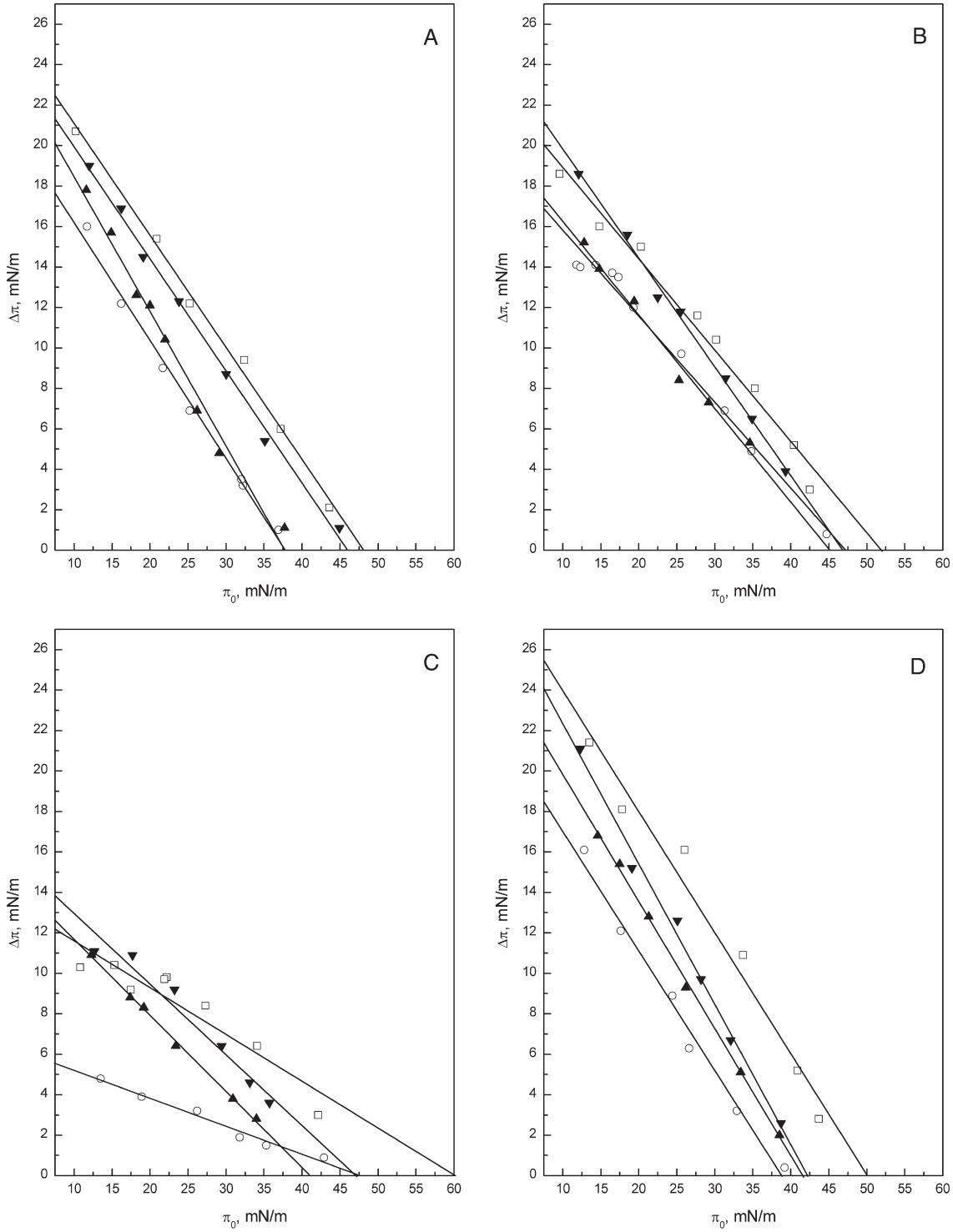

Fig. 1. Penetration of antimicrobial peptides into lipid monolayers residing on low ionic strength buffer (20 mM Hepes, 0.1 mM EDTA, pH 7.0), illustrated as increment in surface pressure ($\Delta\pi$) following the addition of 0.3 μM temporin B (A), temporin L (B), indolicidin (C), or LL-37(F27W) (D) into the subphase as a function of the initial surface pressure π_0. Monolayer compositions were DMPC (○), DMPC:DMPG=8:2 (□), DMPC:PoxnoPC=8.2 (▼), and DMPC:PazePC=8:2 (▲).

assessment of its interactions with model membranes by fluorescence spectroscopy [12]. Importantly, this mutant has been demonstrated to retain essentially identical antimicrobial activity as wild type LL-37 (as verified for *Bacillus subtilis* and *Staphylococcus aureus*). This mutant further adopts similar α-helical secondary structure upon binding to liposomes and intercalates into phospholipid monolayers equally well as the wild type LL-37, as determined by circular dichroism and monolayer penetration measurements, respectively [12].

Irrespective of their exact mode of action, discrimination between the membranes of the target and host organisms represents a pivotal issue regarding AMP function on molecular level. To this end, the lack acidic phospholipids from the outer leaflet of eukaryotic plasma membranes might provide a general mechanism of host cell recognition [22]. This notion is supported by a wealth of experimental evidence from studies with model phospholipid membranes, such as monolayers [23] and large or giant unilamellar vesicles [14]. Yet, cellular membranes undergo upon infection and inflammation a drastic modification due to lipid oxidation resulting in the formation of a myriad of phospholipids with modified acyl chains containing various functional groups [24]. Importantly, also the membranes of invading bacteria are exposed to reactive oxygen species (ROS) secreted by activated leukocytes at sites of infection. In the present study we demonstrate that the inclusion of aldehyde group bearing oxidatively modified phospholipid PoxnoPC into zwitterionic DMPC matrix in Langmuir monolayers or large unilamellar vesicles results in augmented membrane association of four AMPs, temporins B and L, indolicidin, and F27W mutant of LL-37, rivaling that observed in the presence of an equal mole fraction of the negatively charged PG. Notably, in physiological saline the efficiency of PG in promoting membrane interactions of the above peptides was considerably reduced, whereas the effect due to PoxnoPC remained essentially unaffected. In contrast, another phospholipid oxidation product, PazePC bearing a carboxylic function was found to possess negligible ability to promote membrane association of AMPs. The above data suggest a functional role of oxidized phospholipids in serving as molecular targets for AMPs, possibly relating in addition to their membrane perturbing properties to their capability to modulate both innate and adaptive immunity via different mechanisms including induction of cytokine production, stimulation of leukocyte chemotaxis, and promotion of monocyte-derived dendritic cell maturation [10].

Materials and methods

Materials

1-Palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine (PazePC), 1-palmitoyl-2-(9′-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine (PoxnoPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), and 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DMPG, Na^+-salt) were from Avanti Polar Lipids (Alabaster, AL). NaCl, Hepes, EDTA, and methoxyamine hydrochloride were from Sigma (St. Louis, MO). Indolicidin was from Bachem (Bubendorf, Switzerland), temporin B from Synpep (Dublin, CA), and temporin L and LL-37(F27W) from Caslo (Lyngby, Denmark). The purity of the peptides was >95% for indolicidin, temporin B, and LL-37, and >97% for temporin L as verified by high performance liquid chromatography analyses provided by their respective suppliers. The purity of the lipids was checked by thin-layer chromatography on silicic acid-coated plates (Merck, Darmstadt, Germany) using chloroform/methanol/water/ammonia (65:20:2:2, v/v) as the eluent. Examination of the plates after iodine staining revealed no impurities. Concentrations of the phospholipid stock solutions were determined gravimetrically with a high precision electrobalance (Cahn Instruments, Cerritos, CA).

Penetration of the peptides into lipid monolayers

Appropriate amounts of lipid stock solutions were mixed in chloroform to obtain the desired compositions, which were subsequently spread onto air/buffer interface in a magnetically stirred circular Teflon wells with a subphase volume of 1.2 ml (Multiwell plate, Kibron Inc., Espoo, Finland). Surface pressure (π) was monitored with a Wilhelmy wire attached to a computer-controlled Langmuir type film balance (μTroughX, Kibron) using the embedded features of the control software (FilmWare 3.57, Kibron), essentially as described previously [25]. After stabilization of the applied monolayer to an initial value of surface pressure π_0 the peptides were injected into the subphase (0.3 μM final concentration) where after the increment in π ($\Delta\pi$) due to their intercalation into the film was followed. The data are represented as $\Delta\pi$ vs π_0, yielding upon least-squares linear fitting straight lines with negative slopes, the *x*-axis intercept of which represent the critical packing pressure π_c, i.e. the maximum value of initial pressure at which the peptides intercalate into the monolayer [26,27]. All measurements were performed at ambient temperature of ~23 °C.

Preparation of large unilamellar vesicles (LUVs)

Appropriate amounts of lipid stock solutions were mixed in chloroform to obtain the desired compositions. The solvent was removed under a stream of nitrogen and the lipid residue was subsequently maintained overnight under a reduced pressure. The dry lipid film was hydrated at 50 °C in 20 mM Hepes, 0.1 mM EDTA, pH 7.0 or the same buffer containing 150 mM NaCl, to yield a lipid concentration of 2 mM. The resulting dispersions were extruded through a 100 nm pore size polycarbonate filter (Millipore Corp., Bedford, MA) using a Liposofast low pressure homogenizer thermostated at 50 °C with a circulating water bath (Avestin, Ottawa, Canada) to obtain large unilamellar vesicles with average diameter of ~80–100 nm as determined by dynamic light scattering [28,29].

Fluorescence spectroscopy

Peptides were added to a suspension of LUVs (total lipid concentration of 400 μM) in indicated buffer maintained at 25 °C with continuous stirring in a 1 cm path length quartz cuvette yielding a final concentration of 4 μM in a total volume of 2 ml (peptide/lipid ratio of 1:100). After 1 h of equilibration, fluorescence emission spectra were recorded with Perkin-Elmer LS 50B fluorescence spectrometer with both emission and excitation band passes set at 5 nm and using excitation wavelength of 280 nm. The obtained spectra were corrected for the contribution of light scattering in the presence of vesicles.

Quenching of Trp emission by acrylamide

In order to reduce absorbance by acrylamide, excitation of Trp at 295 nm instead of 280 nm was used [30]. Proper aliquots of 3 M acrylamide solution were added to the peptide in the presence or absence of liposomes at a peptide/lipid molar ratio of 1:100. The fluorescence intensity values obtained were corrected for dilution, and the scatter contribution was derived from acrylamide titration of a vesicle blank. The data were analyzed in terms of the Stern–Volmer equation [31],

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F represent the fluorescence intensities in the absence and the presence of the quencher Q, respectively, and K_{SV} is the Stern–Volmer quenching constant, which is a measure of the quenching efficiency and proportional to the accessibility of Trp to acrylamide. On the premise that acrylamide does not significantly partition into the bilayer [30], the value for K_{SV} can be considered to be a reliable reflection of the bimolecular rate constant for the collisional quenching of the Trp residues present in the aqueous phase. Accordingly, K_{SV} is determined by the amount of non-vesicle-associated free peptide, as well as the fraction of the peptide residing on the surface of the bilayer.

Results

In order to pursue the interactions of AMPs with membranes containing oxidized phospholipids we first measured the penetration of

Table 1
Monolayer critical packing pressures (π_c) and estimated increase of surface pressure from initial value of 10 mN/m following peptide addition ($\Delta\pi_{max}$) for temporins B and L, indolicidin, and LL-37(F27W)

	Lipid composition	π_c (mN/m)		$\Delta\pi_{max}$ (at 10 mN/m)	
		No salt	150 mM NaCl	No salt	150 mM NaCl
Temporin B	DMPC	37.7	36.3	16.1	17.7
	X_{DMPG}=0.2	48.0	40.5	21.0	18.1
	$X_{PoxnoPC}$=0.2	45.8	50.6	20.0	20.4
	X_{PazePC}=0.2	37.7	38.5	18.5	17.9
Temporin L	DMPC	47.1	45.2	15.8	15.9
	X_{DMPG}=0.2	51.9	45.9	18.9	18.9
	$X_{PoxnoPC}$=0.2	46.7	49.2	19.9	20.6
	X_{PazePC}=0.2	45.1	44.2	16.3	17.5
Indolicidin	DMPC	47.2	32.3	5.2	12.3
	X_{DMPG}=0.2	60.1	49.2	11.7	5.8
	$X_{PoxnoPC}$=0.2	47.0	44.1	12.9	12.2
	X_{PazePC}=0.2	40.9	42.4	11.7	8.0
LL-37(F27W)	DMPC	38.8	39.0	18.5	21.7
	X_{DMPG}=0.2	50.0	45.6	25.5	23.6
	$X_{PoxnoPC}$=0.2	42.2	39.7	24.0	26.2
	X_{PazePC}=0.2	41.5	36.9	21.4	23.6

Data were taken from Figs. 1 and 2.

temporins B and L, indolicidin, and LL-37(F27W) into lipid monolayers. More specifically, the insertion of these AMPs into monolayers with different initial surface pressures π_0 was observed by recording the increase in the surface pressure $\Delta\pi$ following the addition of the peptides into the subphase. Importantly, in order to avoid possible influence of different phospholipid phases we used, in addition to the two secondary products of lipid peroxidation PoxnoPC or PazePC (at $X=0.2$), DMPC and DMPG ($X=0.2$), which under the conditions employed form liquid expanded ("fluid") films [32], similarly to the unsaturated lipids such as POPC and POPG commonly found in biomembranes. Further, as no significance for structural features other than the acidic headgroup have been found for the interaction of AMPs with liquid expanded membranes, the above saturated lipids can be concluded to be representative also for unsaturated phospholipids, e.g. POPC and POPG.

In a low ionic strength subphase both temporins B and L were highly membrane active producing significant increment in surface

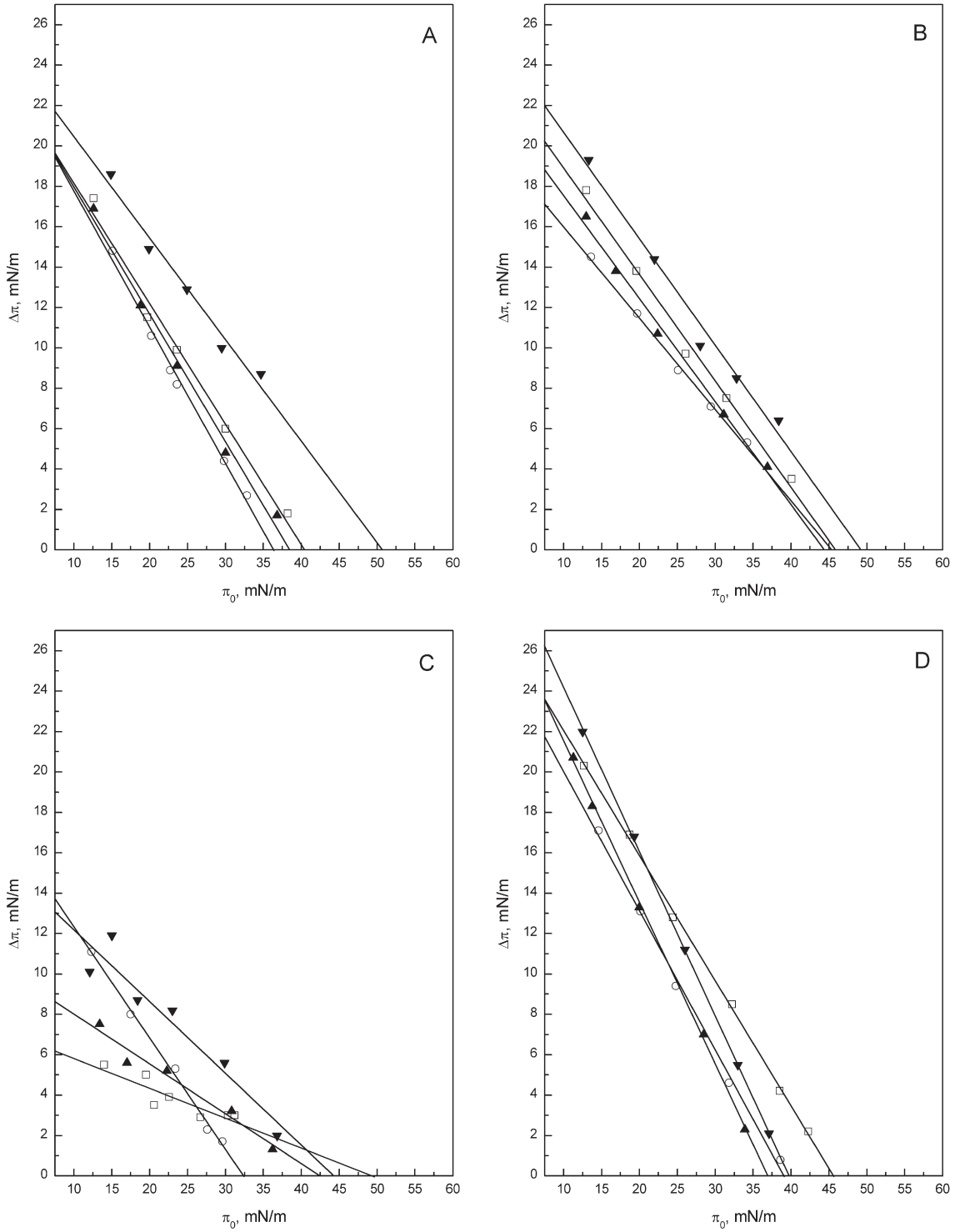

Fig. 2. Penetration of antimicrobial peptides into lipid monolayers residing on buffer with 150 mM NaCl, shown as increment in surface pressure ($\Delta\pi$) following the addition of 0.3 μM temporin B (A), temporin L (B), indolicidin (C), or LL-37(F27W) (D) into the subphase as a function of the initial surface pressure π_0. Monolayer compositions were DMPC (○), DMPC:DMPG=8:2 (□), DMPC:PoxnoPC=8.2 (▼), and DMPC:PazePC=8:2 (▲).

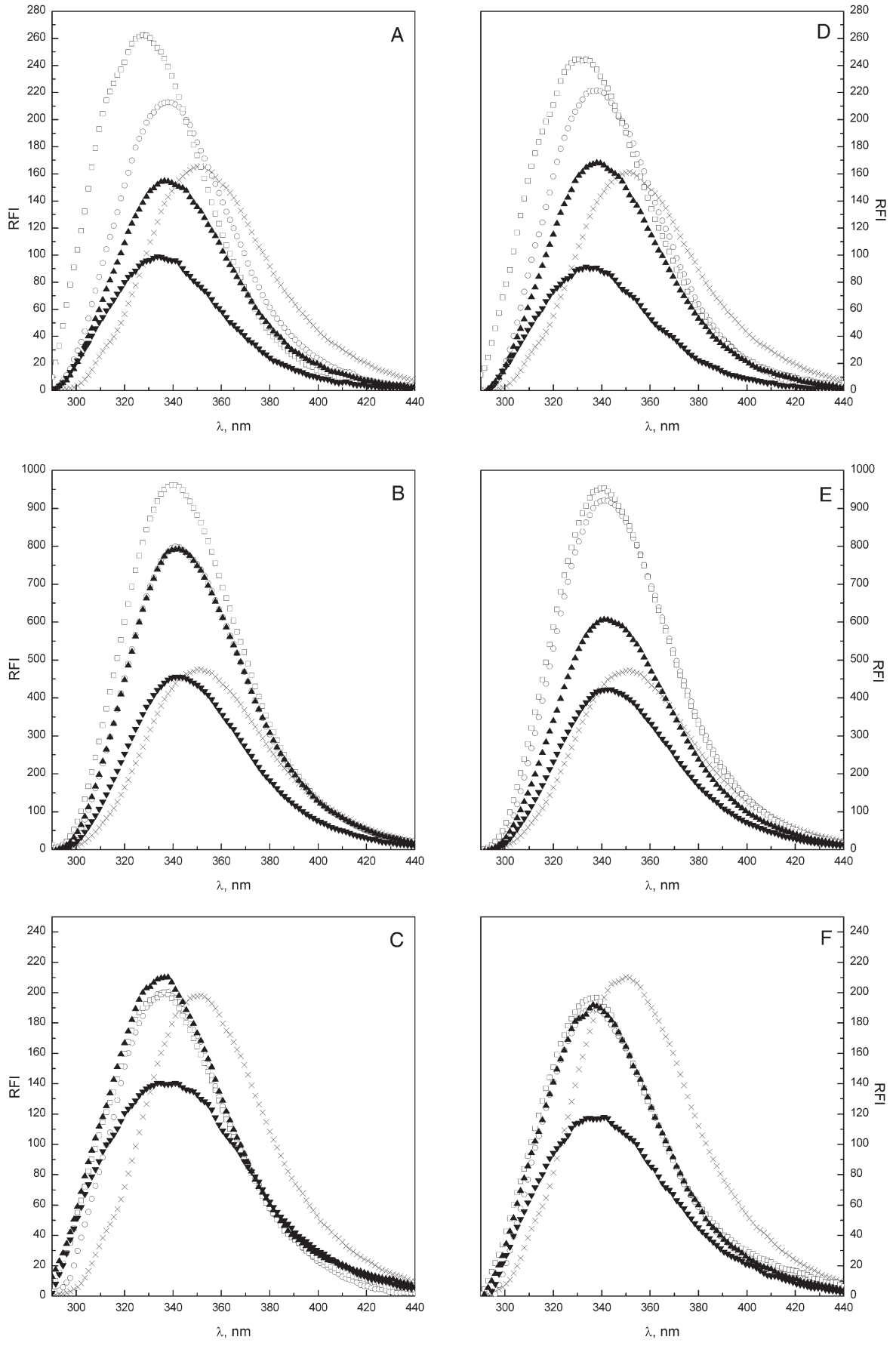

Fig. 3. Tryptophan fluorescence spectra of temporin L (A), indolicidin (B), and LL-37(F27W) (C) in low ionic strength buffer or in buffer with 150 mM NaCl (×) (D, E, and F, respectively) and in the presence of liposomes composed of DMPC (○), DMPC:DMPG=8:2 (□), DMPC:PoxnoPC=8:2 (▼), and DMPC:PazePC=8:2 (▲).

pressure for all four monolayer compositions (Fig. 1A and B, Table 1). Also indolicidin intercalated into the monolayers, albeit somewhat less effectively (Fig. 1C), whereas LL-37(F27W) displayed membrane activity comparable to the temporins. As expected from their positive net charges (+2, +3, +4, and +6 for temporin B, temporin L, indolicidin, and LL-37(F27W), respectively) all four peptides used in the present study interact preferably with monolayers containing acidic phospholipids [13,23]. In keeping with previous studies, highest values of π_c were recorded for films with $X_{DMPG}=0.20$. However, while PazePC contains an ionizable carboxylic function in its *sn*-2 acyl with a net negative charge, only indolicidin displayed augmented penetration into monolayers containing this oxidatively modified phospholipid ($X=0.20$) below surface pressure of ~35 mN/m.

Interestingly and unexpectedly, the intercalation of all peptides into monolayers containing the aldehyde group bearing PoxnoPC was considerably enhanced over neat DMPC, this oxidatively modified phospholipid being almost as effective as DMPG in promoting peptide penetration. With DMPG and PoxnoPC ($X=0.20$) the value of π_c for temporin B increased from ≈38 mN/m measured for neat DMPC up to ≈48 and ≈46 mN/m, respectively (Table 1). For temporin L there was less variation in π_c between different monolayer compositions, with DMPG ($X=0.20$) yielding the highest value of ≈52 mN/m. In the case of indolicidin and LL-37(F27W), incorporation of DMPG into the monolayer resulted in clearly higher values for π_c (≈60 and ≈50 mN/m, respectively) compared to other lipid compositions. Notably, for all four peptides both DMPG and PoxnoPC enhanced considerably the intercalation at low surface pressures as evidenced by values of $\Delta\pi$ at $\pi_0=10$ mN/m (Table 1).

The above experiments were conducted in a low ionic strength medium. Accordingly, it was of interest to study the monolayer penetration using a subphase with physiological saline. Intriguingly, the two temporins and indolicidin intercalated into PoxnoPC containing monolayers as efficiently as from a low ionic strength subphase, and only a minor decrement in π_c was noted for LL-37(F27W). Instead, as expected, their penetration into DMPG containing monolayers decreased due to screening of the electrostatic interactions by salt (Fig. 2). More specifically, in 150 mM NaCl temporin B inserted into neat DMPC monolayers up to π_0≈36 mN/m, while its penetration was lightly augmented by both PazePC and DMPG ($X=0.20$) with the value for π_c increasing up to ≈38 and ≈40 mN/m, respectively. Notably, the intercalation of temporin B into a monolayer containing PoxnoPC was enhanced with π_c increasing up to ≈51 mN/m, a value significantly higher than measured in a low ionic strength medium (≈46 mN/m, Table 1).

In contrast to temporin B, with 150 mM NaCl in the subphase the presence of PazePC or DMPG ($X=0.20$) into the monolayers enhanced the penetration of temporin L only at low surface pressures (≈10–15 mN/m), while the π_c values for these monolayers remained nearly identical (≈45 mN/m). Similarly to temporin B, PoxnoPC promoted the monolayer penetration of temporin L yielding π_c≈49 mN/m. In physiological NaCl the membrane intercalation of indolicidin into neat DMPC monolayers was considerably reduced, with π_c decreasing from 47 to 32 mN/m. However, concomitantly the penetration at lower surface pressures was promoted, exceeding that observed in the presence of DMPG. Also for monolayers containing PazePC the magnitude of penetration was reduced, albeit less than for DMPG. Similarly to temporins B and L, the most effective intercalation of indolicidin was found into monolayers containing PoxnoPC. With the exception of neat DMPC, the introduction of 150 mM NaCl into the subphase led to diminished intercalation of LL-37(F27W) into phospholipid monolayers. Again, highest reduction in π_c was observed for monolayers containing DMPG ($X=0.20$). The penetration of LL-37(F27W) into PoxnoPC containing monolayers was slightly attenuated in the presence of physiological saline. However, as seen for indolicidin, the penetration at lower surface pressure was promoted exceeding that observed for DMPG.

In order to obtain further insight into the interaction of AMPs with these two oxPLs we used the intrinsic Trp fluorescence of temporin L, indolicidin, and LL-37(F27W). To allow the comparison with the above monolayer data the same phospholipids were used and temperature maintained at 25 °C, where the LUVs are above their main transition and in liquid disordered ("fluid") state. Emission spectra of these three peptides were recorded in the absence as well as in the presence of LUVs of different lipid compositions (viz. DMPC as such and with DMPG, PoxnoPC, and PazePC, each at $X=0.20$) both in buffer only and in 150 mM NaCl. In aqueous solution the Trp residue(s) of the peptides emit with a maximum at ~352–354 nm, indicating them to reside in a polar environment [33,34]. A relocation of Trp residues into a less polar environment such as the hydrocarbon phase of lipid membranes causes changes in Trp fluorescence intensity and emission maximum [30,35]. Representative fluorescence spectra of temporin L (Fig. 3) in low ionic strength reveal, upon the addition of DMPC LUVs, a blue shift in λ_{max} by 14 nm (Table 2), together with an increase in the emission intensity, both indicative of association of the peptide with the vesicles. More pronounced increase in both intensity and blue shift (24 nm) were observed following the addition of vesicles containing DMPG, in keeping with previous studies [14]. For LUVs containing PazePC ($X_{PazePC}=0.2$), the blue shift was smaller (15 nm) compared to $X_{DMPG}=0.2$ while the emission intensity was attenuated compared to neat DMPC vesicles. Similar blue shift by 18 nm together with augmented decrement in tryptophan emission was observed also for PoxnoPC ($X_{PoxnoPC}=0.2$, Fig. 2 and Table 2). In physiological saline the emission intensity after the addition of DMPG containing vesicles decreased, in contrast to increase for PazePC and DMPC. Interestingly, the impact of PoxnoPC was identical irrespective of the increase in ionic strength (Fig. 4).

The blue shift for indolicidin was almost completely insensitive to vesicle lipid composition and ionic strength (Figs. 3 and 4). Instead, considerable variation in emission intensity was observed. Both with and without 150 mM NaCl the addition of DMPG containing vesicles resulted in the highest tryptophan emission. The fluorescence intensity in the presence of vesicles containing $X_{PazePC}=0.2$ equaled that measured for neat DMPC LUVs under low ionic strength. In the presence of LUVs with $X_{PoxnoPC}=0.2$, as well as $X_{PazePC}=0.2$ and in physiological saline, Trp emission intensity was lower than for neat DMPC vesicles (Table 2). With the exception of a marked decrease with PoxnoPC containing vesicles, essentially no changes in fluorescence quantum yield were observed following the binding of LL-37(F27W) to liposomes. As for indolicidin, the blue shift of emission

Table 2
Maximum emission wavelengths of Trp fluorescence of temporin L, indolicidin, and LL-37(F27W) (λ_{max}) and its blueshift compared to value recorded in aquous buffer ($\Delta\lambda_{max}$) induced by liposomes of indicated compositions

	LUV composition	λ_{max} (nm)		$\Delta\lambda_{max}$ (nm)		RFI	
		No salt	150 mM NaCl	No salt	150 mM NaCl	No salt	150 mM NaCl
Temporin L	–	352	352	–	–	166	161
	DMPC	338	337	14	15	214	222
	$X_{DMPG}=0.2$	328	332	24	20	263	245
	$X_{PoxnoPC}=0.2$	334	335	18	17	99	91
	$X_{PazePC}=0.2$	337	338	15	14	155	168
Indolicidin	–	352	352	–	–	475	473
	DMPC	341	341	11	11	802	922
	$X_{DMPG}=0.2$	340	340	12	12	960	953
	$X_{PoxnoPC}=0.2$	342	342	10	10	456	420
	$X_{PazePC}=0.2$	341	342	11	10	791	607
LL-37(F27W)	–	352	352	–	–	197	210
	DMPC	337	336	15	16	201	189
	$X_{DMPG}=0.2$	337	336	15	16	201	197
	$X_{PoxnoPC}=0.2$	338	337	14	15	142	118
	$X_{PazePC}=0.2$	337	336	15	16	211	192

Data were taken from Figs. 3 and 4.

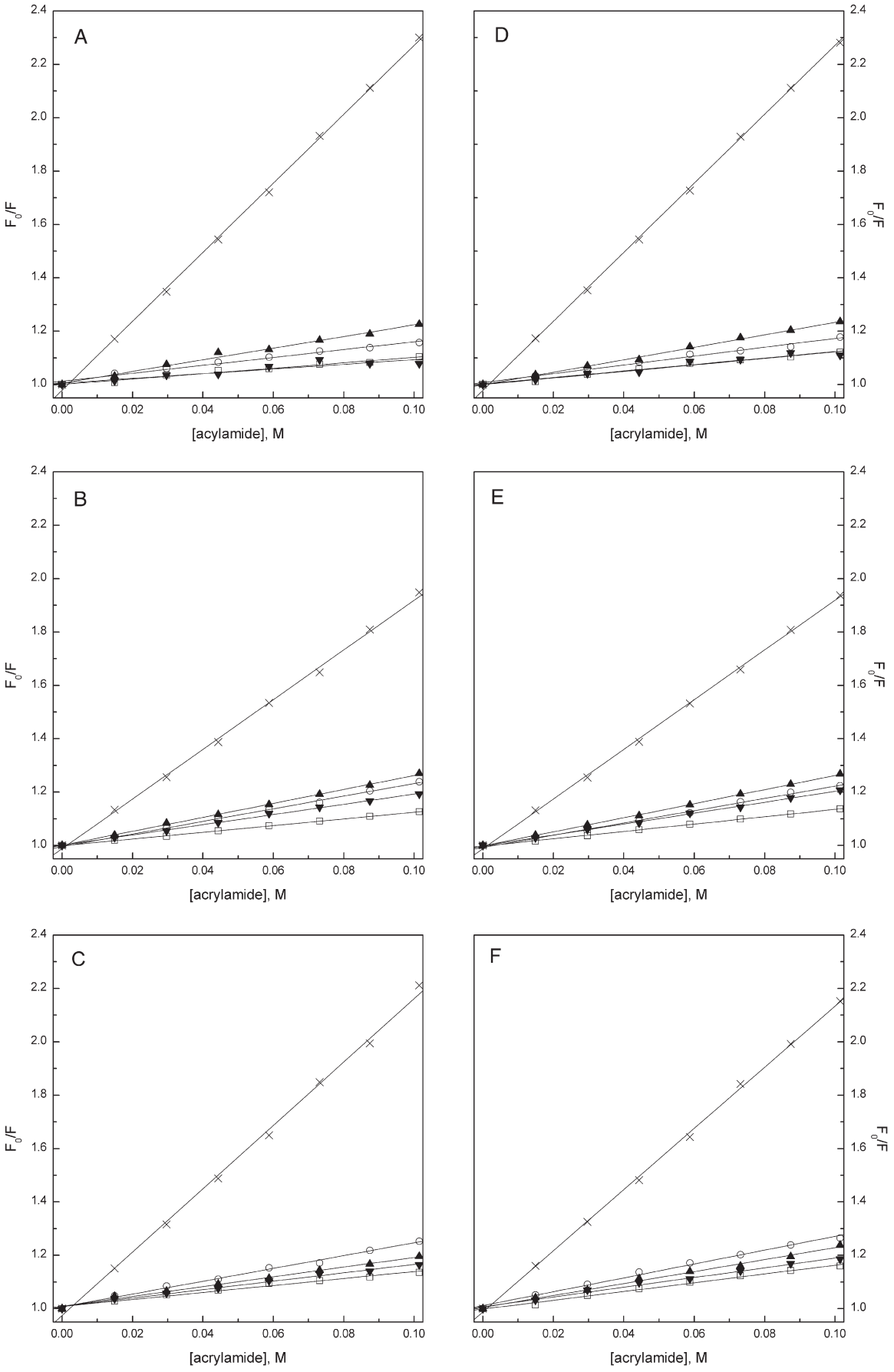

Fig. 4. Stern–Volmer plots for acrylamide quenching of fluorescence of the Trp residues of temporin L (A), indolicidin (B), and LL-37(F27W) (C) in low ionic strength buffer or in buffer with 150 mM NaCl (×) (C, E, and F, respectively) and in the presence of liposomes composed of DMPC (○), DMPC:DMPG=8:2 (□), DMPC:PoxnoPC=8:2 (▼), and DMPC:PazePC=8:2 (▲).

Table 3
The Stern–Volmer quenching constants K_{SV} (M^{-1}) of acrylamide for temporin L, indolicidin, and LL-37(F27W) in the absence and the presence of vesicles of the indicated compositions

		K_{SV} (M^{-1})	
	LUV composition	No salt	150 mM NaCl
Temporin L	–	12.93±0.16	12.90±0.11
	DMPC	1.48±0.08	1.65±0.07
	X_{DMPG}=0.2	1.01±0.06	1.22±0.05
	$X_{PoxnoPC}$=0.2	0.88±0.14	1.22±0.11
	X_{PazePC}=0.2	2.18±0.09	2.34±0.05
Indolicidin	–	9.31±0.15	9.29±0.10
	DMPC	2.37±0.04	2.30±0.06
	X_{DMPG}=0.2	1.25±0.01	1.37±0.02
	$X_{PoxnoPC}$=0.2	1.91±0.03	2.04±0.03
	X_{PazePC}=0.2	2.62±0.03	2.63±0.02
LL-37(F27W)	–	11.87±0.23	11.45±0.14
	DMPC	2.40±0.07	2.59±0.09
	X_{DMPG}=0.2	1.31±0.07	1.64±0.05
	$X_{PoxnoPC}$=0.2	1.58±0.07	1.82±0.07
	X_{PazePC}=0.2	1.83±0.06	2.24±0.07

maximum of this peptide did not vary with ionic strength or liposome composition.

In order to verify the insertion of these three peptides into the hydrophobic region of the bilayers, indicated by the above changes in the Trp emission spectra, quenching by acrylamide was used [31]. Importantly, this quencher is devoid of electrostatic interactions with the headgroups of negatively charged lipids [33] and because of being highly water soluble it provides a measure of the solvent exposure of the Trp residues of the peptides. Stern–Volmer plots of the Trp quenching by acrylamide recorded in the absence and in the presence of vesicles of indicated lipid compositions reveal in all cases a concentration-dependent decrease in fluorescence (Figs. 3 and 4). However, decrease in fluorescence is significantly reduced in the presence of liposomes, demonstrating reduced accessibility of Trp to the quencher, and thus insertion of the peptides into the vesicles.

At low ionic strength the values of K_{SV} for temporin L show a major decrease with neat DMPC vesicles while slightly better access of acrylamide to Trp is seen for LUVs with X_{PazePC}=0.20. Instead, compared to DMPC the values for K_{SV} were further decreased in the presence of DMPG and PoxnoPC containing LUVs. Similar changes in K_{SV} values were evident also for indolicidin and LL-37(F27W) following incubation with liposomes, with the exception of the binding of the latter peptide to PazePC containing vesicles resulting in less efficient quenching compared to neat DMPC LUVs (Table 3). At 150 mM NaCl a general increase in quenching efficiency of Trp fluorescence was observed for all three peptides indicating decreased membrane partitioning and/or relocation of the Trp residue(s) closer to the interface following increase in ionic strength. Again, however, lowest K_{SV} values were measured for liposomes containing DMPG or PoxnoPC (Table 3).

Discussion

AMPs bind avidly to membranes containing acidic phospholipids and, accordingly, their primary targets have been concluded to be the negatively charged lipids, abundant in the outer surface of bacteria [36–38]. Our findings reported here provide further evidence for their mechanisms of action on cells being more complex. Accordingly, our monolayer data demonstrate at physiological ionic strength efficient intercalation of four AMPs into PoxnoPC containing films, up to the critical packing density yielding values for π_c of 40 (LL-37), 45 (indolicidin) and 50 mN/m (temp L, temp B), thus exceeding the equilibrium lateral pressure of ~33 mN/m estimated for biomembranes [39]. Furthermore, the magnitude of surface pressure increase $\Delta\pi$ clearly exceeds for all these peptides the effect observed using the acidic phospholipid, DMPG (Table 1). It was also of interest that binding to the negatively charged oxidized lipid PazePC was significantly weaker than to the zwitterionic PoxnoPC, approaching the insertion of temporins B and L into DMPC.

The above somewhat unprecedented interaction could be confirmed using Trp fluorescence to monitor the binding of temporin L, indolicidin and LL-37(F27W) to liposomes containing PoxnoPC. PoxnoPC is zwitterionic with no net charge. Accordingly, one of the factors promoting its association with AMPs could be efficient hydrogen bonding to the aldehyde moiety. The latter resides in a highly anisotropic membrane environment and at $X_{PoxnoPC}$=0.2 its local content is high. In addition, being contained in a hydrocarbon chain and thus accommodated in a low dielectric milieu the strength of H-bonds formed should be enhanced [40]. Another and more likely possibility is Schiff base formation between the aldehyde group and the primary amines of the peptides, as demonstrated for PoxnoPC and apolipoprotein AI [41]. To asses this we performed monolayer experiments with 1 mM methoxyamine in the subphase (with 150 mM NaCl) as an aldehyde scavenger [42], prior to the injection

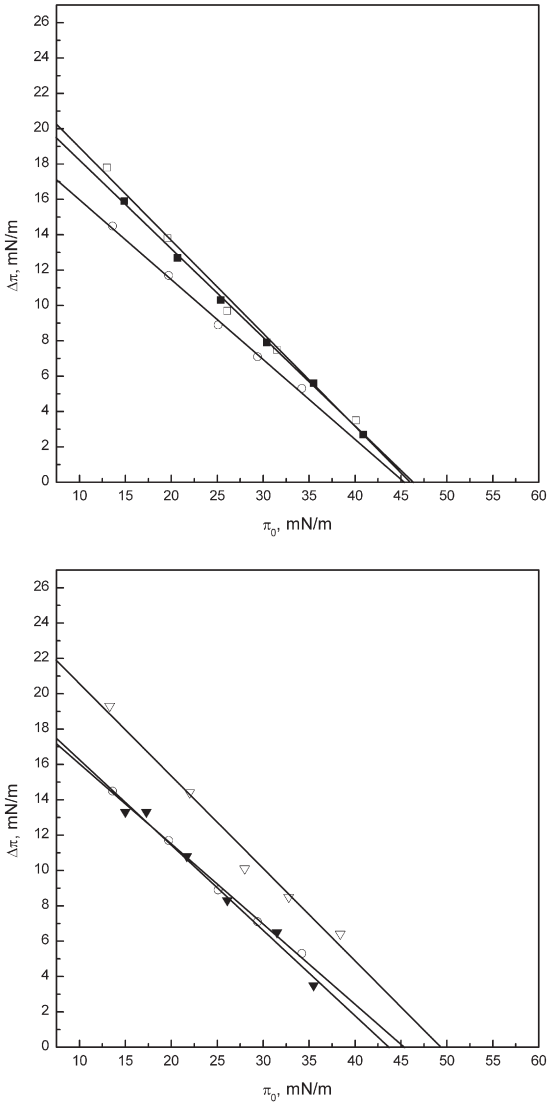

Fig. 5. Penetration of temporin L into lipid monolayers residing on 20 mM Hepes, 0.1 mM EDTA, 150 mM NaCl, pH 7.0 and in the absence (open symbols) or in the presence (closed symbols) of 1 mM methoxyamine, depicted as the increment in surface pressure ($\Delta\pi$) following the addition of 0.3 μM temporin L into the subphase as a function of the initial surface pressure π_0. Monolayer compositions were DMPC (○), DMPC:DMPG=8:2 (□, ■), DMPC:PoxnoPC=8:2 (▽, ▼).

of temporin L. The latter was chosen as it is the most surface active of the four peptides studied. Accordingly, an inhibitory effect should for this peptide override also the nonspecific hydrophobicity driven membrane partitioning. Importantly, the intercalation of temporin L into monolayers containing acidic phospholipid DMPG is unaffected by methoxyamine (Fig. 5). In contrast, for PoxnoPC containing monolayers markedly decreased intercalation of temporin L was observed. Similar results were obtained in a low ionic strength medium (data not shown). Hence, the Schiff base adduct formation between the amino groups of the peptides and the aldehyde function is likely to be involved in the PoxnoPC–peptide interaction, as reported previously for other phospholipid aldehydes [43,44]. Finally, irrespective of the exact molecular level mechanisms involved the cooperative nature of processes in phospholipid membranes can be further anticipated to promote efficient association of AMPs with PoxnoPC.

Trp fluorescence data show that this residue of temporin L becomes in a PoxnoPC containing bilayer accommodated in a hydrophobic milieu, with a 18 nm blue shift in λ_{max}. For comparison the values for $\Delta\lambda_{max}$ in DMPG (X=0.20) and neat DMPC are 24 and 14 nm, respectively (Table 2). Yet, the quantum yield for W4 of temporin L in PoxnoPC is reduced, being ~56% of the value recorded in buffer with 150 mM NaCl in the absence of liposomes. The lower quantum yield could result from H-bonding to the vicinity of W4 or, alternatively, the existence of two populations of W4, one in a hydrophobic milieu and another with a low quantum yield polar environment. The latter mechanism is unlikely. As Trp of the temporin L is poorly accessible to the water soluble quencher, acrylamide, it is feasible that this residue resides within the membrane, most likely in an environment also containing the aldehyde moiety of PoxnoPC. To this end, equal K_{SV} values (1.22) were measured for W4 with PoxnoPC and DMPG containing bilayers, revealing limited access of the water soluble quencher acrylamide to this residue. Quantitatively similar data were obtained by fluorescence spectroscopy for indolicidin. Yet, because of its five Trps and non-helical conformation the discussion of its topology when bound to PoxnoPC containing membrane on the basis of these data alone would be ambiguous. Yet, efficient intercalation of indolicidin into PoxnoPC containing monolayers, particularly at higher packing densities is certainly unexpected and is likely to result from cooperative hydrogen bonding of this peptide to the aldehyde group of the PoxnoPC, similarly to temporins B and L.

The Trp fluorescence data for LL-37(F27W) reveal that upon association with phospholipid bilayers also Trp of this peptide resides in a hydrophobic milieu, with a blue shift in emission maximum of ~15 nm measured for all lipid compositions (Table 2). However, in contrast to the three other peptides only minor changes in fluorescence quantum yield are seen between peptide free in solution and bound to neat DMPC and DMPG or PazePC containing LUVs. A plausible explanation to this is quenching arising from peptide conformational changes upon membrane association bringing the single Trp in closer proximity to one of the charged residues of LL-37 (F27W). Similarly to temporin L and indolicidin decrease in emission intensity in the presence of PoxnoPC containing LUVs was evident for LL-37 (F27W), attributable to direct quenching of Trp by the aldehyde moiety or alternatively self-quenching caused by PoxnoPC promoted aggregation of LL-37(F27W) monomers.

Our results raise several interesting issues. First of all, it is obvious that interactions of peptides and proteins with specific oxPL structures should be studied in detail, in particular as elevated levels of these lipids are found in pathological conditions such as infection, inflammation, and cancer [45–47]. Phosphatidylcholines bearing a *cis*-9 double bond represent a major fraction in all biomembranes and yield PoxnoPC and PazePC upon peroxidation. Accordingly, while no quantitative data are available on the amounts of these oxPLs in biomembrane following ROS attack, it seems likely that their contents in membranes are significant. These oxPLs are also found in oxidized low-density lipoprotein (oxLDL), and are suggested to be involved in the development of atherosclerosis [48–50]. Koppaka and Axelsen have demonstrated the paradigm for amyloid-forming peptides, Aβ, to associate with oxidatively damaged PCs [51,52]. Our own preliminary results also show the human islet amyloid polypeptide, IAPP to bind to oxidized PCs (Domanov, Y. A., Mattila, J.-P., Varis, T., Sabatini, K., and Kinnunen, P. K. J., unpublished data). It is tempting to speculate that secondary products of phospholipid oxidation may represent novel molecular targets mediating the cytotoxic effects of peptides and proteins forming amyloid-type fibrils.

The possible contribution of ROS to bacterial killing has remained unclear. Neutrophils represent an essential component in resolution of acute microbial infections. Following phagocytosis of bacteria, neutrophil granules containing among others antimicrobial proteins and peptides such as lysozyme and defensins, fuse with the formed phagosome exposing the engulfed bacteria to their content. Concomitantly O_2^- generated by NADPH oxidase residing in the phagosomal membrane is converted into hydrogen peroxide by superoxide dismutase inside the phagosome [53]. It has been suggested that O_2^- generation is required to release and activate certain cationic proteases inside the phagosome [54]. In the light of the present results it seems possible that ROS and the action of AMPs could be interrelated, with ROS induced lipid oxidation in phagolysosome generating high affinity AMP binding sites on the bacterial membrane. Studies exploring these issues are currently in progress in our laboratory.

Acknowledgments

The authors wish to thank Drs. Yegor Domanov and Juha-Matti Alakoskela for critical reading of the manuscript. The expert technical assistance of Kristiina Söderholm is appreciated. HBBG is supported by Finnish Academy, Marie Curie Training Network, and Sigrid Jusèlius Foundation.

References

[1] T. Ganz, R.I. Lehrer, Antimicrobial peptides of vertebrates, Curr. Opin. Immunol. 10 (1998) 41–44.
[2] R.E. Hancock, M.G. Scott, The role of antimicrobial peptides in animal defences, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 8856–8861.
[3] M. Zasloff, Antimicrobial peptides of multicellular organisms, Nature 415 (2002) 389–395.
[4] http://aps.unmc.edu/AP/main.php.
[5] J. He, R. Eckert, T. Pharm, M.D. Simanian, C. Hu, D.K. Yarbrough, F. Qi, M.H. Anderson, W. Shi, Novel synthetic antimicrobial peptides against *Streptococcus mutans*, Antimicrob. Agents Chemother. 51 (2007) 1351–1358.
[6] R.E. Hancock, R. Lehrer, Cationic peptides: a new source of antibiotics, Trends. Biotechnol. 16 (1998) 82–88.
[7] B. Bechinger, K. Lohner, Detergent-like actions of linear amphipathic cationic antimicrobial peptides, Biochim. Biophys. Acta 1758 (2006) 1529–1539.
[8] H. Zhao, A. Jutila, T. Nurminen, S.A. Wickström, J. Keski-Oja, P.K.J. Kinnunen, Binding of endostatin to phosphatidylserine-containing membranes and formation of amyloid-like fibers, Biochemistry 44 (2005) 2857–2863.
[9] H. Zhao, R. Sood, A. Jutila, S. Bose, G. Fimland, J. Nissen-Meyer, P.K.J. Kinnunen, Interaction of the antimicrobial peptide pheromone Plantaricin A with model membranes: implications for a novel mechanism of action, Biochim. Biophys. Acta 1758 (2006) 1461–1474.
[10] D.M. Bowdish, D.J. Davidson, R.E. Hancock, A re-evaluation of the role of host defence peptides in mammalian immunity, Curr. Prot. Peptide Sci. 6 (2005) 35–51.
[11] N. Mookherjee, R.E. Hancock, Cationic host defence peptides: innate immune regulatory peptides as a novel approach for treating infections, Cell. Mol. Life Sci. 64 (2007) 922–933.
[12] R. Sood, Y. Domanov, M. Pietiäinen, V.P. Kontinen, P.K.J. Kinnunen, Binding of LL-37 to model biomembranes: insight into target vs host cell recognition, Biochim. Biophys. Acta 1778 (2008) 983–996.
[13] H. Zhao, A.C. Rinaldi, A. Di Giulio, M. Simmaco, P.K.J. Kinnunen, Interaction of the antimicrobial peptides temporins with model biomembranes. Comparison of Temporins B and L, Biochemistry 41 (2002) 4425–4436.
[14] H. Zhao, P.K.J. Kinnunen, Binding of the antimicrobial peptide temporin L to liposomes assessed by Trp fluorescence, J. Biol. Chem. 277 (2002) 25170–25177.
[15] M.L. Mangoni, A.C. Rinaldi, A. Di Giulio, G. Mignogna, A. Bozzi, D. Barra, M. Simmaco, Structure–function relationships of temporins, small antimicrobial peptides from amphibian skin, Eur. J. Biochem. 267 (2000) 1447–1454.
[16] A. Rozek, C.L. Friedrich, R.E. Hancock, Structure of the bovine antimicrobial peptide indolicidin bound to dodecylphosphocholine and sodium dodecyl sulfate micelles, Biochemistry 39 (2000) 15765–15774.
[17] J. Malm, O. Sorensen, T. Persson, M. Frohm-Nilsson, B. Johansson, A. Bjartell, H. Lilja, M. Stahle-Backdahl, N. Borregaard, A. Egesten, The human cationic antimicrobial

protein (hCAP-18) is expressed in the epithelium of human epididymis, is present in seminal plasma at high concentrations, and is attached to spermatozoa, Infect. Immun. 683 (2000) 4297–4302.
[18] K. Hase, L. Eckmann, J.D. Leopard, N. Varki, M.F. Kagnoff, Cell differentiation is a key determinant of cathelicidin LL-37/human cationic antimicrobial protein 18 expression by human colon epithelium, Infect. Immun. 70 (2002) 953–963.
[19] D.M. Bowdish, D.J. Davidson, Y.E. Lau, K. Lee, M.G. Scott, R.E. Hancock, Impact of LL-37 on anti-infective immunity, J. Leukoc. Biol. 77 (2005) 451–459.
[20] Z. Oren, J.C. Lerman, G.H. Gudmundsson, B. Agerberth, Y. Shai, Structure and organization of the human antimicrobial peptide LL-37 in phospholipid membranes: relevance to the molecular basis for its non-cell-selective activity, Biochem. J. 341 (1999) 501–513.
[21] K.A. Henzler Wildman, D.K. Lee, A. Ramamoorthy, Mechanism of lipid bilayer disruption by the human antimicrobial peptide, LL-37, Biochemistry 42 (2003) 6545–6558.
[22] M.R. Yeaman, N.Y. Yount, Mechanisms of antimicrobial peptide action and resistance, Pharmacol. Rev. 55 (2003) 27–55.
[23] H. Zhao, J.-P. Mattila, J.M. Holopainen, P.K.J. Kinnunen, Comparison of the membrane association of two antimicrobial peptides, magainin 2 and indolicidin, Biophys. J. 81 (2001) 2979–2991.
[24] G.O. Fruhwirth, A. Loidl, A. Hermetter, Oxidized phospholipids: from molecular properties to disease, Biochim. Biophys. Acta 1772 (2007) 718–736.
[25] K. Sabatini, J.-P. Mattila, F.M. Megli, P.K.J. Kinnunen, Characterization of two oxidatively modified phospholipids in mixed monolayers with DPPC, Biophys. J. 90 (2006) 4488–4499.
[26] R. Verger, F. Pattus, Spreading of membranes at the air/water interface, Chem. Phys. Lipids 16 (1976) 285–291.
[27] H. Brockman, Lipid monolayers: why to use half of a membrane to characterize protein–membrane interactions? Curr. Opin. Struck. Biol. 9 (1999) 438–443.
[28] N. Berger, A. Sachse, J. Bender, R. Schubert, M. Brandl, Filter extrusion of liposomes using different devices: comparison of liposome size, encapsulation efficiency, and process characteristics, Int. J. Pharm. 223 (2001) 55–68.
[29] J.-P. Mattila, K. Sabatini, P.K.J. Kinnunen, Oxidized phospholipids as potential novel drug targets, Biophys. J. 93 (2007) 3105–3112.
[30] A.I.P.M. De Kroon, M.W. Soekarjo, J. De Gier, B. De Kruijff, The role of charge and hydrophobicity in peptide–lipid interaction: a comparative study based on Tryptophan fluorescence measurements combined with the use of aqueous and hydrophobic quenchers, Biochemistry 29 (1990) 8229–8240.
[31] M.R. Eftink, C.A. Ghiron, Fluorescence quenching of indole and model micelle systems, J. Phys. Chem 80 (1976) 486–493.
[32] M.T. Lamy-Freund, K.A. Riske, The peculiar thermo-structural behavior of the anionic lipid DMPG, Chem. Phys. Lipids 122 (2003) 19–32.
[33] E. Breukink, C. van Kraaij, A. van Dalen, R.A. Demel, R.J. Siezen, B. de Kruijff, O.P. Kuipers, The orientation of nisin in membrane, Biochemistry 37 (1998) 8153–8162.
[34] W.K. Surewicz, R.M. Epand, Role of peptide in lipid–peptide interactions: a fluorescence study of the binding of pentagastrin-related pentapeptides to phospholipid vesicles, Biochemistry 23 (1984) 6072–6077.
[35] R.W. Cowgill, Fluorescence and protein structure. X. Reappraisal of solvent and structural effects, Biochim. Biophys. Acta 133 (1967) 6–18.
[36] Y. Shai, Mode of action of membrane active antimicrobial peptides, Biopolymers 66 (2002) 236–248.
[37] Y. Shai, Mechanism of the binding, insertion and destabilization of phospholipids bilayer membranes by α-helical antimicrobial and cell non-selective membrane-lytic peptides, Biochim. Biophys. Acta 1462 (1999) 55–70.
[38] A. Tossi, L. Sandri, A. Giangaspero, Amphipathic, alpha-helical antimicrobial peptides, Biopolymers 55 (2000) 4–30.
[39] R.A. Demel, W.S.G. van Kessel, R.F. Zwaal, B. Roelofsen, L.L. van Deenen, Relation between various phospholipase actions on human red cell membranes and the interfacial phospholipid pressure in monolayers, Biochim. Biophys. Acta 406 (1975) 97–107.
[40] S.O. Shan, S. Loh, D. Herschlag, The energetics of hydrogen bonds in model systems: implications for enzymatic catalysis, Science 272 (1996) 97–101.
[41] Z. Ahmed, A. Ravandi, G.F. Maguire, A. Kuksis, P.W. Connelly, Formation of apolipoprotein AI-phosphatidylcholine core aldehyde Schiff base adducts promotes uptake by THP-1 macrophages, Cardiovasc. Res. Jun. 58 (2003) 712–720.
[42] J. Bieschke, Q. Zhang, D.A. Bosco, R.A. Lerner, E.T. Powers, P. Wentworth Jr., J.W. Kelly, Small molecule oxidation products trigger disease-associated protein misfolding, Acc. Chem. Res. 39 (2006) 611–619.
[43] U.P. Steinbrecher, Oxidation of human low density lipoprotein results in derivatization of lysine residues of Apolipoprotein B by lipid peroxide decomposition products, J. Biol. Chem. 262 (1987) 3603–3608.
[44] J.-P. Kurvinen, A. Kuksis, A. Ravandi, O. Sjövall, H. Kallio, Rapid complexing of oxoacylglycerols with amino acids, peptides and aminophospholipids, Lipids 34 (1999) 299–305.
[45] B. Halliwell, J.M. Gutteridge, Role of free radicals and catalytic metal ions in human disease: an overview, Methods. Enzymol 186 (1990) 1–85.
[46] M. Gago-Dominguez, J.E. Castelao, J.M. Yuan, R.K. Ross, M.C. Yu, Lipid peroxidation: a novel and unifying concept of the etiology of renal cell carcinoma (United States), Cancer Causes Control 13 (2002) 287–293.
[47] P. Cejas, E. Casado, C. Belda-Iniesta, J. De Castro, E. Espinosa, A. Redondo, M. Sereno, M.A. Garcia-Cabezas, J.A.F. Vara, A. Domınguez-Caceres, R. Perona, M. Gonzalez-Baron, Implication of oxidative stress and cell membrane lipid peroxidation in human cancer (Spain), Cancer Causes Control 15 (2004) 707–719.
[48] J.A. Berliner, J.W. Heinecke, The role of oxidized lipoproteins in atherogenesis, Free Radic. Biol. Med 20 (1996) 707–727.
[49] S.S. Davies, A.V. Pontsler, G.K. Marathe, K.A. Harrison, R.C. Murphy, J.C. Hinshaw, G.D. Prestwich, A.S. Hilaire, S.M. Prescott, G.A. Zimmerman, T.M. McIntyre, Oxidized alkyl phospholipids are specific, high affinity peroxisome proliferator-activated receptor gamma ligands and agonists, J. Biol. Chem. 276 (2001) 16015–16023.
[50] Z. Ahmed, A. Ravandi, G.F. Maguire, A. Emili, D. Draganov, B.N. La Du, A. Kuksis, P.W. Connelly, Apolipoprotein A-I promotes the formation of phosphatidylcholine core aldehydes that are hydrolyzed by paraoxonase (PON-1) during high density lipoprotein oxidation with a peroxynitrate donor, J. Biol. Chem. 276 (2001) 24473–24481.
[51] V. Koppaka, C. Paul, I.V. Murray, P.H. Axelsen, Early synergy between Abeta42 and oxidatively damaged membranes in promoting amyloid fibril formation by Abeta40, J. Biol. Chem. 278 (2003) 36277–36284.
[52] V. Koppaka, P.H. Axelsen, Accelerated accumulation of amyloid beta proteins on oxidatively damaged lipid membranes, Biochemistry 32 (2000) 10011–10016.
[53] A. Mayer-Scholl, P. Averhoff, A. Zychlinsky, How do neutrophils and pathogens interact? Curr. Opin. Microbiol. 7 (2004) 62–66.
[54] D. Roos, C.C. Winterbourn, Immunology. Lethal weapons, Science 296 (2002) 669–671.