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The effect of a membrane potential on the interaction of mastoparan X, a mitochondrial presequence, and several regulatory peptides with phospholipid vesicles

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Recently the pH gradient evoked by a K^+ diffusion potential was shown to translocate a synthetic monobasic amphipathic hexapeptide across the bilayer of lipid vesicles (De Kroon, A.I.P.M., Vogt, B., Van 't Hof, R., De Kruijff, B. and De Gier, J. (1991) Biophys. J. 60, in press). Here this observation is extended by studying the effect of a membrane potential on a set of bioactive peptides. The panel of peptides comprises the toxin mastoparan X, a tryptophan-containing analogue of the presequence of the mitochondrial protein cytochrome oxidase subunit IV (preCoxIV(1–25)W^{18}), and the regulatory peptides ACTH(1–24), α-MSH, ACTH(1–10), dynorphin A, bombesin, and LHRH. The interaction of these peptides with phospholipid vesicles has been measured using the intrinsic tryptophan residue as fluorescent probe. In the absence of a K^+ diffusion potential only mastoparan X and the presequence show considerable binding to vesicles consisting of phosphatidylcholine (PC). In contrast, under these conditions all peptides display affinity for vesicles consisting of the acidic phospholipid cardiolipin (CL), the extent of which depends on the net positive charge of the peptide. Application of a K^+ diffusion potential to large unilamellar vesicles (LUV) consisting of PC results in a time dependent tryptophan fluorescence increase for mastoparan X, which is accelerated upon incorporating increasing amounts of CL into the LUV. A similar fluorescence increase in response to a K^+ diffusion potential was observed for the above model peptide. Yet the mechanism resulting in the fluorescence increase of mastoparan X is completely different from that of the hexapeptide. Binding experiments indicate that a membrane potential-induced enhanced binding of the peptide to the outer surface of the vesicles contributes to the fluorescence increase. PreCoxIV(1–25)W^{18}, dynorphin A, and ACTH(1–24) show fluorescence responses upon applying a membrane potential that are consistent with that of mastoparan X, whereas the other peptides tested do not respond up to a LUV CL content of 50%. The results tentatively suggest that the membrane potential only affects a peptide when it has the ability to adopt a stable membrane bound conformation.

Introduction

The interaction of a peptide with the lipid phase of biological membranes is thought to precede and to facilitate the implementation of its physiological action in a number of biological processes. Factors that determine this interaction are the membrane surface charge which depends on the membrane lipid composition, the peptide's charge and hydrophobicity, and possibly also the membrane potential that exists across most biomembranes. In the present study the effect of a

Abbreviations: CL, bovine heart cardiolipin; diS-C_2-(5), 3,3'-diethylthiadicarbocyanine iodide; DNS-PE, *N*-(5-dimethylamlnonaphthalene-1-sulfonyl)-dipalmitoylphosphatidylethanolamine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; naf, normalized accessibility factor; PC, (egg yolk) phosphatidylcholine; R_i, lipid/peptide molar ratio; RET, resonance energy transfer; TPP^+, tetraphenylphosphonium ion.

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membrane potential on the interaction of a number of representative biologically active peptides with lipid vesicles of different phospholipid compositions, has been investigated.

Mastoparan is a toxic tetradecapeptide isolated from wasp venom that stimulates exocytosis from several mammalian cells, e.g. of histamine from mast cells [1], serotonin from platelets [2] and insulin from pancreatic islets [3] via a mechanism that involves activation of GTP binding regulatory proteins (G-proteins). Mastoparan has a strong membrane affinity and adopts an amphipathic a-helix conformation when membrane bound [4,5]. This conformation has been proposed to be a prerequisite for a direct interaction with the G-proteins [6,7]. The membrane potential has been suggested to transfer mastoparan across the plasma membrane to the site of the G-proteins [6]. Other toxic activities of mastoparan related to its tendency to assume an α-helical configuration, are the inhibition of calmodulin via peptide–protein interaction [8], the activation of phospholipase A_2 [9] and the enhancement of mitochondrial respiration [10] via interaction with membrane lipids.

The import into mitochondria of most mitochondrial proteins after their synthesis in the cytosol, requires the presence of a transient N-terminal extension, the presequence, which is recognized by a proteinaceous receptor system (for review see Ref. 11). Like mastoparan these presequences have the ability to form an amphipathic α-helix [12], a structure which renders them ideally suited for interaction with the membrane lipids [13–15]. For the import of most precursor proteins the presence of a membrane potential across the mitochondrial inner membrane is essential. This opens up the possibility that interaction of the presequence with the membrane lipids involving the membrane potential, plays a role at some stage of the mitochondrial protein import process.

The membrane lipid phase has been proposed to catalyze the binding of regulatory peptides such as neuropeptides and hormones to their receptor [16,17]. According to this mechanism the membrane would provide a matrix for fast two-dimensional diffusion of these amphiphilic peptides. In addition, adsorption to a membrane/water interface may modulate the peptides' conformation and affect its orientation, thus further facilitating a productive interaction with the appropriate receptor [18,19]. It is obvious that a membrane potential may influence these interactions.

The main objective of this paper is to investigate the effect of a membrane potential on the interaction of phospholipid vesicles with peptides that are representative for these classes of bioactive peptides. First, the affinity of the peptides for vesicles consisting of zwitterionic and acidic phospholipids is characterized in measurements based on tryptophan fluorescence. Tryptophan fluorescence quenching experiments using brominated phospholipids provide information on the membrane topology of the peptides. Consequently the choice of the peptides studied was also determined by the presence of a tryptophan residue. Next, a valinomycin-induced K^+ diffusion potential is applied to large unilamellar phospholipid vesicles in order to explore the influence of a transmembrane potential (negative inside) on the membrane interaction of these positively charged peptides. Previously, similar studies performed on synthetic model peptides [20,21], revealed that the pH gradient evoked by the K^+ diffusion potential drives the uptake into vesicles of hydrophobic peptides containing a single ionizable group. A model peptide containing more than one charged moiety was not accumulated inside the vesicles, but showed behaviour consistent with a slightly enhanced vesicle binding in response to the potential, which was independent of the pH gradient. The results obtained for the bioactive peptides will be discussed in the light of these model peptide data.

Mastoparan X (Table I) is a natural analogue of

TABLE I

Amino acid sequence and net charge of the peptides

Peptide	Amino acid sequence [a]	Net charge [b]
Mastoparan X	H_3N^+ I N W K$^+$ G I A A M A K$^+$ K$^+$ L L *CO NH$_2$*	4+
preCoxIV(1–25)W^{18}	H_3N^+ M L S L R$^+$ Q S I R$^+$ F F K$^+$ P A T R$^+$ T W C S S R$^+$ Y L L *COO$^-$*	5+
ACTH(1–24)	H_3N^+ S Y S M E$^-$ H$^+$ F R$^+$ W G K$^+$ P V G K$^+$ K$^+$ R$^+$ R$^+$ P V K$^+$ V Y P *COO$^-$*	6+/7+
α-MSH	*N-Ac* S Y S M E$^-$ H$^+$ F R$^+$ W G K$^+$ P V *CO NH$_2$*	1+/2+
ACTH(1–10)	H_3N^+ S Y S M E$^-$ H$^+$ F R$^+$ W G *COO$^-$*	0/1+
Dynorphin A	H_3N^+ Y G G F L R$^+$ R$^+$ I R$^+$ P K$^+$ L K$^+$ W D$^-$ N Q *COO$^-$*	4+
Bombesin	Py Q R$^+$ L G N Q W A V G H$^+$ L M *CO NH$_2$*	1+/2+
LHRH	Py H$^+$ W S Y G L R$^+$ P G *CO NH$_2$*	1+/2+

[a] The one letter code for amino acids is used, Py denotes pyroglutamic acid; the positions of the charged residues are indicated; the tryptophan (W) residues are underlined.

[b] For peptides containing His (H) two values of the net charge are given in view of the pK_a value of 6.5 of the His side chain.

mastoparan containing a tryptophan residue [1] and has similar properties [4,7,8]. The presequence of cytochrome oxidase subunit IV (preCoxIV(1–25)) has been synthesized with tryptophan replacing the leucine residue at position 18 which is localized at the hydrophobic side of the putative amphipathic α-helix [13]. The effects exerted on mitochondrial respiration and membrane potential, and the inhibition of the import of the precursor of cytochrome oxidase subunit IV are similar for preCoxIV(1–25) and its tryptophan containing analogue preCoxIV(1–25)W^{18} (Van Heerde, W. and Nicolay, K., unpublished results). Dynorphin A is a neuropeptide belonging to the group of the opioid peptides. Based on correlations between pharmacological activity and data concerning the interaction with phospholipid bilayers, Schwyzer [22] has proposed that the selection of the proper receptor subtype by the opioid peptides is mediated by the membrane lipid phase. Furthermore two analogues of the hormone and neuropeptide adrenocorticotropin, ACTH(1–24) and ACTH(1–10), and the structurally related hormone α-melanotropin, α-MSH (see Table I), for which a similar receptor selection mechanism has been proposed [23] are included in this study. Bombesin and luteinizing hormone releasing hormone (LHRH) are other tryptophan-containing regulatory peptides of which the interaction with the membrane lipid phase may catalyze receptor binding [24–27].

Materials and Methods

Phospholipids

Egg-yolk phosphatidylcholine (PC) and bovine heart cardiolipin (CL) were isolated and purified as described [28,29]. Dioleoylphosphatidylcholine (DOPC) was synthesized as described [30] and converted to dioleoylphosphatidylglycerol (DOPG) by phospholipase D mediated base exchange [31]. The synthesis and characterization of the brominated phosphatidylcholines (BrPC), 1-palmitoyl-2-(2-bromohexadecanoyl) phosphatidylcholine (2-BrPC) and 1-palmitoyl-2-(*n*,*n* + 1-dibromostearoyl)phosphatidylcholine (6,7-Br_2PC, 9,10-Br_2PC, and 11,12-Br_2PC) has been described [20]. *N*-(5-Dimethylaminonaphthalene-1-sulfonyl)dipalmitoylphosphatidylethanolamine (DNS-PE) was obtained from Molecular Probes (Eugene, OR).

Peptides

Mastoparan, mastoparan X, ACTH(1–24), α-MSH, ACTH(1–10), dynorphin A, bombesin, and LHRH were purchased from Bissendorf Biochemicals GmbH (Hannover, F.R.G.). The presequence of yeast cytochrome oxidase subunit IV (preCoxIV(1–25) and its tryptophan-containing analogue (preCoxIV(1–25)W^{18}) were prepared by solid phase synthesis by Mr. D. Olshevski (University of California, San Diego) and purified by reverse-phase HPLC on a Nucleosil C4 column (Macherey-Nagel, Düren, F.R.G.) eluted with linear water-acetonitrile gradients containing 0.1% (v/v) TFA. The identity of these peptides was confirmed by analysis of the amino acid composition. Both peptides were at least 90% pure as judged from the HPLC profiles.

Peptide stock solutions (approx. 1 mM) were prepared in water and stored at −20°C in aliquots which were used only once. The concentration of the tryptophan containing peptides was determined using $\epsilon^{280} = 5600\ M^{-1}\ cm^{-1}$. The concentration of mastoparan and preCoxIV(1–25) were determined by the BCA protein assay (Pierce, Rockford, IL) using known amounts of mastoparan X and preCoxIV(1–25)W^{18} as standard, respectively.

Vesicle preparation and the generation of ion gradients

Small unilamellar vesicles (SUV) were prepared by hydrating a dry lipid film in a buffer containing 100 mM NaCl, 10 mM Tris-HCl (pH 7.5), 2 mM EDTA, followed by sonication as described [20].

Ion gradients were applied to large unilamellar vesicles (LUV) or multilamellar vesicles (MLV). The LUV were prepared in 150 mM K_2SO_4, 20 mM Hepes (pH 7.0) 2 mM EDTA by an extrusion technique using 400 nm polycarbonate filters [21,32]. LUV were passed through a Sephadex G-50 column eluted with 150 mM Na_2SO_4, 20 mM Hepes (pH 7.0) 2 mM EDTA in order to generate a K^+_{in}/Na^+_{out} gradient. PC MLV were prepared by hydration of a dry lipidfilm in 100 mM K_2SO_4, 20 mM Hepes (pH 7.0). In this case the ion gradient was applied by resuspending the MLV in 100 mM Na_2SO_4, 20 mM Hepes (pH 7.0) after centrifugation for 30 min at $37000 \times g$. This procedure was repeated to remove residual external K^+ ions.

Vesicle concentrations were determined by phosphorus analysis [33]. Concentrations and amounts of CL are always expressed on phosphorus basis.

Determination of peptide phospholipid vesicle interaction

The 'spontaneous' interaction of the peptides with vesicles consisting of either zwitterionic or acidic phospholipid, was characterized by measuring changes in emission intensity and in the wavelength of maximum emission (λ_{max}) of the peptides' intrinsic tryptophan in SUV titration experiments, and by determining the accessibility of the tryptophan residue to aqueous and hydrophobic quenchers. The effect of a K^+ diffusion potential on the peptide membrane interaction was investigated using LUV (or MLV) since these model membrane systems are able to maintain stable ion gradients over a longer period of time [32].

Peptide–SUV interaction. Tryptophan fluorescence emission spectra were recorded on an SLM-Aminco SPF-500C fluorimeter using an excitation wavelength

of 280 nm with a band width of 5 nm and an emission band width of 7.5 nm. For peptides containing (a) tyrosine residue(s) an excitation wavelength of 295 nm (band width 5 nm) was applied to minimize contributions from tyrosine fluorescence; in this case the emission band width was set at 10 nm. Small aliquots of concentrated SUV suspensions were successively added to 3 μM peptide in 100 mM NaCl, 10 mM Tris-HCl (pH 7.5), 2 mM EDTA, under continuous stirring at room temperature. After 10 min of equilibration an emission spectrum was recorded and the fluorescence intensity at 340 nm read. Spectra were corrected for a vesicle blank before determining λ_{max}. Fluorescence intensities were corrected for the vesicle blank, for the dilution due to SUV addition, and for the inner filter effect as described [20], and are expressed as F/F_o with F the corrected fluorescence intensity measured in the presence, and F_o the fluorescence intensity measured in the absence of SUV.

In titrations of mastoparan X and preCoxIV(1–25)W[18] with CL or DOPG SUV, respectively, vesicle aggregation was apparent from an increased absorbance at 295 nm, which interfered with the measurement of fluorescence intensities. The absorbance increased up to the lipid/peptide molar ratio corresponding to charge stoichiometry ($R_i \approx 4$–5), beyond which it decreased and stabilized at a level higher than that of the vesicle blank. The fluorescence data obtained in these titrations were corrected using data derived from SUV titrations of mastoparan and preCoxIV(1–25), respectively, both of which do not contain tryptophan and induce similar extents of aggregation, as was established in absorbance measurements (data not shown). Titrations of ACTH(1–24) with CL SUV revealed an increased vesicle absorbance as compared to the blank up to a R_i of 6–7, beyond which the absorbance immediately returned to the level of the vesicle blank (data not shown). The other peptides did not affect the absorbance of the SUV. Throughout all SUV titration experiments the contribution of scattering to the fluorescence signal never exceeded 15% of the total signal.

The change in fluorescence intensity (F/F_o) was used to determine the affinity parameter $K_d \cdot n$ where K_d represents the apparent dissociation constant and n the number of lipid molecules constituting one peptide binding side, as has been described [20,34]. When the obtained F/F_o values after application of the above corrections did not allow a reliable $K_d \cdot n$ assessment due to the occurrence of vesicle aggregation, $K_d \cdot n$ was determined from the changes in λ_{max} as described [34].

An additional method to assess the relative affinity of the peptides for the phospholipid vesicles is the determination of the peptides' accessibility to the aqueous quenchers of tryptophan fluorescence, I^- and acrylamide, in the absence and presence of SUV [20,35,36]. Quenching data were analyzed according to the Stern-Volmer equation for collisional quenching ($F_o/F = 1 + K_{SV}[Q]$), yielding K_{SV}- and K_q-values for titrations with KI and acrylamide, respectively, as described previously [20]. Briefly, K_q values derive from a linear fit according to the Stern-Volmer equation up to an acrylamide concentration of 60 mM, disregarding the contribution of static quenching which causes a deviation from linearity that becomes apparent at higher concentrations of acrylamide [36]. Since K_{SV} and K_q are different for each peptide the normalized accessibility factor (naf), which is defined as the quotient of the quenching constant in the presence and that in the absence of SUV [20], was applied to enable comparison of the different peptides' residual accessibility to the quenchers in the presence of SUV.

Quenching by brominated phosphatidylcholines (BrPC) was measured by incorporating various concentrations of BrPC into PC SUV (10, 25, 40 mol%) and into CL (DOPG) SUV (10, 20, 30 mol%) and determining the relative fluorescence change (F/F_o) as described above, at lipid/peptide ratios (R_i) of 100 and 50, respectively, for each of the concentrations of the different BrPC. The data were analyzed according to the Stern-Volmer equation by plotting F_o/F vs. [BrPC] and determining the K_{SV} values. In the case of BrPC incorporation into CL (DOPG) SUV F_o was derived from CL (DOPG) SUV containing the corresponding amount of DOPC. The incorporation of DOPC into CL (DOPG) SUV up to 30 mol% gave rise to an increase of the fluorescence level by $\approx$ 10% for all peptides tested as was observed previously [20]. This observation is accounted for by an effect of the phospholipid headgroups on the quantum yield of the fluorophore [20,37].

Peptide–vesicle interaction under the influence of a transbilayer K^+ diffusion potential. The effect of a K^+ diffusion potential on the peptide–vesicle interaction was determined either continuously by monitoring changes in tryptophan fluorescence or changes in tryptophan-dansylPE resonance energy transfer (RET) or discontinuously by determining peptide–vesicle binding in centrifugation experiments or in a minicolumn gelfiltration assay.

The fluorescence measurements were carried out as described [21] on a Perkin-Elmer MPF 3 fluorimeter, under continuous stirring and at room temperature using an excitation wavelength of 280 nm (band pass 6 nm). The tryptophan fluorescence emission of 2 μM peptide in the presence of 300 μM LUV was monitored at 340 nm (band pass 11 nm). A K^+ diffusion potential was generated by adding valinomycin (Boehringer, Mannheim, F.R.G.) from an ethanolic stock solution (10 μg/ml or 100 μg/ml) to LUV exhibiting a K^+_{in}/Na^+_{out} gradient. The effect of the transbilayer pH

gradient was assessed by the simultaneous addition of the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; Sigma, St. Louis, MO; 10 μg/ml in ethanol). The ion gradient was dissipated by adding tryptophan-*N*-formylated gramicidin [38] from a 0.5 mM solution in DMSO. Unlike gramicidin, this derivative does not contribute to the tryptophan fluorescence intensity. The fluorescence data were corrected for a vesicle blank (scatter), that constituted approx. 20% of the total signal.

A more sensitive detection of ion gradient-induced peptide–vesicle interaction was accomplished by incorporating 2 mol% DNS-PE into the LUV and measuring changes in resonance energy transfer (RET) between the tryptophan-dansyl donor-acceptor pair [39]. DNS-PE fluorescence was monitored at 520 nm upon addition of 20 μM peptide to 200 μM LUV. Other conditions were as stated for the tryptophan fluorescence measurements. Application of the assay conditions to ACTH(1–24) and LUV consisting of PC/CL 1:1 revealed the occurrence of aggregation. Measurement of the change in absorbance at 295 nm carried out in parallel, showed that the maximum level of aggregation was reached within 30 s after adding the peptide to the LUV independently of the presence of a K^+ diffusion potential. A correction was applied for the contribution of scattering which never exceeded 10% of the total signal.

Peptide-vesicle association in the absence or presence of a K^+ diffusion potential was assayed by passing samples withdrawn from incubations containing 1 mM PC LUV and 0.1 mM peptide, through Sephadex G-50 minicolumns [32]. The amounts of phospholipid and associated peptide present in the filtrate were quantitated by phosphorus analysis and tryptophan fluorescence measurements as described [21]. The application of [^{3}H]tetraphenylphosphonium bromide (TPP^+, New England Nuclear) and [^{14}C]methylamine (MeAm, NEN) in this assay enabled the parallel quantitation of the effect exerted by the peptides on the membrane potential ($\Delta\psi$) and the transmembrane pH gradient (ΔpH) [32,40]. The values of $\log([TPP^+]_{in}/[TPP^+]_{out})$ which served as a measure of $\Delta\psi$, and of $\log([MeAm]_{in}/[MeAm]_{out})$ which yielded ΔpH, were determined as previously described [21].

Alternatively the effect of a K^+ diffusion potential on the association of mastoparan X with PC vesicles was determined by centrifugation. In order to efficiently spin down the vesicles, MLV and buffers containing 100 mM instead of 150 mM K_2SO_4/Na_2SO_4 were used. Aliquots withdrawn from incubations of 3 mM PC MLV and 0.1 mM mastoparan X were centrifuged for 30 min at 37000 $\times g$. The percentage of peptide bound was determined by relating the amount of peptide remaining in the supernatant to that in a non-centrifuged sample. Quantitation of the peptide was accomplished by measuring the tryptophan fluorescence intensities of samples solubilized in 0.5% (w/v) sodium cholate containing buffer [21].

DiS-C_2-(5) (Molecular Probes) was routinely used for a fast qualitative measurement of $\Delta\psi$ [41]. The rate and the extent of the fluorescence quenching of diS-C_2-(5) (1.5 μM in Na_2SO_4 buffer, λ_{ex} = 622 nm, λ_{em} = 660 nm) upon addition of 25 μM PC LUV or 5 μM LUV consisting of a PC/CL mixture, withdrawn from a peptide-vesicle association experiment, provided information on the relative effects of the peptides on membrane potential stability.

Circular dichroism

Changes in secondary structure of some of the peptides upon addition of phospholipid SUV were measured by circular dichroism (CD) on a Jasco-600 spectropolarimeter controlled by a Laser 386 computer. CD spectra were recorded at room temperature using quartz cells of 0.2 mm path length at a peptide concentration of 50 μM in 100 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. DOPC and DOPG SUV were added to a R_i of 50 and 20, respectively. A lineshape analysis of the CD spectra was carried out as described [42].

Results

'Spontaneous' peptide–vesicle interaction

Before examining the effect of a K^+ diffusion potential on the membrane interaction of the peptides, the affinity of the peptides for vesicles consisting of either zwitterionic (PC) or acidic phospholipids (CL/DOPG) was studied in a comparative way using tryptophan fluorescence. Fig. 1 compares the increases in fluorescence quantum yield of mastoparan X and preCoxIV(1–25)W^{18} upon titration with PC SUV. The

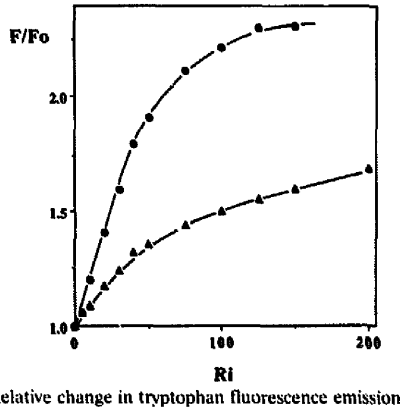

Fig. 1. Relative change in tryptophan fluorescence emission intensity at 340 nm (F/F_0) upon titrating 3 μM of the peptides mastoparan X (●) and preCoxIV(1–25)W^{18} (▲) with egg PC SUV.

TABLE II

Fluorescence characteristics and binding parameters of the peptides interacting with SUV consisting of zwitterionic or anionic phospholipid

Peptide	Phospholipid	R_i [a]	F/F_o (340 nm)	$\Delta\lambda_{max}$ (nm)	$K_d \cdot n$ [b] (mM)
Mastoparan X	PC	150	2.3	16	0.12
	CL	50	1.8	24	0.003 [c]
preCoxIV(1–25)W[18]	PC	200	1.7	7	0.3
	DOPG	50	1.5	8	0.006
ACTH(1–24)	CL	50	1.5	9	0.01 [c]
α-MSH	CL	150	1.4	2	0.2
ACTH(1–10)	CL	150	1.1	0	
Dynorphin A	CL	50	1.6	4	
Bombesin	CL	150	1.3	2	0.3
LHRH	CL	150	1.3	4	0.4

[a] The F/F_o (340 nm) and $\Delta\lambda_{max}$ values were determined at the lipid/peptide molar ratios (R_i) indicated.

[b] The relative error in the $K_d \cdot n$ values obtained from the fluorescence increase (see Methods) amounts to 10%.

[c] For practical reasons $K_d \cdot n$ values were obtained from the change of the wavelength of maximum emission (see Methods); the relative error amounts to 20% in this case.

increase in fluorescence intensity is paralleled by a decrease in λ_{max} (Table II), indicative for the fluorophore entering an environment with a lower dielectric constant. In the absence of lipid all peptides tested in this study, display a λ_{max} of 354 ± 1 nm. Comparison of the affinity parameters $K_d \cdot n$ derived from the fluorescence intensity titration curves (Table II) shows that the affinity of mastoparan X for PC bilayers is larger than that of preCoxIV(1–25)W[18]. The other six peptides do not show significant changes in F/F_o nor

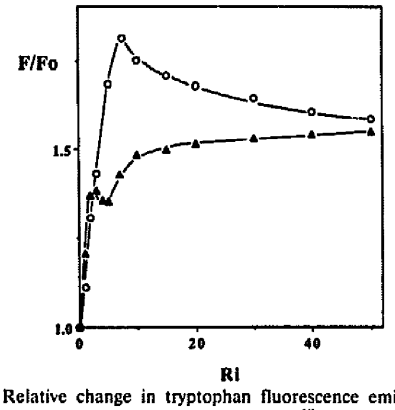

Fig. 2. Relative change in tryptophan fluorescence emission measured in a titration of preCoxIV(1–25)W[18] with DOPG SUV (▲) and in a titration of dynorphin A with CL SUV (○); [peptide] = 3 μM.

in λ_{max} in titrations with PC SUV (data not shown). The virtual lack of interaction of these peptides with zwitterionic phospholipid vesicles is also apparent from their accessibility to the aqueous quenchers I^- and acrylamide which is hardly or not affected when PC SUV are present (Table III). In contrast, the tryptophan residues of mastoparan X and preCoxIV(1–25)W[18] are largely screened from these aqueous quenchers in the presence of PC SUV (Table III).

In Fig. 2 titrations of preCoxIV(1–25)W[18] and dynorphin A with SUV consisting of acidic phospholipids are shown. Titrations with CL and DOPG SUV yielded similar results as judged from the derived $K_d \cdot n$ values, although the absolute numbers of F/F_o were

TABLE III

Accessibility to iodide and acrylamide (aa) of the peptides in the absence and presence of PC and CL SUV [a]

Peptide	K_{SV} (I^-) (M^{-1})	naf (I^-) [b] PC	naf (I^-) [c,d] CL	K_q (aa) (M^{-1})	naf (aa) [b] PC	naf (aa) [c] CL
Mastoparan X	9.4	0.23	0.05	13.1	0.19	0.09
PreCoxIV(1–25)W[18]	10.6	0.25	0.08 [e]	12.7	0.25	0.19 [e]
ACTH(1–24)	9.3	0.95	0.09	14.0	1.01	0.15
α-MSH	7.7	0.99	0.51	13.6		0.49
ACTH(1–10)	6.7	0.97	0.73	16.9		0.68
Dynorphin A	6.8	0.81	0.12	11.7	0.95	0.25
Bombesin	5.9	0.93	0.80	10.8		0.68
LHRH	4.8	0.94	0.79	8.6		0.56

[a] All data are mean values obtained in at least two independent experiments; the relative error in K_{SV} and K_q is less than 3%; the absolute error in the naf amounts to 0.02 for naf values ≤ 0.3 and to 0.06 for naf values > 0.3.

[b] The normalized accessibility factors (naf) were determined at a PC/peptide molar ratio of 200 for preCoxIV(1–25)W[18], of 150 for mastoparan X, α-MSH and bombesin and of 100 for the other peptides.

[c] The naf were determined at a CL/peptide molar ratio of 150 for α-MSH, ACTH(1–10), bombesin and LHRH, and of 50 for the other peptides.

[d] K_{SV} values in the presence of CL SUV were obtained from linear fits up to KI concentrations of 60 mM (ACTH(1–24) and α-MSH), of 100 mM (ACTH(1–10) and LHRH) and of 220 mM (the other peptides).

[e] Measurements were carried out using DOPG instead of CL.

different (data not shown). The titration curve of preCoxIV(1–25)W[18] shows an irregularity at R_i values below 5 due to the occurrence of peptide-induced vesicle aggregation in this range, which is not completely filtered out by the applied corrections (see Methods). Nevertheless $K_d \cdot n$ could be calculated and its value was in good agreement with that determined from the shift in λ_{max} (data not shown). Larger irregularities of the same kind were apparent in titrations of mastoparan X and ACTH(1–24) with CL or DOPG SUV (not shown) necessitating the use of shifts in λ_{max} to determine $K_d \cdot n$ (Table II). Dynorphin A exhibits an atypical fluorescence intensity titration curve in that the fluorescence gradually starts to decline after reaching a maximum value at $R_i \approx 8$ (Fig. 2). As this peptide shows a very small $\Delta\lambda_{max}$ (Table II) no $k_D \cdot N$ could be determined.

The parameters derived from intrinsic fluorescence measurements, and the accessibility to the aqueous quenchers have been summarized in Tables II and III. It is evident that the affinity for negatively charged bilayers of all peptides studied is much larger than that for zwitterionic membranes as a result of electrostatic attraction. The extent of the interaction with negatively charged model membranes is predominantly determined by the net positive charge of the peptides (Table I), that of mastoparan X, preCoxIV(1–25)W[18], ACTH(1–24) and dynorphin A being largest. The affinity for CL SUV of the peptides α-MSH, ACTH(1–10), bombesin, and LHRH with net charges of +1/+2 is comparable to that found for small amphiphilic model peptides with the same net charge, in similar experiments [20]. The $K_d \cdot n$ value obtained for bombesin binding to CL SUV (Table II) agrees with that reported for its binding to DMPS vesicles [24].

The importance of electrostatics for the peptide–vesicle interaction is underscored by the sensitivity of the interaction to changes in ionic strength. In the course of the KI titrations of the peptides in the presence of CL SUV an upward deviation from linearity in the Stern-Volmer plot of I^- quenching was apparent for a number of peptides due to KI-induced peptide-vesicle dissociation. In these cases a linear fit of the quenching data for the entire KI-concentration range is impossible (see Table III). Titrations with CL SUV carried out at 0 mM NaCl accordingly yield much smaller $K_d \cdot n$ and naf (acrylamide) values for these peptides, e.g. for LHRH at 0 mM NaCl a $K_d \cdot n$ of 0.1 mM was found, whereas in the standard buffer with 100 mM NaCl it is 0.4 mM (Table II). The relative KI concentration insensitivity of the vesicle interaction of mastoparan X, preCoxIV(1–25)W[18], and dynorphin A indicates that for these peptides hydrophobic interactions with the lipid contribute to the peptide–vesicle association.

A comparison of the naf values for I^- and acrylamide reveals that the aqueous quenchers yield consistent results in agreement with a previous study [20], except for a small divergence occurring at naf values < 0.3 in the experiments with acidic phospholipid SUV (Table III): In this range I^- yields naf values which are about half the naf values obtained with acrylamide, probably due to electrostatic repulsion of I^- by the negatively charged bilayers.

The decreased accessibility to aqueous quenchers is paralleled by an increased exposure to intramembranous quenchers, in agreement with the tryptophan residue inserting into the bilayer when the peptide binds to the membrane. The overall efficiency of quenching by the brominated phosphatidylcholines reflects the relative affinity of the peptides for the vesicles, whereas a comparison of the relative quenching efficiencies by the bromines at different positions along the acyl chain provides information about the relative membrane insertion depth of the tryptophan residue. The quenching numbers obtained for 2-BrPC are underestimates of the actual values because the bromine content of this phospholipid is only 37% due to the synthesis procedure [20]. Keeping this in mind while knowing that the quenching efficiencies of mono- and dibrominated phospholipids are inherently different [43], this extra reduction in bromine content of 2-BrPC does not interfere with a qualitative interpretation of the comparative sets of data.

The BrPC quenching data depicted in Fig. 3 confirm the greater affinity for PC SUV of mastoparan X as compared to preCoxIV(1–25)W[18] and the similar extent of binding of both peptides to SUV consisting of acidic phospholipids at a lipid/peptide molar ratio of 50. The shape of the quenching profiles, indicates that the tryptophan residue of mastoparan X is able to penetrate deeper into the bilayer, in particular in the case of PC SUV, than that of the presequence, which agrees with the larger fluorescence increases and shifts in λ_{max} observed for mastoparan X (Table II). Furthermore, the tryptophan residue of mastoparan is localized closer to the membrane surface in negatively charged bilayers than in PC bilayers (Fig. 3). Fig. 4a demonstrates the decreasing affinity for CL SUV when going from ACTH(1–24) via α-MSH to ACTH(1–10) and shows that the tryptophan residue of these peptides, when membrane bound, attains a similar localization near the membrane/water interface. The peptides bombesin and LHRH display similarly shaped quenching profiles, whereas for dynorphin A the membrane surface localization of the tryptophan residue is even more outspoken (Fig. 4b) in agreement with its relatively small $\Delta\lambda_{max}$ (Table II). The seemingly aberrant behaviour of the tryptophan fluorescence intensity of dynorphin A in the titration with CL SUV (Fig. 2) may be related to this localization. At low lipid/peptide ratios the high density of peptide on the membrane

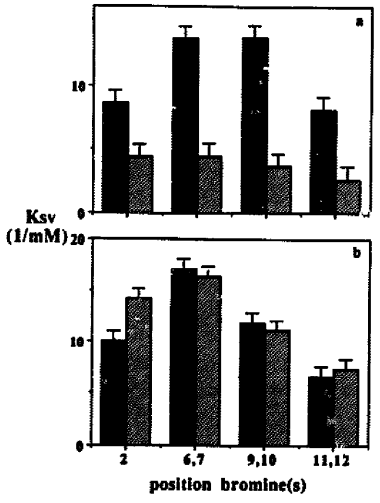

Fig. 3. Depth-dependent quenching by BrPC of mastoparan X (black columns) and preCoxIV(1–25)W[18] (dashed columns). In panel (a) BrPC were incorporated into PC SUV and the quenching constant K_{SV} was determined at $R_i = 100$ as described in the Methods. In panel (b) BrPC were incorporated into CL SUV in the case of mastoparan X and into DOPG SUV for preCoxIV(1–25)W[18]; K_{SV} was determined at $R_i = 50$. Quenching of mastoparan X by BrPC incorporated into DOPG SUV yielded a profile identical to that shown for CL within experimental error. The absolute error is indicated.

surface may change the lipid packing allowing the tryptophan to insert somewhat deeper into the membrane which gives rise to a normal fluorescence increase. When the bound peptide is diluted by adding more vesicles, the disturbance of the lipid packing is diminished, and the tryptophan residue will gradually be expelled to a localization with a higher dielectric constant.

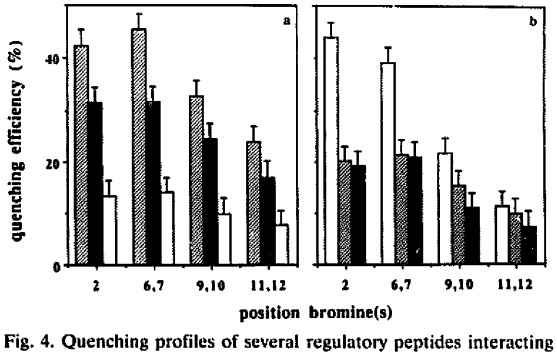

Fig. 4. Quenching profiles of several regulatory peptides interacting with CL SUV at the R_i indicated: ACTH(1–24) ($R_i = 50$, dashed), α-MSH ($R_i = 100$, black) and ACTH(1-10) ($R_i = 100$, open) in panel (a); dynorphin A ($R_i = 50$, open), bombesin ($R_i = 100$, dashed) and LHRH ($R_i = 100$, black) in panel (b). Quenching efficiencies are expressed as $(1 - F/F_0) \cdot 100\%$ with F the fluorescence intensity measured in the presence of SUV containing 30% BrPC and F_0 that in the presence of CL SUV containing 30% DOPC (see Methods) and were calculated for ACTH(1–24) and dynorphin A using the K_{SV} values obtained as described in the Methods. For the other peptides the quenching efficiency was determined as the average of three measurements using CL SUV containing 30% BrPC. The error bars indicate the absolute error of the data.

The effect of a K^+ diffusion potential on the peptide–vesicle interaction

Having examined the influence of the membrane surface charge on the interaction of the different peptides with lipid vesicles, next the effect of a K^+ diffusion potential on the vesicle interaction of the peptides was studied. In these experiments LUV (or MLV) were used instead of SUV as SUV do not maintain stable ion gradients. In general, the binding affinity of peptides for lipid bilayers decreases with decreasing vesicle curvature due to differences in lipid packing (see, for example, Ref. 44). The lower fluorescence intensity of mastoparan X in the presence of PC LUV at $R_i = 150$ (see forthcoming Fig. 7a) as compared to that in the presence of PC SUV (Fig. 1) is in agreement with this notion.

Addition of the K^+ ionophore valinomycin to PC LUV experiencing a K^+_{in}/Na^+_{out} gradient, generates a membrane potential ($\Delta\psi$, negative inside) across the vesicle membrane in excess of 200 mV, which on its turn gives rise to a pH gradient (ΔpH) as a result of the proton permeability of the membrane and the limited buffering capacity of the used buffer [21,40]. Fig. 5 shows the effect of a valinomycin-induced K^+ diffusion potential on the interaction of mastoparan X with PC LUV as it reveals itself in the fluorescence quantum yield of the intrinsic tryptophan residue. The fluorescence intensity of mastoparan X at 340 nm shows a time dependent increase in response to the K^+ diffusion potential, the rate and extent depending on the amount of valinomycin added. The higher fluorescence level reached at the lower valinomycin concentration reflects a greater membrane potential stability as was confirmed by $\Delta\psi$ measurements (data not shown), and excludes any other involvement of the ionophore than to switch on the membrane potential. The valinomycin concentration dependent rate of the fluorescence increase is due to the mixing lag time (cf. 21). The addition of the protonophore FCCP together with valinomycin allows the establishment of electrochemical equilibrium between the electrical potential ($\Delta\psi$) and the induced pH gradient [40]. In the system used, this results in an enhancement of the $\Delta\psi$-induced pH gradient from ≈ 0.8 pH unit, reached 30 min after the addition of valinomycin alone, to ≈ 2.7 pH units, at the expense of 20 mV of the $\Delta\psi$ [21]. As is shown in Fig. 5, FCCP does not affect the response of mastoparan X when added together with valinomycin indicating that the ΔpH component of the K^+ diffusion potential does not contribute to the fluorescence

increase. Dissipation of the membrane potential by tryptophan-*N*-formylated gramicidin [21] results in a very slowly proceeding reduction of the fluorescence quantum yield (Fig. 5). In the absence of ionophores with or without an ion gradient present the fluorescence intensity of mastoparan X slowly decreases which is most likely due to the occurrence of aggregation of the peptide in solution leading to self-quenching of the tryptophan fluorescence (cf. [45,46]).

In order to investigate whether the fluorescence increase of mastoparan X is due to enhanced binding of the peptide to the vesicles, analogously to the fluorescence increase obtained in the SUV titration experiments, a binding experiment was performed. Fig. 6 shows that a K^+ diffusion potential is able to enhance the binding of mastoparan X to PC MLV by some 10% under the conditions used ($R_i = 30$). In the absence of a potential there is already considerable vesicle binding in agreement with the findings of Uzu et al. [47].

The K^+ diffusion potential-induced vesicle uptake of a number of local anaesthetics and biogenic amines [48] and of synthetic model peptides [21] has been conveniently measured in a minicolumn gelfiltration assay. In contrast to the binding experiment described above, application of this assay to mastoparan X did not reveal any significant enhancement of the peptide-LUV association under the influence of a membrane potential, with the level of association ranging from 5

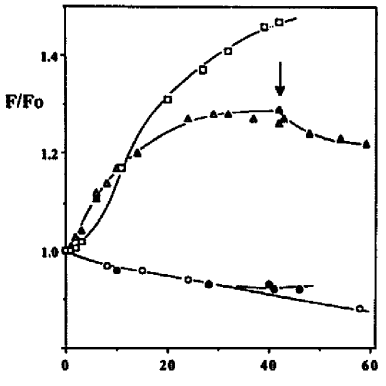

Fig. 5. Time course of the K^+ diffusion potential induced tryptophan fluorescence change at 340 nm of 2 μM mastoparan X added to 300 μM PC LUV. Data are shown for vesicles exhibiting a K^+_{in}/Na^+_{out} gradient with valinomycin added to a 10^{-3} (▲) or a 10^{-4} (□) molar ratio with respect to PC, with both valinomycin and FCCP added to molar ratios of 10^{-3} and $1.3 \cdot 10^{-4}$ with respect to PC, respectively (△), in the absence of any ionophores (●) and for vesicles without an ion gradient (○, K^+_{in}/K^+_{out}). The ionophores were added at $t = 0$. The arrow indicates the addition of tryptophan-N-formylated gramicidin to a 1:400 molar ratio with respect to PC. The fluorescence change is expressed as F/F_0 with F the fluorescence intensity measured and F_0 the fluorescence intensity of mastoparan X in the presence of vesicles at $t = 0$.

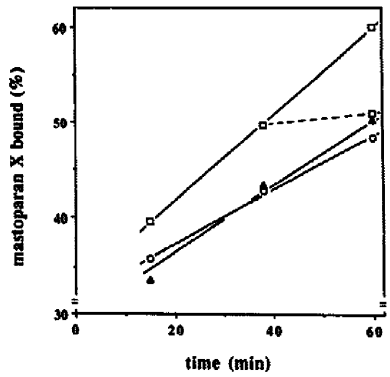

Fig. 6. The influence of a K^+ diffusion gradient potential on the binding of mastoparan X to PC MLV.PC MLV, K^+_{in}/K^+_{out} (○) or K^+_{in}/Na^+_{out} in the absence (▲) and presence of valinomycin (□,drawn line) were incubated at 3 mM with 0.1 mM mastoparan X. At the time points indicated aliquots were centrifuged and the amount of peptide bound was determined (see Methods). Valinomycin was added at $t = 0$ to a molar ratio of 10^{-4} with respect to PC. At $t = 39$ min the K^+ diffusion potential was dissipated by adding tryptophan-*N*-formylated gramicidin at a 1:400 molar ratio with respect to PC (□, dashed line). The absolute error of the data is less than 3%.

to 10%. This discrepancy is not due to complete dissipation of the membrane potential by mastoparan X under the conditions of this assay ($R_i = 10$) as evidenced by the TPP^+ data, which reveal a decrease from a $\log([TPP^+]_{in}/[TPP^+]_{out})$ value of ≈ 3.8 in the absence [21] to ≈ 3.0 in the presence of mastoparan X at $R_i = 10$, 20 min after the addition of peptide and valinomycin (valinomycin/PC = 10^{-3}) to the LUV. Although mastoparan X has a destabilizing effect on $\Delta\psi$, it is insufficient to account for the absence of any enhanced peptide–vesicle association observed in the gelfiltration assay. Apparently the increased association of mastoparan X with the vesicles is reversed upon passing the vesicles through the Sephadex column.

A negative membrane surface charge greatly enhances the affinity of mastoparan X for phospholipid vesicles (Table II). Fig. 7a compares the tryptophan fluorescence increases recorded for mastoparan X in response to a K^+ diffusion potential induced by valinomycin (valinomycin/phospholipid = 10^{-4}) and applied to PC LUV without or with 10% or 25% CL incorporated. The greater affinity of the peptide for vesicles carrying a negative surface charge is reflected in the higher initial ($t = 0$) fluorescence intensity when more CL is incorporated. The effect of a negative surface charge on the $\Delta\psi$ response is two-fold: the rate of the induced fluorescence increase is drastically enhanced and consequently the plateau level is reached earlier, yet the difference between the initial and maximal fluorescence intensity level becomes smaller with increasing CL contents. Qualitatively similar results were

obtained for preCoxIV(1–25)W^{18} (Fig. 7b). Compared to mastoparan X the rates of the fluorescence increase for this peptide are slower. Since the overall lipid affinity of preCoxIV(1–25)W^{18} is less than that of mastoparan X (Tables II and III), this observation agrees with the correlation between lipid affinity and rate of the $\Delta\psi$-induced fluorescence increase. In the absence of a $\Delta\psi$, the preCoxIV(1–25)W^{18} fluorescence level gradually decreases as was observed for mastoparan X above.

The other six peptides do not show any response to a $\Delta\psi$ applied to PC LUV, neither do they affect the stability of the ion gradient (data not shown) in agreement with the absence of any interaction (Table III). Upon incorporating increasing amounts of CL into the PC LUV up to 50%, only the peptide dynorphin A starts to show a $\Delta\psi$-dependent tryptophan fluorescence increase from a CL content of 25% (data not shown). Its rate and extent are less than that observed for preCoxIV(1–25)W^{18} under the same conditions (Fig. 7b).

The sensitivity of detecting a $\Delta\psi$ effect on peptide–vesicle interaction was enhanced by using a RET assay which previously was shown to accurately monitor the K^+ diffusion potential-induced vesicle uptake of a synthetic model peptide [21]. The assay measures resonance energy transfer from the peptide's intrinsic tryptophan to a dansyl moiety present in the phospholipid head group region, and has the advantage that much larger amounts of peptide can be used while allowing the detection of a $\Delta\psi$ effect even if only a minor subpopulation of the total amount of peptide present is affected.

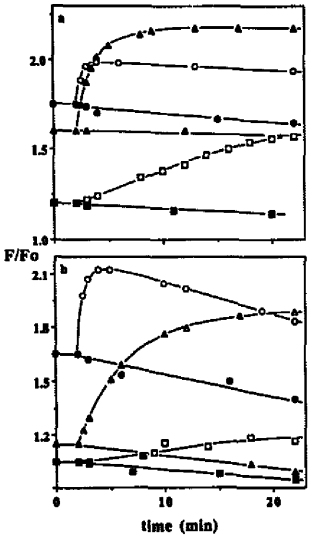

Fig. 7. Tryptophan fluorescence change at 340 nm of mastoparan X (panel a) and preCoxIV(1–25)W^{18} (panel b) upon addition of LUV consisting of PC (□), PC/CL 9:1 (△) and PC/CL 3:1 (○) and experiencing a K^+_{in}/Na^+_{out} gradient in the absence (closed symbols) and presence of valinomycin which was added at $t = 2$ min to a 10^{-4} valinomycin/phospholipid molar ratio (open symbols). The fluorescence measured, F, is related to the F_0, the fluorescence level of the peptide in the absence of vesicles.

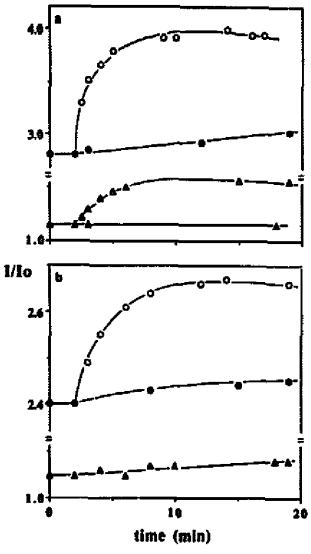

Fig. 8. The effect of a valinomycin-induced K^+ diffusion potential on the interaction of dynorphin A (panel a) and ACTH(1–24) (panel b) with LUV consisting of PC/CL 3:1 (△) and PC/CL 1:1 (○), monitored by tryptophan – DNS-PE RET. Valinomycin was added at $t = 2$ min to a 10^{-4} valinomycin/phospholipid molar ratio (open symbols). The closed symbols show the fluorescence development recorded in the presence of vesicles experiencing a K^+_{in}/Na^+_{out} gradient in the absence of valinomycin. The fluorescence intensity I is related to I_0, the fluorescence intensity emitted by the vesicles in the absence of peptide. Note the different scales on the y-axis in panels a and b.

Fig. 8 shows the effect of $\Delta\psi$ on the interaction of dynorphin A and ACTH(1–24) with vesicles consisting of PC and CL in ratios of 3:1 and 1:1 as detected by the RET assay. The initial level of fluorescence reflects the binding to the vesicles as such and shows a strong surface charge dependence for both peptides in agreement with the results obtained in the SUV titrations. Dynorphin A displays a strong $\Delta\psi$ response at both lipid compositions whereas ACTH(1–24) exhibits a relatively modest fluorescence increase only at the higher CL content (Fig. 8). DiS-C_2-(5) analysis revealed no difference in the dissipation of $\Delta\psi$ by these two peptides. For α-MSH, ACTH(1–10), bombesin and LHRH no $\Delta\psi$-enhanced RET was detectable up to a vesicle CL content of 50%, neither was a DNS-PE fluorescence increase detectable upon adding these peptides to the vesicles (data not shown).

Discussion

The effect of a membrane potential on the interaction of some biologically active peptides with phospholipid vesicles has been investigated. The main observation made is that a membrane potential affects peptide–membrane interaction in a peptide specific way depending on the lipid composition of the vesicles. Before considering in detail the membrane potential-induced effects, the membrane affinity and membrane topology of the peptides measured in the absence of a membrane potential will be discussed.

Mastoparan X is a peptide that shows strong affinity for both zwitterionic and acidic model membranes as evidenced by the increased tryptophan fluorescence intensity, the large shift of λ_{max}, the loss in accessibility to aqueous quenchers, and the exposure to intramembraneous quenchers in the presence of the SUV. The brominated PC quenching profiles obtained for mastoparan X demonstrate that its tryptophan residue inserts relatively deep into the bilayer when compared to that of the other peptides studied. The different shapes of the profiles obtained for PC and CL SUV suggest that the negative surface charge of the latter fixes the positive charges of the peptide, thus preventing a deeper insertion as is observed for the peptide when it is bound to PC SUV (Fig. 3). Interestingly, the tryptophan residue of the related peptide melittin was found to insert deeper into CL bilayers than into PC bilayers [49].

The preference of the mitochondrial presequence analogue preCoxIV(1–25)W^{18} to interact with acidic phospholipid vesicles as compared to PC vesicles (Tables II, III) has been established previously using a preCoxIV(1–25) analogue with a net charge of +6, the additional positive charge resulting from a blocked C-terminus (cf. Table I; [50]). The lipid affinity of the presequence is about half that of mastoparan X (Tables II and III). Compared to the tryptophan in mastoparan X, that of preCoxIV(1–25)W^{18} attains a localization closer to the membrane/water interface, particularly in PC bilayers as judged from the BrPC quenching profiles (Fig. 3). The differences in lipid interaction between preCoxIV(1–25)W^{18} and mastoparan x are probably due to the greater average hydrophobicity of the toxin as compared to that of the presequence (cf. Ref. 20).

The lipid interaction of the regulatory peptides depends largely on electrostatic attraction since these positively charged peptides only bind to vesicles containing acidic phospholipids to an extent that is more or less proportional to their net positive charge, in agreement with earlier reports [51,52]. Once electrostatically bound to the membrane surface, hydrophobic interactions may contribute to the binding to different extents as can be inferred from the differences in sensitivity to ionic strength found for the binding of these peptides to CL SUV. The preferential localization of the tryptophan residue near the membrane/water interface which is observed for all peptides tested in this study, appears to be a general feature of this amino acid when present in amphiphilic peptides [20,53].

The relative affinity of the opiomelanocortins, ACTH(1–24), α-MSH, and ACTH(1–10), for CL bilayers follows the order predicted on theoretical grounds by Schwyzer [23]. The bilayer topology of the tryptophan residue is virtually identical for these three peptides as judged from the relative BrPC quenching efficiencies (Fig. 4a). The BrPC quenching profile (Fig. 4b) and the very small $\Delta\lambda_{max}$ (Table II) obtained for the tryptophan of dynorphin A suggest a membrane surface localization for this residue as expected based on its position in the primary sequence in between two charged amino acids (Table I). The finding that the vesicle binding of dynorphin A is relatively insensitive to ionic strength, points to stabilization of the binding by hydrophobic interactions, that may be provided by the nine N-terminal amino acid residues inserting into the bilayer as an α-helix [51,54,55].

Having summarized the contributions of electrostatic and hydrophobic interactions, the influence exerted by a third parameter, i.e. the presence of a membrane potential, on the interaction of the peptides with phospholipid vesicles will be discussed. The results obtained here, for mastoparan X in particular, will be compared with those obtained in previous studies where the effect of a K^+ diffusion potential on the vesicle interaction of synthetic model peptides has been analyzed [21,56]. Using the minicolumn gelfiltration assay, it was shown that a K^+ diffusion potential when applied to LUV is able to enhance the vesicle association of a hydrophobic hexapeptide with a single positive charge originating from the N-terminal amino group, AIMLWAme$^+$ (one letter code), to a level of 30% of the amount of peptide available. This K^+ diffusion potential-induced association of the peptide is paralleled by an increase in tryptophan fluorescence. Using vesicles with an asymmetric transbilayer distribution of brominated phosphatidylcholine it was demonstrated that this peptide is translocated and becomes inserted into the inner leaflet of the vesicle membrane. The observation that the rate and the extent of the fluorescence increase of this peptide were enhanced upon decreasing the internal volume of the used vesicles, inferred the existence of a partitioning equilibrium of the peptide between the internal aqueous compartment and the membrane. The uptake was for the most part attributed to the pH gradient evoked by the K^+ diffusion potential, and proceeded via membrane translocation of the deprotonated neutral form.

Mastoparan X displays an increase in tryptophan

fluorescence intensity upon applying a K^+ diffusion potential to PC LUV, which shows a strong resemblance to that obtained for the hexapeptide. However, the nature of the fluorescence increase of mastoparan X differs from that of the hexapeptide. In contrast to that of the hexapeptide, the fluorescence increase of mastoparan X does not depend on the ΔpH component of the K^+ diffusion potential (Fig. 5), nor does it depend on the internal volume of the vesicles (data not shown). Furthermore no $\Delta\psi$-enhanced vesicle association of mastoparan X could be measured in the minicolumn assay. These results rule out a large scale accumulation of the toxin inside the vesicles, as was found for the hexapeptide.

These differences inevitably lead to the conclusion that a membrane potential is able to exert yet another and more direct effect on peptide–lipid interaction, which is unrelated to the concurring pH gradient. The $\Delta\psi$-induced fluorescence increase of mastoparan X is either due to enhanced binding of the peptide to the outer membrane leaflet of the vesicles, or to a change in conformation or orientation of the membrane-bound peptide. The increased binding of mastoparan X to MLV experiencing a $\Delta\psi$ supports the first possibility, although it should be kept in mind that for technical reasons this experiment was carried out at concentrations different from those in the fluorescence measurements. The observation that no enhanced peptide-vesicle binding is detectable in the minicolumn gelfiltration assay, indicates that the binding is reversible. However, the very slow fluorescence decrease that is recorded upon dissipating $\Delta\psi$ (Fig. 5), points to a very slow release of the peptide bound by $\Delta\psi$, which is hard to reconcile with this reversibility of the binding, and therefore argues for the second possibility. Based on the present data, neither of the two possible explanations can be ruled out. The increased binding to the MLV in response to $\Delta\psi$ infers that at least part of the fluorescence is due to enhanced peptide-vesicle binding. The initial rate of the $\Delta\psi$-induced fluorescence increase becomes faster as the CL content of the vesicles is increased (Fig. 7a), indicating that the rate of the $\Delta\psi$ effect, whatever its precise nature, is enhanced upon increasing the peptide's affinity for the vesicles.

It has been proposed that a membrane potential electrophoretically transfers mastoparan across the plasma membrane thus enabling the activation of the intracellularly located G-proteins [6]. The data presented here argue against the occurrence of a large scale electrophoresis of mastoparan X across a lipid bilayer, but do not exclude the possibility that a few mastoparan X molecules are able to cross the bilayer in response to the $\Delta\psi$.

The effect of $\Delta\psi$ on the tryptophan fluorescence of preCoxIV(1–25)W^{18} is fully comparable to that observed for mastoparan X, although the initial rates of the by-induced fluorescence increase are smaller for the presequence (Fig. 7). This difference is explained by the greater membrane affinity of mastoparan X. Similar results were found for dynorphin A. This peptide does not show significant interaction with pure PC bilayers, and accordingly no $\Delta\psi$-induced fluorescence increase was detectable when using PC LUV. However, upon raising the CL content of the LUV to 25%, thereby increasing the affinity of dynorphin A for the vesicles, a $\Delta\psi$-induced tryptophan fluorescence increase became apparent.

Compared to dynorphin A, ACTH(1–24) shows a very modest response to $\Delta\psi$ (Fig. 8), which is not discernible in tryptophan fluorescence measurements and can only be revealed by the RET assay when applied to PC/CL 1:1 LUV (Fig. 8). The different $\Delta\psi$ responses observed for dynorphin A and ACTH(1–24) may be due to the binding of ACTH(1–24) to the vesicles being already almost saturated before the $\Delta\psi$ is applied, or to the membrane bound state of ACTH(1–24) being less susceptible to $\Delta\psi$ than that of dynorphin A. For α-MSH, ACTH(1–10), bombesin, and LHRH no $\Delta\psi$ effect has been detected up to a vesicle CL content of 50%. It can not be excluded that these peptides do respond to $\Delta\psi$ when the negative surface charge of the vesicles is further increased.

The $\Delta\psi$-induced fluorescence responses observed for the bioactive peptides are consistent with those observed for synthetic model peptides containing two positively charged groups. One of these, the pentapeptide $RMLWAme^{2+}$, showed a relatively modest $\Delta\psi$-induced increase in RET when the CL content of the vesicles was raised to 50% [21]. It is concluded that peptides can only respond to a membrane potential when they already have the ability to interact with the membrane, in other words, when they can sense the attraction by the electric field created by the membrane potential across the lipid bilayer. A model peptide with a positive charge at each end of the molecule which has virtually the same membrane affinity characteristics as $RMLWAme^{2+}$, does not respond to $\Delta\psi$ under the same conditions. The different mode of membrane anchoring of this peptide has been proposed to render it insusceptible to $\Delta\psi$ [21].

Based on the present results and on data derived from the literature we put forward the hypothesis that the ability of a peptide to adopt an α-helical conformation plays a decisive role in determining the susceptibility of a peptide to the $\Delta\psi$.

Mastoparan X which displays the largest $\Delta\psi$ response, has a very strong tendency to change from an unordered structure in aqueous solution into an α-helical conformation upon binding to a membrane. CD measurements yielded α-helix contents for mastoparan X of 6% in the absence, and 44% and 60% in the

presence of DOPC SUV ($R_i = 50$) and DOPG SUV ($R_i = 20$), respectively (data not shown). These data agree with an earlier ^{1}H-NMR study [5] and are in accordance with CD data obtained for mastoparan [4,7].

Like mastoparan X, preCoxIV(1–25) is reported to adopt an α-helical conformation in negatively charged lipid bilayers, but in contrast to the toxin it assumes an unordered extended structure in bilayers consisting of PC [57]. The apparently somewhat smaller tendency of the presequence to adopt an α-helical structure as compared to mastoparan X may explain the smaller $\Delta\psi$ effects observed for the former. Literature data suggest that the ability of a presequence to adopt an α-helical structure determines its susceptibility to $\Delta\psi$. Recently it was shown that a synthetic peptide corresponding to preCoxIV(1–22) which competitively inhibits the import into mitochondria of the entire preCoxIV precursor protein without dissipating $\Delta\psi$ [58], is imported into mitochondria in a $\Delta\psi$-dependent manner [59]. In contrast, the binding of the pre-ornithine transcarbamylase presequence to phospholipid vesicles was found to be insensitive to a K^+ diffusion potential [15]. Accordingly, this presequence was shown to be imported into mitochondria in the absence of a $\Delta\psi$, even though the import of the entire precursor does require the mitochondrial $\Delta\psi$ [60]. The apparent lack of membrane potential susceptibility of this presequence may very well be related to its preference to adopt a β-pleated instead of an α-helical structure, also when membrane bound [61]. Obviously, studies on entire precursor proteins are required to elucidate the role of membrane potential in mitochondrial protein import.

For dynorphin A and ACTH(1–24), both of which respond to $\Delta\psi$, the adoption of α-helical structure in the presence of lipids has been reported [55,62,63]. The lack of a $\Delta\psi$ response for bombesin and LHRH correlates with the finding that neither of these peptides adopts a defined secondary structure upon binding to vesicles or micelles [25,27].

Summarizing, a $\Delta\psi$ has been demonstrated to affect the interaction of physiologically active peptides with phospholipid vesicles. From the coherence of the results obtained for the different peptides tested with the mastoparan X data, it can be surmised that the membrane potential is able to enhance the amount of membrane bound peptide, provided that the peptide already has a basic level of affinity for the membrane. Conceivably the membrane potential may play a similar role in the relevant biological processes. A $\Delta\psi$-induced change in the orientation or conformation of the membrane-bound form of the peptide, as was recently demonstrated for the voltage-dependent (α-helical) segment of a colicin [64], may also contribute to the $\Delta\psi$-induced fluorescence changes. Based on the data the hypothesis has been put forward that the ability of a peptide to assume an α-helical conformation renders it susceptible to a membrane potential: in these cases $\Delta\psi$ may act on the α-helical dipole moment rather than on the peptide's positive charge (cf. Ref. 65).

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Recently, a K^+ diffusion potential-induced increase in the extent of binding of mastoparan X to PC LUV at the concentrations used in the tryptophan fluorescence measurements, was demonstrated in centrifugation experiments. The binding of dynorphin A to LUV consisting of PC/CL (3 : 1) was found to be enhanced in the presence of a K^+ diffusion potential (Leenhouts, J.M. and De Kroon, A.I.P.M., unpublished observations).

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