# Ion gradient-induced membrane translocation of model peptides



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ABSTRACT The $K^+$ diffusion potential-induced association of synthetic model peptides carrying a single positive charge originating from the $NH_2$-terminal amino function with large unilamellar vesicles (LUV) consisting of phosphatidylcholine (PC) has been reported previously (de Kroon, A. I. P. M., J. de Gier, and B. de Kruijff. 1989. *Biochim. Biophys. Acta.* 981:371–373). To determine the vesicle localization of the associated peptides, fluorescence measurements utilizing the peptides' tryptophan residue as intrinsic fluorescent probe were performed. The application in these measurements, of vesicles that exhibit an asymmetric transbilayer distribution of brominated PC which is a quencher of tryptophan fluorescence, unequivocally demonstrated that the peptide $H_3N^+$-AIMLWA-Ome ($AIXme^+$) is accumulated in the interface of the inner leaflet of the vesicle membrane in response to the valinomycin-induced $K^+$ diffusion potential (negative inside). The relative contributions of the membrane potential ($\Delta\psi$) and the pH gradient ($\Delta$pH, acidic inside) induced by the $K^+$ diffusion potential, to the process have been assessed. An analysis of the pH and $\Delta$pH dependencies of the process demonstrated that the $K^+$ diffusion potential-induced peptide accumulation is largely determined by a redistribution of peptide according to the transbilayer pH gradient, in agreement with a translocation across the vesicle membrane of the neutral, deprotonated form of the peptide. The general validity of the mechanism proposed for the vesicle-uptake of $AIXme^+$ has been examined by extending the experiments to peptide analogues with a single negative charge and to peptides with two positive charges, and by investigating the effect of incorporating the acidic phospholipid cardiolipin (CL) into the LUV. The incorporation of CL appeared not to affect the $K^+$ diffusion, potential-induced vesicle uptake of $AIXme^+$. The peptide $H_3N^+$-RMLWA-Ome ($RXme^{2+}$) showed a small $\Delta$pH independent fluorescence response to the $\Delta\psi$ upon raising the CL content of the vesicles to 25%.

## INTRODUCTION

Peptide-lipid interactions have been implied to play a role in biological processes that involve membrane binding or transmembrane passage of peptides and proteins (see Sargent and Schwyzer, 1986; Jones et al., 1990). The study of peptide-lipid interactions in model systems contributes to the understanding of the mechanisms of these processes. Some important factors governing the mode and the extent of peptide-lipid bilayer interaction are the hydrophobicity of the peptide, the charge of the peptide, and related to the latter the surface charge of the membrane. In a recent model system study, the contribution of each of these factors to the membrane affinity and membrane topology of a set of related synthetic peptides has been investigated using the fluorescence properties of the peptides' intrinsic tryptophan residue (de Kroon et al., 1990).

Another important parameter likely to influence the interaction of peptides or proteins with the lipid phase of biomembranes is provided by the electrochemical gradients existing across most biological membranes. We have studied this possibility in model systems that consist of the synthetic peptides already mentioned, and of phospholipid vesicles exhibiting ion gradients that give rise to membrane potentials ($\Delta\psi$) and pH gradients ($\Delta$pH).

Previously, the effect of a valinomycin-induced $K^+$ diffusion potential (negative inside) on the vesicle-association of the peptides $AXme^+$ and $AIXme^+$ (see Table 1 for the abbreviations) has been reported (de Kroon et al., 1989). Both peptides carry a single positive charge arising from the $NH_2$-terminal amino group and show a very modest affinity for phosphatidylcholine (PC) vesicles as such (see also de Kroon et al., 1990). However, upon application of a $K^+$ diffusion potential, the peptide-vesicle association as assayed by minicolumn gel filtration, is strongly increased, with the extent of the association depending on the applied $K^+$ gradient and on the hydrophobicity of the peptide.

This result raised a number of questions that have been addressed in the present paper. The question of the localization of the associated peptide molecules has been elucidated in tryptophan fluorescence measurements employing PC vesicles that exhibit an asymmetric transbilayer distribution of brominated PC. Brominated

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TABLE 1 **Peptides used**

| Abbreviation* | Sequence |
|---|---|
| $AXme^+$ | $^+$N-Ala-Met-Leu-Trp-Ala-Ome |
| $AIXme^+$ | $^+$N-Ala-Ile-Met-Leu-Trp-Ala-Ome |
| $RXme^{2+}$ | $^+$N-Arg$^+$-Met-Leu-Trp-Ala-Ome |
| $AXetN^{2+}$ | $^+$N-Ala-Met-Leu-Trp-Ala-ONH $(CH_2)_2NH_3^+$ |
| $bocAX^-$ | *t*-Boc-Ala-Met-Leu-Trp-Ala-O$^-$ |
| $bocAIX^-$ | *t*-Boc-Ala-Ile-Met-Leu-Trp-Ala-O$^-$ |

*The abbreviation X denotes the sequence Met-Leu-Trp-Ala. Other amino acids are indicated by the one-letter symbols; boc indicates the coupling of a *tert*-butyloxycarbonyl group to the amino-terminus, me, methylation of the carboxyl group, and etN, the attachment of diaminoethane to the COOH-terminus.

phospholipids are short range quenchers of tryptophan fluorescence (Bolen and Holloway, 1990; de Kroon et al., 1990), and have been used previously in a similar experimental approach to solve the membrane topology of cytochrome $b_5$ (Everett et al., 1986).

The mechanism of the $K^+$ diffusion potential-induced peptide-vesicle association was addressed by determining the relative contributions to the process of the membrane potential itself and of the pH gradient evoked by the membrane potential, while varying the pH of the system. Similar studies on the $K^+$ diffusion potential-induced vesicle-uptake of the local anaesthetic dibucaine and the biogenic amine dopamine revealed a major contribution to the uptake by the concurring transmembrane pH gradient (Mayer et al., 1988; Bally et al., 1988).

The generality of the ion gradient-induced peptide-vesicle association was examined by extending the experiments to peptides carrying more than one positive charge, and to vesicles with a negative surface charge as is often found in biomembranes. Furthermore, the applicability of the findings was tested in systems of the opposite polarity, which consist of anionic peptide analogues and vesicles exhibiting reversed ion gradients (i.e., $Na_{in}^+/K_{out}^+$). A mechanism for the ion gradient–induced peptide-vesicle association is proposed and the relevance of this study for some biological processes is indicated.

## MATERIALS AND METHODS

### Peptides

The synthesis, modification, purification and nomenclature of the model peptides employed has been described previously (de Kroon et al., 1990). The peptides and their nomenclature are summarized in Table 1. Stock solutions of peptides were prepared in dimethylsulfoxide (DMSO), the concentrations were determined using $\epsilon^{280} = 5.6$ $mM^{-1}cm^{-1}$.

### Phospholipids

Egg-phosphatidylcholine (PC) and beef heart cardiolipin (CL) were purified as described (Van Duijn et al., 1984; Smaal et al., 1985). The synthesis, purification, and characterization of the brominated phosphatidylcholines (BrPC), 1-palmitoyl-2-(2-bromohexadecanoyl)-phosphatidylcholine (2-BrPC), 1-palmitoyl-2-(*n,n* +-dibromostearoyl)phosphatidylcholine (6,7-$Br_2$PC, 9,10-$Br_2$PC, 11,12-$Br_2$PC) and 1,2-bis-(9, 10-dibromostearoyl)phosphatidylcholine ($Br_4$PC) has been described (de Kroon et al., 1990). *N*-(5-dimethylaminonaphtalene-1-sulfonyl)dipalmitoyl-phosphatidylethanolamine (DNS-PE) was purchased from Molecular Probes Inc. (Eugene, OR).

### Miscellaneous

Phosphatidylcholine-specific transfer protein (PCTP) was purified from bovine liver (Westerman et al., 1983) and stored at 0.8 mg/ml in 50% glycerol containing buffer at −20°C. *N*-(11-(9-carbazole)undecanoyl)glucosaminic acid (CUGA) was synthesized by coupling glucosaminic acid (Sigma Chemical Co., St. Louis, MO) to 11-(9-carbazole)undecanoic acid (Molecular Probes Inc.) according to Everett et al. (1986). The product revealed a single fluorescent spot upon thin layer chromatography and was not further purified. A ~1-mM stock solution of CUGA in water was prepared on weight basis. Tryptophan-N-formylated gramicidin (NFG) was prepared as reported (Killian et al., 1985). Valinomycin was purchased from Boehringer Mannheim Corp. (Mannheim, Germany). Carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP) was obtained from Sigma. 3,3′-Diethyl-thiadicarbocyanine iodide [diS-$C_2$-(5)] was obtained from Molecular Probes. [$^3$H]Tetraphenyl phosphoniumbromide ($TPP^+$), [$^{14}$C]methylamine hydrochloride (MeAm) and [$^{14}$C]inuline were purchased from New England Nuclear (Boston, MA). [$^3$H]Cholesteryl-oleoylether and [$^{14}$C]dioleoylphosphatidylcholine (DOPC) were from Amersham Corp. (Arlington Heights, IL). All other chemicals were analytical grade or better.

### Preparation of large unilamellar vesicles (LUV) and generation of ion gradients

Dry lipid films were hydrated in the appropriate buffer by vortexing. The dispersions were frozen and thawed ten times and subsequently extruded ten times through 400 nm or 200 nm pore size polycarbonate filters as described (Hope et al., 1985). When using egg PC at a concentration of 20 mM, this procedure in our hands yielded LUVET 400 with a trapped volume of 3.0 ± 0.1 μl/μmol and LUVET 200 with a trapped volume of 2.0 ± 0.1 μl/μmol respectively, as determined by trapping [$^{14}$C]inuline (Mayer et al., 1986*a*). Tournois et al. (1990) found that at least 80% of the lipids of LUVET 400 resides in the outermost bilayer. LUVET 400 were used in all experiments unless stated otherwise.

Transmembrane $K^+_{in}/Na^+_{out}$ chemical gradients were obtained by preparing the vesicles in 150 mM $K_2SO_4$, 20 mM Hepes adjusted to pH 7.0 with KOH, and removing the untrapped buffer by passing the vesicles through a Sephadex G 50 (medium) column eluted with 150 mM $Na_2SO_4$, 20 mM Hepes adjusted to pH 7.0 with NaOH (de Kroon et al., 1989). To increase the buffering capacity, some experiments were carried out in phosphate buffer. Vesicles were prepared in 200 mM $KP_i$ and the external $K^+$ was replaced by $Na^+$ on a Sephadex column eluted with 200 mM $NaP_i$. The desired pH values in the range 6–8 were obtained by mixing the appropriate amounts of the mono- and dibasic salt. When pH values in the range 7.5–9.0 were required, 20 mM Tricine was used as buffer. When using CL containing vesicles,

2 mM EDTA was included in the buffers. Transmembrane $Na_{in}^+/K_{out}^+$ gradients were created by diluting vesicles prepared in 150 mM $Na_2SO_4$, and 10 mM each of Hepes, MES, and glutamic acid (abbreviated as 10 mM HMG) at the desired pH in the range 4–7, into 150 mM $K_2SO_4$, 10 mM HMG at the same pH.

Phospholipid concentrations of vesicle preparations were determined by the method of Rouser et al. (1970). Amounts and concentrations of CL are always expressed on phosphorus basis. When applying phosphate buffer, vesicles were radiolabeled with [$^{14}$C]DOPC or [$^3$H]cholesteryloleoylether to enable quantitation.

## Determination of peptide-vesicle association

Peptide-vesicle association was assayed either discontinuously in a direct binding assay employing gelfiltration through minicolumns, or on-line, in fluorescence experiments utilizing the tryptophan residue as an intrinsic fluorescent probe.

In the first method, 0.1 mM of peptide from a ~20-mM stock solution in DMSO was added at time zero to a 1-mM vesicle suspension exhibiting the appropriate ion gradient (lipid/peptide molar ratio, $R_i$, of 10). Where employed, valinomycin was added at $t$ = 0 from a 0.1 or 1 mg/ml solution in ethanol, and FCCP from a 0.01 mg/ml solution in ethanol. At several time points after starting the incubation 150-μl aliquots were withdrawn and within 10 s centrifuged through 1.5-ml Sephadex G 50 (medium) minicolumns (2 min, 300 · $g$) to separate free from associated peptide (Hope et al., 1985). The minicolumns were always preequilibrated with the external buffer used in the peptide-vesicle incubation. Quantitation of vesicle-associated peptide was accomplished by analysis of the filtrates for phospholipid by phosphorus determination or scintillation counting, and for peptide by measuring the tryptophan fluorescence intensity in 0.5% (wt/vol) sodium cholate containing buffer using an SLM-Aminco SPF-500 C fluorimeter and 0.5-ml cuvettes (de Kroon et al., 1989).

Ion gradient-induced peptide-lipid vesicle interaction was monitored continuously by recording the peptide's fluorescence emission at 340 nm (band pass 11 nm) on a fluorimeter (MPF3, Perkin-Elmer Corp., Norwalk, CT) under continuous stirring and at room temperature. The excitation wavelength was 280 nm (band pass 6 nm). Unless stated otherwise, 2 μM of peptide from a 2-mM stock solution in DMSO was added at $t$ = 0 to 300 μM LUV of the appropriate composition and exhibiting an ion gradient ($R_i$ = 150). Under these conditions the vesicle blank (scatter) constituted ~20% of the total signal. When required valinomycin and FCCP were added at $t$ = 0. NFG instead of gramicidin was used to dissipate ion gradients as it does not contribute to the fluorescence signal. Where applied this compound was added to a 1/400 NFG/phospholipid ratio from a 0.5-mM solution in DMSO. Fluorescence changes are always expressed as $F/F_0$ with $F$ the measured fluorescence and $F_0$ the fluorescence at time zero, both corrected for the vesicle blank.

To enable a more sensitive detection of possible ion gradient–induced changes in peptide-vesicle interaction in cases where the methods described above were not conclusive, a tryptophan-dansyl resonance energy transfer assay (Vaz et al., 1977) was applied. In these experiments 2% (mol/mol) DNS-PE was incorporated into the vesicles. The time dependent change of DNS-PE fluorescence upon addition of 20 μM peptide to 200 μM LUV ($R_i$ = 10) was recorded at 520 nm. Other conditions were as stated above for the recording of tryptophan fluorescence. A correction was applied for a vesicle blank, i.e., vesicles devoid of DNS-PE, which never exceeded 5% of the total signal.

## Determination of Δψ and ΔpH

Membrane potentials (negative inside) and transmembrane pH gradients (acidic inside) were assessed by measuring the transbilayer distribution of [$^3$H]TPP$^+$ and [$^{14}$C]MeAm employing the minicolumn separation method described above (Rottenberg, 1979; Hope et al., 1985; Redelmeier et al., 1989). [$^3$H]TPP$^+$ was added at 1 μCi/ml (corresponds to 0.026 nM) and [$^{14}$C]MeAm at 0.24 μCi/ml (5.8 μM) final concentrations to the vesicle suspensions in the presence or absence of peptide. A correction was made for the background association of the probes, which was obtained by applying the same conditions to the vesicles in the absence of an ion gradient. This background association never exceeded 1.5% of the total amount of TPP$^+$ present, for MeAm it never exceeded 0.5%. Calibration of the TPP$^+$ probe by varying the external $K^+$ concentration and applying the Nernst equation (Rottenberg, 1979), revealed that this probe overestimated the value of Δψ when applied to the LUVET 400 system (data not shown), probably due to additional probe binding to the vesicle interior (Lolkema et al., 1982; cf Redelmeier et al., 1989). Therefore, and because of the inherent inaccuracy of the method at high potentials, the TPP$^+$ data were not converted into membrane potential values. Instead, log ($[TPP^+]_{in}/[TPP^+]_{out}$) was used as a relative measure for the membrane potential. The methylamine distribution was translated to a ΔpH value according to $\Delta pH = \log([MeAm]_{in}/[MeAm]_{out})$. Redelmeier et al. (1989) have demonstrated that the MeAm distribution provides an accurate measure of the pH gradient between the interior and exterior aqueous compartments.

For a fast routine determination of Δψ the fluorescent carbocyanine probe diS-$C_2$-(5) was used (Sims et al., 1974). Briefly, 25 μM PC from a peptide-vesicle association experiment was added to 1.5 μM diS-$C_2$-(5) in the appropriate buffer and the response of the fluorescence intensity ($\lambda_{ex}$ = 622 nm, $\lambda_{em}$ = 660 nm) was recorded. This method enabled a qualitative assessment of the degree of dissipation of the membrane potential. The incorporation of BrPC into the vesicles did not interfere with this method.

## Preparation and characterization of vesicles with an asymmetric transbilayer distribution of BrPC

Following Everett et al. (1986), the membrane topology of the associated peptides was determined using vesicles with an asymmetric transbilayer distribution of brominated PC, which is a quencher of tryptophan fluorescence. PCTP was used to randomize the BrPC composition of the outer leaflets of donor and acceptor vesicles (Dawidowicz and Rothman, 1976). LUV were used as acceptor vesicles and sonicated vesicles (SUV) as donor vesicles to enable separation by centrifugation. Depending on the experimental conditions LUVET 400 and LUVET 200 were applied.

### $PC_{out}/Br_4PC_{in}LUV$

$Br_4$-PC LUVET 200, 5 μmol, labeled with 1.0 μCi (10 nmol) of the exchangeable marker [$^{14}$C]DOPC was incubated with a 20-fold excess of (100 μmol) eggPC SUV labeled with 20 μCi (0.4 nmol) of the unexchangeable marker [$^3$H]cholesteryloleoylether and prepared as described elsewhere (de Kroon et al., 1990), in the presence of 2.5 μg/ml PCTP at a total lipid concentration of 0.5 mM overnight at room temperature under continuous stirring. Both LUV and SUV were prepared in 150 mM $K_2SO_4$, 20 mM Hepes pH 7.0 and collected from respectively the pellet and the supernatant of a 1 h 170,000 · $g_{max}$ centrifugation using a 60-Ti rotor in an ultracentrifuge (L5-65, Beckman Insts., Carlsbad, CA). After the incubation the LUV were

recovered by centrifugation at the same speed and the incubation was repeated with freshly prepared SUV. After pelleting, the LUV were resuspended in $K_2SO_4$ buffer and centrifuged 1 h at 140,000 · $g_{max}$ to remove copelleted SUV. The final pellet was carefully rinsed twice with 150 mM $Na_2SO_4$, 20 mM Hepes pH 7.0, before resuspending it in this buffer to create a $K^+/Na^+$ ion gradient. Subsequently, these LUV were used in the fluorescence experiments.

Typically this procedure resulted in a LUV preparation (yield: 60%) containing a SUV contamination amounting to 10% of the total lipid. The asymmetry of the LUV was estimated by two independent methods. Scintillation counting demonstrated the exchange of 45% of the [$^{14}$C]DOPC initially present in the LUV. Provided that the exchange equilibrium attained for DOPC and $Br_4$PC is similar, and assuming a 47/53 phospholipid distribution between inner and outer leaflet of the LUVET 200 based on the vesicle size (Mayer et al., 1986*a*), this would indicate that the asymmetric LUV retain 15% $Br_4$PC in their outer leaflet.

Everett et al. (1986) have introduced the fluorescent amphiphile CUGA as a suitable asymmetry probe. LUVET 200 with different amounts of $Br_4$PC incorporated were used to calibrate the quenching of CUGA fluorescence. 20 μM of vesicles was added to 2.5 μM CUGA in buffer under continuous stirring at room temperature and the fluorescence intensity ($\lambda_{ex}$ = 300 nm, $\lambda_{em}$ = 353 nm) was read after 2 min. Taking into account the SUV contamination of the LUV preparation, it could be shown by the CUGA method that the asymmetric LUV contained at most 20% $Br_4$PC in the outer leaflet, in good agreement with the number deduced from scintillation counting.

## 6,7-$Br_2PC_{out}/PC_{in}$LUV

Briefly, 4 μmol eggPC LUV and 9 μmol eggPC/6,7-$Br_2$PC 3/1 (mol/mol) SUV labeled with both 2 μCi [$^{14}$C]DOPC and 4 μCi [$^3$H]cholesteryloleolether were incubated overnight with PCTP as described above. LUV and SUV had been precentrifuged for 1 h at 260,000 · $g_{max}$ and, respectively, the pellet and supernatant were used. To efficiently spin down the PC LUV, the incubation was carried out in 100 mM $K_2SO_4$ instead of 150 mM $K_2SO_4$ buffer. For the same reason, LUVET 400 were used and not LUVET 200. 6,7-$Br_2$PC and not $Br_4$PC was used and incorporated to only 25% in the donor SUV to avoid copelleting of SUV. After one round of exchange, the pellet fraction of a 260,000 · $g_{max}$ centrifugation was passed through a Sephadex G50 column eluted with 100 mM $Na_2SO_4$, 20 mM Hepes pH 7.0 to generate the ion gradient before use in the fluorescence experiments.

The recovery of LUV in the pellet fraction was 60% and the contamination with SUV amounted to 30% of the total lipid. Based on scintillation counting of pellet and supernatant it was calculated that 12% of the [$^{14}$C]DOPC initially present in the SUV had been exchanged from SUV to LUV. Under the assumption made above for exchange efficacy and taking a phospholipid distribution between inner and outer leaflet of 48/52 for LUVET 400, this number would correspond to a 13% 6,7-$Br_2$PC content in the outer leaflet of the resulting asymmetric LUV. Using CUGA as asymmetry probe after calibration with 6,7-$Br_2$PC containing LUVET 400 in the presence of 30% SUV containing 25% 6,7-$Br_2$PC, the 6,7-$Br_2$PC content of the LUV outer membrane leaflet was determined to be at least 8%.

# RESULTS

## Localization

In assessing the localization of the associated peptides, advantage was taken of the fluorescent properties of the intrinsic tryptophan residue. Fig. 1 shows that the $K^+$ diffusion potential-enhanced vesicle association of $AIXme^+$ (Table 1) is apparent from an increased fluorescence intensity at 340 nm in on-line tryptophan fluorescence measurements. The increased emission intensity which is accompanied by a blue shift of the wavelength of maximum emission (data not shown), indicates that the tryptophan enters a different, more hydrophobic environment. In the absence of an ion gradient, the addition of PC LUV to $AIXme^+$ to a lipid/peptide molar ratio of 150 results in an increase of the fluorescence intensity of <10%, reflecting the low affinity of this peptide for PC bilayers (de Kroon et al., 1990).

The rate and the extent of the fluorescence increase induced by the addition of valinomycin to PC LUV exhibiting a $K^+/Na^+$ gradient, depend on the amount of valinomycin added (shown for val/PC = $10^{-4}$ and $10^{-3}$ in Fig. 1). Whereas, at the lower valinomycin concentration, the fluorescence increase initially proceeds more slowly, the maximum emission intensity attainable is greater than at the higher valinomycin concentration. The higher fluorescence intensity reached reflects that the membrane potential is larger and more stable at the lower valinomycin concentration, as was confirmed by $TPP^+$ distribution measurements (data not shown). The

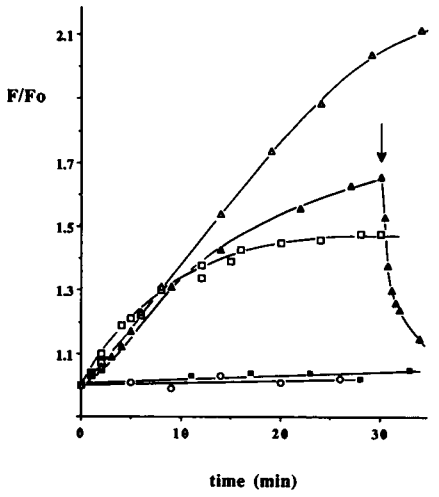


FIGURE 1 Time course of the ion gradient induced tryptophan fluorescence change at 340 nm of 2 μM $AIXme^+$ added to 300 μM PC LUV. LUV were prepared in 150 mM $K_2SO_4$, 20 mM Hepes pH 7.0 and the untrapped buffer was replaced by 150 mM $Na_2SO_4$, 20 mM Hepes pH 7.0 (see Methods). Data are shown for vesicles experiencing a $K^+/Na^+$ gradient in the absence of ionophores (■), with valinomycin added to a $10^{-3}$ (□) or $10^{-4}$ (▲) molar ratio with respect to PC, with both valinomycin and FCCP added to molar ratios of $10^{-4}$ and $1.3 \cdot 10^{-4}$ with respect to PC (△), and for vesicles with $K^+$ on both sides of the membrane (○). The ionophores were added at $t$ = 0. The arrow indicates the addition of NFG to a 1:400 NFG/PC molar ratio.

dependence of the initial kinetics of the fluorescence increase on the valinomycin concentration is most likely due to the difference in lagtime for achieving a homogeneous valinomycin-vesicle distribution and was observed earlier for the membrane potential probe safranine O (Woolley et al., 1987). These data allow the conclusion that the ionophore only contributes to the peptide-vesicle association by switching on the membrane potential. The addition of valinomycin together with the proton ionophore FCCP at a low concentration (FCCP/PC = $1.3 \cdot 10^{-4}$) further enhances the fluorescence increase (Fig. 1) and provides a first clue for a role of the pH gradient in the process. The addition of FCCP alone does not significantly affect the fluorescence level. The reversibility of the peptide-vesicle association is evidenced by the fast drop in fluorescence intensity upon dissipation of the ion gradient by NFG (Fig. 1; cf de Kroon et al., 1989).

The susceptibility of tryptophan fluorescence to quenching by brominated PC incorporated into the LUV experiencing a membrane potential shows that the peptide AIXme$^+$ is inserted into the membrane. The quenching profile depicted in Fig. 2 demonstrates that AIXme$^+$ attains an interfacial localization. It should be stated that the quenching efficiency by 2-BrPC is an underestimate of its actual value as the bromine content of this lipid is only 37% (de Kroon et al., 1990). Furthermore, a comparison of the quenching efficiency of 2-BrPC to that of the dibromo-PC is inherently impeded by the different number of bromines per molecule (Bolen and Holloway, 1990).

To distinguish between the possibility that the peptide adsorbs at the outer vesicle surface and the possibility that it accumulates at the interface of the inner membrane leaflet after translocating across the bilayer, vesicles with an asymmetric transbilayer distribution of BrPC were used in the fluorescence measurements (Fig. 3).

Upon addition of valinomycin to asymmetric vesicles containing 100% $Br_4$PC in the inner, and at least 80% PC in the outer membrane leaflet, a decrease of AIXme$^+$ fluorescence is apparent that can be enhanced in the presence of FCCP (Fig. 3 *a*) and reversed by adding NFG (data not shown). In the control experiments using PC LUV with a symmetric transbilayer distribution of 20% $Br_4$PC, a fluorescence increase is observed (Fig. 3 *a*)

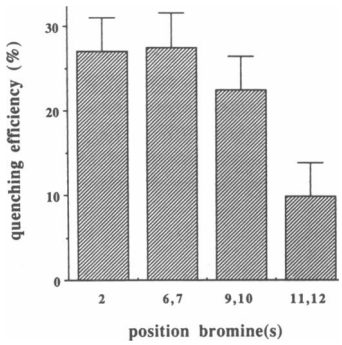


FIGURE 2 Depth dependent quenching efficiency of AIXme$^+$ fluorescence at 30% (mol/mol) brominated PC incorporation into PC LUV experiencing a valinomycin-induced $K^+$ diffusion potential. Fluorescence intensities, $F$, were measured 30 min after the addition of valinomycin to a $10^{-3}$ molar ratio with respect to PC and the quenching efficiency was calculated according to $(1-F/F_0) \cdot 100\%$ with $F_0$ the fluorescence intensity measured in the absence of brominated PC. The error bars indicate the absolute error. DiS-$C_2$-(5) measurements revealed no significant differences in membrane potential stability upon comparison of LUV with and without 30% of each of the BrPC incorporated.

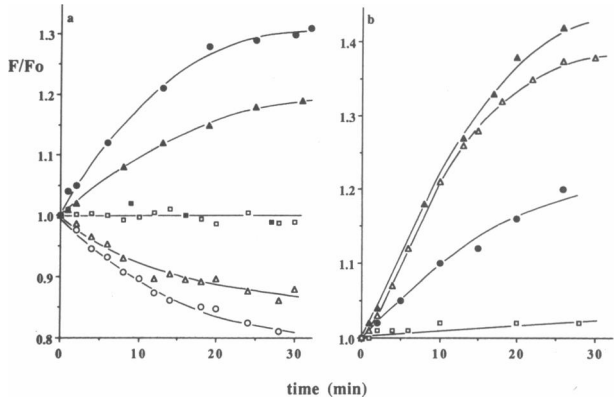


FIGURE 3 A $K^+$ diffusion potential applied to PC LUV induces the quenching of AIXme$^+$ fluorescence by BrPC present in the inner membrane leaflet (*a*) but not by BrPC exclusively present in the outer membrane leaflet (*b*). (*a*) Fluorescence change of AIXme$^+$ in response to LUVET 200 with an asymmetric transbilayer distribution of 100% $Br_4$PC in the inner membrane leaflet and $\leq$20% $Br_4$PC and $\geq$80% PC in the outer membrane leaflet in the absence of an ion gradient (□), in the presence of a $K_{in}^+/Na_{out}^+$ gradient after addition of valinomycin to a $10^{-4}$ molar ratio with respect to PC (△) and after addition of both valinomycin and FCCP to molar ratios of $10^{-4}$ and $1.3 \cdot 10^{-4}$ with respect to PC (○). The corresponding closed symbols represent identical conditions applied to symmetric LUVET 200 with 20% $Br_4$PC incorporated. (*b*) Fluorescence change of AIXme$^+$ upon addition to LUVET 400 containing 6,7-$Br_2$PC exclusively in the outer membrane leaflet and experiencing a $K_{in}^+/Na_{out}^+$ gradient without (□) and with valinomycin added at $t = 0$ (△). For comparison the fluorescence response to PC LUVET 400 (▲) and 12.5% 6,7-$Br_2$PC containing symmetric LUVET 400 (●) exhibiting a valinomycin-induced $K^+$ diffusion potential is shown; 128 μM 25% 6,7-$Br_2$PC containing SUV ($K_{in}^+/Na_{out}^+$) was present in these experiments to mimic the SUV contamination of the asymmetric LUV preparation (see Methods). The use of 100 mM $K_2SO_4/Na_2SO_4$ instead of 150 mM in this experiment (see Methods) did not affect the fluorescence measurements. The val/PC molar ratio was $2 \cdot 10^{-4}$; other concentrations as in Fig. 1.

which is less than that obtained for PC LUV, as expected in view of the data presented in Fig. 2. Taken together, these data allow the conclusion that at least part of the peptide's fluorescence is quenched by bromines in the inner bilayer leaflet.

Conversely, the membrane potential induces a fluorescence increase when applied to asymmetric vesicles containing 8–13% 6,7-$Br_2$PC exclusively in the outer leaflet, to an extent that is similar to that obtained in the control experiment for PC LUV and greater than that recorded for symmetric 12.5% 6,7-$Br_2$PC containing vesicles (Fig. 3 *b*).

The lack of fluorescence quenching by BrPC in the outer membrane leaflet (Fig. 3 *b*) infers that the entire quenching by BrPC (Figs. 2, 3 *a*) must occur in the inner leaflet, with the associated peptide consequently residing in the interface of the inner membrane leaflet.

Reexamination of the bilayer asymmetry of CUGA showed that no redistribution (flip-flop) of $Br_4$PC occurs during the peptide uptake experiments. Other control experiments concern the stability of the ion gradient maintained by the asymmetric $Br_4PC_{in}/PC_{out}$ vesicles. Membrane potential measurements employing diS-$C_2$-(5) carried out after the incubation with the peptide, yielded responses which were indistinguishable for the asymmetric vesicles and the symmetric 20% $Br_4$PC containing LUV (data not shown). The two vesicle types used in the experiments described above (Fig. 3) yield different $AIXme^+$ fluorescence responses upon applying a $K^+$ diffusion potential (see Discussion).

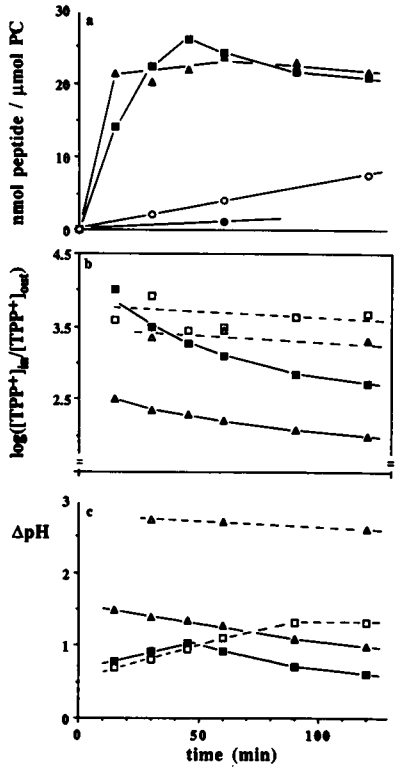


FIGURE 4 (*a*) Time course of ion gradient induced vesicle accumulation of $AIXme^+$ assayed by the minicolumn method. Aliquots were taken from incubations of 0.1 mM $AIXme^+$ and 1 mM PC LUV with $K^+$ at both sides of the membrane (●) or exhibiting a $K^+/Na^+$ gradient in the absence (○) and presence of valinomycin (■, val/PC = $10^{-3}$) and in the presence of both FCCP and valinomycin (▲, FCCP/PC = $1.3 \cdot 10^{-4}$, val/PC = $10^{-3}$). (*b*, *c*) In parallel, the effect of $AIXme^+$ on $\Delta\psi$ (*b*) and $\Delta$pH (*c*) was determined. For this purpose [$^3$H]$TPP^+$ and [$^{14}$C]MeAm were included in incubations with (*closed symbols, drawn lines*) and without (*open symbols, dashed lines*) $AIXme^+$ present. Results are shown for $K_{in}^+/Na_{out}^+$ vesicles with valinomycin (*squares*) and both FCCP and valinomycin present (*triangles*). Conditions as in *a*. The error in the peptide association (*a*) amounts to 10%; the absolute error in the $TPP^+$ (*b*) and MeAm (*c*) distribution data is ± 0.2.

## The roles of $\Delta\psi$, $\Delta$pH, and pH

To elucidate the mechanism by which the peptide attains a localization in the interface of the inner bilayer leaflet, the involvement of the pH gradient and the influence of the pH have been investigated. It is well established that due to the proton permeability of the lipid bilayer, a $K^+$ diffusion potential evokes a transbilayer pH gradient (Nichols et al., 1980; Cafiso and Hubbell, 1983; Redelmeier et al., 1989).

In Fig. 4, the time course of $AIXme^+$ uptake assayed by gel filtration on minicolumns is shown (*a*). In parallel, the concomitant changes in $\Delta\psi$ and $\Delta$pH were determined from $TPP^+$ and MeAm distribution (*b* and *c*). In the absence of ionophores, the $K^+/Na^+$ gradient induces a slowly proceeding uptake of peptide that was not discerned previously (de Kroon et al., 1989). $\Delta\psi$ measurements reveal the gradual development of a potential up to a log ($[TPP^+]_{in}/[TPP^+]_{out}$) value of ~1.4 after 2 h under these conditions (data not shown).

In the absence of peptide, valinomycin induces a stable $\Delta\psi$, corresponding to log ($[TPP^+]_{in}/[TPP^+]_{out}$) ~3.8, that gives rise to the gradual building up of a pH gradient that amounts to ~1.4 pH units after 2 h (Figs. 4 *b* and *c*), in agreement with Mayer et al. (1988). With the uptake of $AIXme^+$, which is present at $R_i = 10$, the membrane potential is gradually dissipated, whereas remarkably, the decline of the pH gradient does not set in until ~50 min after starting the incubation (Fig. 4 *c*). The latter observation suggests that the interaction of the peptide with the vesicle membrane accelerates the development of the $K^+$ diffusion potential-induced pH gradient. The influence of the peptide on the $K^+$ diffusion potential dependent pH gradient is obvious when the induced pH gradient is measured as function of the applied $K^+$ gradient in the absence and presence of the peptide at $R_i = 10$, as shown in Fig. 5. The

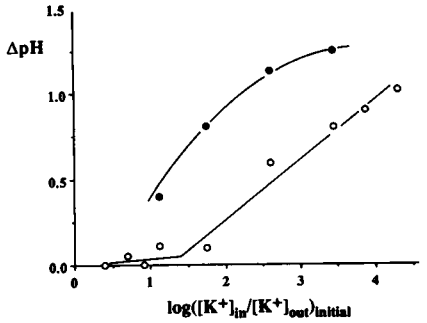


FIGURE 5 ΔpH as a function of the applied transmembrane $K^+$ gradient in the absence (○) and presence of peptide at a lipid/peptide molar ratio of 10 (●). The pH gradient was determined from the MeAm distribution in a 1 mM PC incubation, 30 min after the addition of valinomycin (val/PC = $10^{-3}$). The internal buffer was 150 mM $K_2SO_4$, 20 mM Hepes pH 7.0, the isoosmotic external buffer contained various ratios of $K_2SO_4/Na_2SO_4$, 20 mM Hepes pH 7.0.

apparent threshold dependence on the $K^+$ gradient for the induction of a pH gradient in the absence of peptide, and the increased acidification of the vesicle interior under the influence of the compound taken up, have been reported previously (Mayer et al., 1988; Redelmeier et al., 1989).

FCCP renders the membrane permeable to protons. The presence of both valinomycin and FCCP should give rise to $\Delta\psi$ and ΔpH values corresponding to electrochemical equilibrium between the electrical potential and the induced pH gradient (Redelmeier et al., 1989). Under these conditions, a ΔpH of 2.7 units is measured with the value of $\log([TPP^+]_{in}/[TPP^+]_{out})$ amounting to 3.4 (Figs. 4 *b* and *c*). The discrepancy observed between $\log([TPP^+]_{in}/[TPP^+]_{out})$ and $\log([MeAm]_{in}/[MeAm]_{out})$ may partly be due to membrane binding of $TPP^+$ (see Methods). When both FCCP and valinomycin are present, the rate of the peptide uptake is increased over that occurring in the presence of valinomycin alone, but the maximum level of uptake is similar in both cases. The fast peptide accumulation in the val/FCCP system is paralleled by an immediate reduction of $\Delta\psi$ and ΔpH (Fig. 4). The strong reduction of the ΔpH points to a buffering of the internal pH by the peptide taken up, and was also observed in experiments using PC LUV with applied pH gradients (data not shown).

The data at this point seem to indicate that the pH gradient evoked by the $K^+$ diffusion potential, is the primary cause of the uptake of $AIXme^+$. However, a comparison of the residual pH gradients remaining after the uptake of similar amounts of peptide in response to valinomycin alone and to both FCCP and valinomycin (Figs. 4 *a* and *c*), tentatively suggests a contribution by $\Delta\psi$ to the uptake process other than the mere induction of the pH gradient.

The investigation of this putative additional role for $\Delta\psi$ has been pursued in the fluorescence experiments where a much lower peptide/lipid ratio is used ($R_i$ = 150). These conditions are expected to enable a better distinction between the roles of $\Delta\psi$ and ΔpH, as the profound enhancement by the peptide of the $K^+$ diffusion potential-induced pH gradient to a level which is disguised due to buffering by the internalized peptide, will be minimized. Furthermore, to neutralize the $K^+$ diffusion potential-induced pH gradient, these experiments have been performed in phosphate buffered systems which have a greater buffering capacity than the systems used so far. The experiments were carried out at pH 6, 7, and 8 to assess the role of the protonation degree of the peptide. Upon addition of valinomycin, the phosphate-buffered vesicle systems in the presence of $AIXme^+$ at $R_i$ = 150 display a $\Delta\psi$ characterized by $\log([TPP^+]_{in}/[TPP^+]_{out})$ distributions in excess of 3.4, without any detectable pH gradient until at least 30 min after valinomycin addition, irrespective of the pH. As is shown in Fig. 6, under these conditions a modest $AIXme^+$ fluorescence increase at pH 7.0 is apparent (cf Fig. 1) which is larger than that at pH 8.0 whereas at pH 6.0 there is no fluorescence change.

Upon addition of both valinomycin (val/PC = $10^{-3}$)

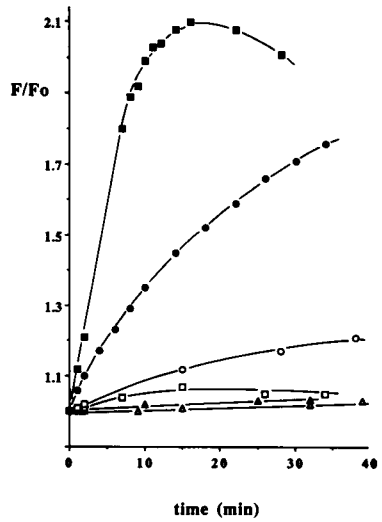


FIGURE 6 $AIXme^+$ fluorescence increases recorded in phosphate buffered systems exhibiting a $K_{in}^+/Na_{out}^+$ gradient at different pH values. LUV were prepared in 200 mM $KP_i$ at pH 6.0 (*triangles*), pH 7.0 (*circles*), or pH 8.0 (*squares*) and the nonenclosed buffer was replaced by 200 mM $NaP_i$ adjusted to the same pH. Valinomycin (val/PC = $10^{-3}$, *open symbols*) or both FCCP and valinomycin (FCCP/PC = $1.3 \cdot 10^{-4}$, val/PC = $10^{-3}$, *closed symbols*) were added at $t$ = 0 to 300 μM PC LUV, 2 μM $AIXme^+$.

and FCCP (FCCP/PC = $1.3 \cdot 10^{-4}$) the rate and the extent of the fluorescence increase at pH 8.0 is tremendously enhanced, at pH 7.0 this enhancement is less dramatic whereas at pH 6.0 no change is observed (Fig. 6). Under these conditions a log ($[TPP^+]_{in}/[TPP^+]_{out}$) value of 3.1 is measured irrespective of the pH, and the induced pH gradients amount to 1.55 at pH 6.0, 1.93 at pH 7.0, and 1.85 at pH 8.0 after 30 min. The data seem to indicate that the relative roles of $\Delta\psi$ and $\Delta$pH in the peptide uptake process depend on the protonation degree of the peptide.

## Peptide and lipid specificity

Qualitatively similar results as reported above for $AIXme^+$ have been found for its pentamerous analogue $AXme^+$. However, the efficiency of peptide uptake is less for this peptide (de Kroon et al., 1989). This difference is also evident from the time course of tryptophan fluorescence changes (data not shown).

To find out whether a $K^+$ diffusion potential (negative inside) also affects the membrane interaction of peptides carrying more than one positive charge, two divalent positively charged peptide analogues of $AXme^+$ were examined. Neither $AXetN^{2+}$, with a $NH_3^+$-moiety at each end of the molecule, nor $RXme^{2+}$ with a $NH_3^+$- and a side chain guanidinium group at the $NH_2$-terminus of the molecule, showed any response to, or effect on a $K^+$ diffusion potential, as judged from both fluorescence and minicolumn experiments in which peptide association and $\Delta\psi$ dissipation ($TPP^+$) were assayed. Not even at external pH values in the range 8–9, where deprotonation is expected to neutralize at least one of the positive charges, was any effect detectable (data not shown).

The introduction of a negative surface charge greatly enhances the affinity of the positively charged peptides for phospholipid bilayers, which could influence the effect of ion gradients on the peptide-membrane interaction. Because previously the interaction of these peptides with SUV consisting of the acidic phospholipid CL has been characterized (de Kroon et al., 1990), this possibility was analyzed by incorporating CL into the LUV and studying the $K^+$ diffusion potential-induced peptide association. The presence of CL tends to enhance the initial rate of the fluorescence increase of $AIXme^+$ (Fig. 7). In the absence of valinomycin, at 50% CL incorporation, $AIXme^+$ fluorescence also increases (Fig. 7). This can probably be accounted for by the increased $K^+$ permeability due to CL incorporation (cf Mayer et al., 1985). $TPP^+$ distribution data revealed that with increasing CL contents the ability of the vesicles to maintain a stable $\Delta\psi$ is impaired (even though this effect was partly masked by enhanced $TPP^+$ binding due to the negative surface charge; data not shown). This deterio-

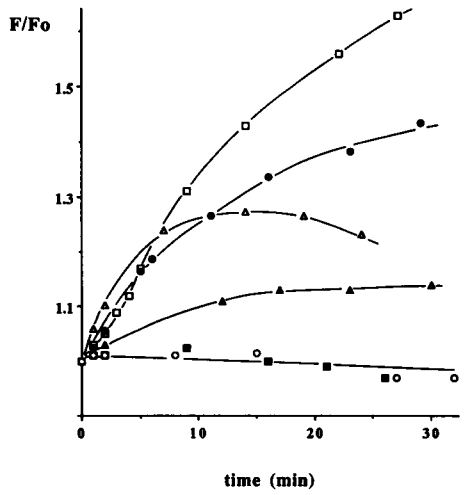


FIGURE 7 The fluorescence change of $AIXme^+$ in response to a valinomycin induced $K^+$ diffusion potential applied to PC (□), PC/CL 3/1 (●), and PC/CL 1/1(△) vesicles compared to that of the peptides $RXme^{2+}$ (■) and $AXetN^{2+}$ (○) in response to PC/CL 1/1 vesicles under the same conditions. The fluorescence increase of $AIXme^+$ in the presence of PC/CL 1/1 without valinomycin added is also shown (▲). Valinomycin was added at $t = 0$ to 300 μM LUV, 2 μM peptide at a valinomycin/phospholipid molar ratio of $10^{-4}$. Buffers as in Fig. 1, containing 2 mM EDTA. The fluorescence increase is related to that at $t = 0$ ($F/F_O$), disregarding the initial fluorescence increase due to spontaneous peptide-vesicle association $(F/F_O)_i$ (1.3 for $RXme^{2+}$ and $AXetN^{2+}$, and 1.2 for $AIXme^+$ in the presence of PC/CL 1/1; 1.1 for $AIXme^+$ in the presence of PC/CL 3/1).

rating stability is reflected in the maximum factor of fluorescence increase becoming smaller as more CL is incorporated, and in the subsequent fluorescence decline observed for PC/CL 1/1 (Fig. 7).

In contrast to $AIXme^+$ the divalent peptides $AXetN^{2+}$ and $RXme^{2+}$ (Table 1) do not show any additional change in fluorescence after the initial increase due to "spontaneous" binding (Fig. 7 legend) upon applying a $K^+$ diffusion potential to vesicles consisting of PC/CL 1/1 at pH 7.0 (Fig. 7). The same negative result was obtained at external pH values up to 8.7 and at PC/CL molar ratios of 3/1 and 9/1 (data not shown).

The RET assay (see Methods) monitors energy transfer from the associated peptide's tryptophan to membrane incorporated DNS-PE and allows the use of much higher peptide concentrations. Application of this assay to $AIXme^+$ reveals an increased energy transfer efficiency in response to a $K^+$ diffusion potential, which is consistent with the tryptophan fluorescence data except for the earlier onset of the fluorescence decline (cf Figs. 7 and 8). The latter is accounted for by the larger amount of peptide destabilizing the ion gradient under the conditions of the RET assay. The divalent peptides in the presence of PC/CL vesicles at a lipid/peptide

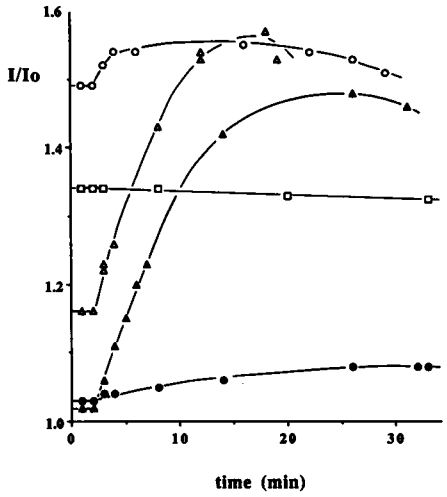


FIGURE 8 The effect of a valinomycin-induced $K^+$ diffusion potential on peptide-lipid vesicle interaction monitored by resonance energy transfer. DNS-PE was incorporated at 2% in PC/CL 3/1 (*closed symbols*) and PC/CL 1/1 vesicles (*open symbols*). At $t = 0$ the peptides AIXme$^+$ (*triangles*), RXme$^{2+}$ (*circles*) and AXetN$^{2+}$ (*squares*) were added at a concentration of 20 μM to 200 μM LUV exhibiting a $K_{in}^+/Na_{out}^+$ gradient. Valinomycin was introduced at $t = 2$ min (val/PC = $10^{-4}$). The fluorescence intensity $I$ is related to $I_0$ the fluorescence emitted by the vesicles in the absence of peptide.

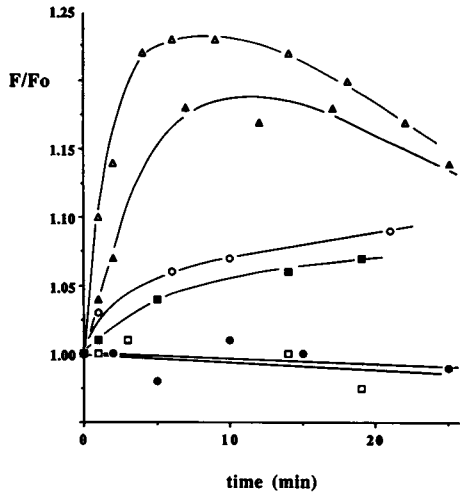


FIGURE 9 Effect of a reversed ion gradient, $Na_{in}^+/K_{out}^+$, on the tryptophan fluorescence of anionic peptides at pH 5.0. Peptides were added at 2 μM to PC LUV prepared in 150 mM $Na_2SO_4$, 10 mM HMG pH 5.0 and diluted to 300 μM in 150 mM $K_2SO_4$, 10 mM HMG pH 5.0. Data are shown for bocAX$^-$ in the absence (●) and presence of valinomycin (○) and for bocAIX$^-$ under the same conditions (■, ▲), and with both FCCP and valinomycin present (△) and in the absence of an ion gradient (□, $Na_{in}^+/Na_{out}^+$). Valinomycin and FCCP were added at $t = 0$ to ratios of $10^{-4}$ and $1.3 \cdot 10^{-4}$ with respect to PC, respectively.

molar ratio of 10 revealed a small membrane potential driven energy transfer increase for RXme$^{2+}$ which was larger at 50% than at 25% CL incorporation (Fig. 8). In contrast, the peptide AXetN$^{2+}$ appears not to be susceptible to membrane potential up to PC/CL ratios of 1/1 although it does show significant vesicle association, apparent from the initial fluorescence level ($I/I_0$ at $t = 0$ in Fig. 8). FCCP does not affect the valinomycin induced RET changes of the divalent peptides (data not shown).

To examine whether oppositely charged peptide analogue of AIXme$^+$ are likewise affected by ion gradients with a reversed polarity, the anionic peptides bocAX$^-$ and bocAIX$^-$ were tested using vesicles exhibiting a $Na^+$ inside/$K^+$ outside gradient. Fig. 9 shows the results at pH 5: the peptide bocAIX$^-$ shows a valinomycin induced fluorescence increase that is enhanced in the presence of FCCP, and that can be reversed by NFG (data not shown); also in the absence of valinomycin the ion gradient gives rise to a gradual fluorescence increase. The less hydrophobic peptide boxAX$^-$ displays consistent changes in fluorescence although to a lesser extent (Fig. 9). At pH 4 the rate, but not the extent, of the fluorescence increase is enhanced, whereas at pH 6 there is hardly a significant membrane potential effect detectable (data not shown). As was found for AIXme$^+$ and AXme$^+$ the fluorescence responses of the anionic peptides are highly dependent on the pH of the system as well as on the hydrophobicity of the peptide.

## DISCUSSION

The $K^+$ diffusion potential-enhanced vesicle association of peptides carrying a single positive charge, previously established in a gel filtration assay (de Kroon et al., 1989) is paralleled by an increase of the fluorescence emission intensity of the peptides' intrinsic tryptophan residue. The first method allows the quantitation of the amount of peptide associated. The second method enables the localization of the associated peptide molecules in the vesicle.

Quenching of tryptophan fluorescence by brominated phospholipids that serve as a molecular ruler of insertion depth, demonstrated that the associated peptide is preferentially localized in the membrane-solution interface. The accumulation of peptide in the interface of the inner and not the outer membrane leaflet of the PC vesicles was unambiguously demonstrated in two complementary experiments, in which asymmetric PC vesicles were used either containing BrPC in the inner and eggPC in the outer leaflet of the bilayer, or containing BrPC exclusively in the outer leaflet of the membrane (Fig. 3). The results of these experiments imply that the entire fluorescence increase observed for AIXme$^+$ in response to a $K^+$ diffusion potential is due to the

internalized peptide and not to peptide adsorbed at the outside of the vesicles.

A comparison of the $AIXme^+$ fluorescence responses in the presence of LUV differing in trapped volume (LUVET 400 and LUVET 200, data not shown) reveals a faster and larger fluorescence increase for the smaller vesicle type. This is consistent with the fluorescence increase reflecting the internal peptide concentration dependent partioning of peptide into the bilayer. Estimating from the data that under the conditions of the fluorescence experiments (lipid/peptide ratio of 150) the amount of accumulated peptide is in the order of 50% of the total amount of peptide present, a theoretical peptide concentration of ~1 mM inside the LUVET 400 (trapped volume: 3 μl/μmol) can be calculated. At this peptide concentration considerable membrane partitioning is expected (cf de Kroon et al., 1990). The rapid return of the fluorescence intensity to its initial level upon dissipation of the ion gradient rules out the formation of large peptide aggregates within the vesicles.

In the minicolumn gelfiltration experiments (lipid/peptide ratio of 10) the calculated, theoretical internal peptide concentration attained upon application of a $K^+$ diffusion potential would amount to ~10 mM. Consequently the effects exerted by the membrane inserted peptide on the ion gradient stability are much more profound under these conditions than under the conditions of the fluorescence assay.

The peptide was shown to affect the ion gradients in two ways: (*a*) It increases the ion permeability of the lipid bilayer resulting in the acceleration both of the $\Delta\psi$-induced proton gradient development (Figs. 4 *c* and 5), and of the eventual dissipation of the ion gradients (Fig. 4). (*b*) The internalized peptide increases the pH inside the vesicles (Fig. 4 *c*). The latter is also apparent from the data presented in Fig. 10, where the $AIXme^+$ fluorescence intensity read 30 min after the addition of valinomycin is plotted against the $K^+$ gradient applied to the PC LUV, for lipid/peptide molar ratios of 250 and 150. There is a striking difference in curve shape under these two conditions. Whereas at 500 μM PC, the shape of the curve is sigmoidal resembling that obtained for potential sensitive fluorescence probes such as safranine (Woolley et al., 1987), at 300 μM the $K^+$ gradient dependence of the fluorescence increase displays a distinct "dip." This characteristic is probably due to the buffering capacity of the internalized peptide that counterbalances the induced pH gradient in that particular range of the initially applied $\Delta\psi$. At 500 μM PC this effect is not apparent because in the range of $K^+$ gradients studied the maximum attainable internal concentration of peptide is too low to affect $\Delta$pH, due to the larger internal volume available. This result confirms the

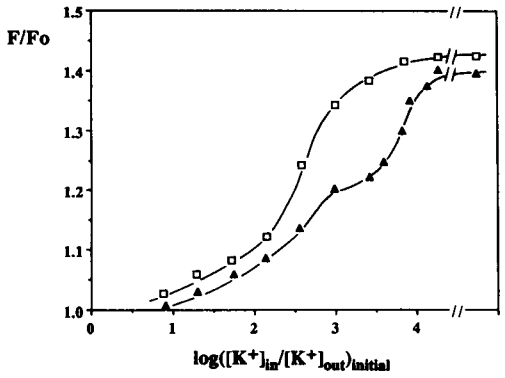


FIGURE 10 The $K^+$ diffusion potential-induced $AIXme^+$ fluorescence increase 30 min after the addition of valinomycin plotted against the applied $K^+$ gradient. Cuvette solutions contained 2 μM $AIXme^+$ and either 300 μM (△) or 500 μM PC LUV (□). Other conditions as in Fig. 5.

importance of the pH gradient for the peptide uptake and underscores the sensitivity of the fluorescence assay. Likewise, the $\Delta$pH probe MeAm which is used at a relatively high concentration (see Methods) may affect the internal pH of the vesicles if the buffering capacity in the vesicle lumen is limited.

With respect to the mechanism of the peptide accumulation, the obvious importance of the pH gradient implies that membrane translocation of the deprotonated form provides a major, if not the only contribution to the process. Proton-gradient driven vesicle uptake of weak bases due to the high permeability of the neutral form is well established for a large number of compounds containing an amino function (Nichols and Deamer, 1976; Mayer et al., 1986*b*; Bally et al., 1988). In several cases however, the uptake of these compounds in response to a $K^+$ diffusion potential could not be entirely accounted for by the $\Delta\psi$-induced pH gradient, suggesting that $\Delta\psi$ may be able to drive the uptake of the protonated, charged form of the molecules (Mayer et al., 1988; Bally et al., 1988).

The fluorescence experiments employing phosphate buffered systems suggest that the relative contributions of $\Delta\psi$ and $\Delta$pH to the uptake of the peptide depend strongly on the pH and consequently on the protonation degree of $AIXme^+$ (Fig. 6). The typical $pK_a$ value of a $NH_2$-terminal amino group in a peptide is 8.0 (Stryer, 1981). At pH 6, where most of the peptide molecules are in the protonated, charged state, no significant peptide uptake occurs, irrespective of the presence of a pH gradient, inferring that only membrane translocation of the uncharged molecule can occur. Accordingly, at the higher pH values in the presence of both valinomycin and FCCP, the efficiency of peptide uptake reflects the

deprotonation degree of the peptide. If only valinomycin is present, the uptake is drastically reduced. However, interestingly, under these conditions the fluorescence increase recorded at pH 7 is distinctly larger than that at pH 8.

The latter result could be interpreted as a direct influence of $\Delta\psi$ on the charged peptide, that can only reveal itself if the peptide is translocation competent, i.e., when the pH allows the peptide to deprotonate. The possibility that the fluorescence increase at pH 7 originates from peptide adsorbed to the outside surface of the vesicle is considered highly unlikely in view of the virtual absence of a fluorescence increase at pH 6 and in view of the results obtained using the asymmetric vesicles containing BrPC exclusively in the outer leaflet (Fig. 3 *b*).

The data are consistent with the following uptake mechanism. The $K^+$ diffusion potential driven vesicle-accumulation of peptides with a single ionizable amino function is primarily the result of a redistribution of a weak base across the bilayer in response to the $\Delta$pH induced by the $\Delta\psi$. This mechanism infers that the neutral species of the peptide crosses the bilayer and explains the strong pH dependence of the process. The efficiency of transbilayer diffusion will depend on the permeability coefficient of the peptide. The membrane permeability coefficient of a solute, $P_m$, on its turn, depends on the solute's partition coefficient $\alpha$ ($=c_m/c_a$, where $c_m$ and $c_a$ denote the peptide's concentrations in the membrane and aqueous phase, respectively) according to $P_m = \alpha \cdot D_m/\delta_m$, where $D_m$ is the solute's diffusion coefficient in the membrane and $\delta_m$ the thickness of the membrane's hydrophobic core (Andersen, 1989). Possibly, $\Delta\psi$ is able to attract the protonated form of the peptide promoting its insertion into the membrane. This would shift to some extent the dynamic equilibrium between the free and membrane partitioned form of the protonated peptide in favor of the latter, leading to an enhancement of peptide permeation if the outside pH permits deprotonation. Without a $\Delta$pH present, the translocated peptide is expected to immediately flow back to the external aqueous phase. Nevertheless, a small $\Delta\psi$-induced net inward flux can be detected which is greater at pH 7 than at pH 8 (Fig. 6), in agreement with the notion that $\Delta\psi$ would affect the charged species of the peptide.

An alternative explanation for the effects that so far have been attributed to $\Delta\psi$ takes into account the existence of unstirred layers adjacent to the membrane. These are likely to influence, and to be influenced by, an accumulation of peptide in the membrane-water interface. It is conceivable that in the presence of a $K^+$ diffusion potential, the pH in the stagnant aqueous solution adjacent to the bilayer is lower than the pH of the bulk solution inside the vesicle which is measured by MeAm. This difference may be larger at pH 7 than at pH 8, simply because it takes more protons to create a local pH difference at pH 8 than at pH 7. In addition, once the peptide has been taken up, appreciable pH differences are likely to develop across the unstirred layers as a result of the concentration polarization of the unionized species (Gutknecht and Tosteson, 1973; Andersen, 1989), that limit the permeation of the peptide. The latter phenomenon probably accounts for the development of the fluorescence increase in the absence of a bulk $\Delta$pH at pH 7 and pH 8 (Fig. 6), irrespective of the first cause of the uptake being a direct $\Delta\psi$ effect or the initial pH gradient between the unstirred layers.

The introduction of a negative surface charge by incorporation of CL into the vesicles will increase the peptide's concentration at the membrane surface leading to an enhanced partitioning of $AIXme^+$ into the membrane (de Kroon et al., 1990), which is expected to promote the $K^+$ diffusion potential-induced peptide uptake, provided that the changes in ion gradient stability do not interfere. On the contrary, the lower surface pH arising from the negative surface charge (MacDonald et al., 1976) is likely to inhibit the deprotonation of the peptide that is required for uptake. The experiment reveals an $AIXme^+$ fluorescence response of which the initial rate appears to increase with increasing CL contents up to 50% of the total lipid (Fig. 7), indicating that under these conditions the effect of the negative surface charge on the partition coefficient of the peptide is greater than the concomitant effects on the surface pH and the stability of the ion gradient. The effect of the lower surface pH on peptide permeation is apparent from the rate of the NFG-induced decline of the $AIXme^+$ fluorescence, which reflects the peptide's release and which is slower at higher CL contents (data not shown).

The lower uptake efficiency of $AXme^+$ into PC LUV as compared to $AIXme^+$ is accounted for by the dependence of the bilayer partitioning on the peptide's hydrophobicity (de Kroon et al., 1989; 1990). Likewise, the fluorescence increase recorded for the anionic peptide $bocAIX^-$ is larger than that for its less hydrophobic analogue $boxAX^-$ in the presence of vesicles exhibiting "reversed," i.e., $Na_{in}^+/K_{out}^+$ gradients (Fig. 9). The results obtained in this system comply in all respects with the mechanism proposed for the uptake of $AIXme^+$. The pH dependence of the $K^+$ gradient induced fluorescence increase again reflects that only the neutral, protonated, species of the peptides can pass the bilayer, and is in agreement with the $pK_a$ value of 3.1 of the COOH-terminal carboxyl moiety of peptides (Stryer, 1981). The extents of the fluorescence increases obtained at pH 5 (Fig. 9) and at pH 4 (data not shown) are

smaller than those obtained for $AIXme^+$ in the system of opposite polarity for reasons inherent to the system: (*a*) The inward leakage of $K^+$ leads to faster dissipation of the ion gradient than the outward leakage in the reversed system. (*b*) The $\Delta\psi$-induced $\Delta pH$ will be smaller in the relevant pH range as more protons will have to permeate in order to generate a pH gradient. (*c*) The anionic peptides exert a stronger influence on the membrane ion permeability for ions than $AIXme^+$ because of a more efficient membrane partitioning resulting from their greater hydrophobicity (de Kroon et al., 1990). The faster initial rate of the fluorescence increase of the anionic peptides as compared to that of $AIXme^+$ (cf Figs. 1 and 9) and the occurrence of a fluorescence increase of $bocAIX^-$ without valinomycin present, are accordingly explained by the hydrophobicity of the peptides.

The absence of any ion gradient-induced response of the divalent positively charged peptides $AXetN^{2+}$ and $RXme^{2+}$ as detected by the gel filtration assay and by the tryptophan fluorescence measurements, is interpreted as the inability of these peptides to move across the lipid bilayer under the conditions tested. The most likely explanation for this translocation incompetence is provided by the combination of a lack of partitioning into pure PC bilayers by these peptides (de Kroon et al., 1990) and the impossibility to deprotonate to the neutral form which is due to the $pK_a$ value of $\sim 12.0$ of the arginine side chain (Stryer, 1981) in $RXme^{2+}$, and probably caused by the low surface pH of the CL containing vesicles in the case of $AXetN^{2+}$.

However, under the conditions of the RET assay, with CL incorporated into the LUV and with a large amount of peptide present, an increase in tryptophan-dansylPE energy transfer in response to the $K^+$ diffusion potential is apparent for $RXme^{2+}$, which is small and fast compared to that observed for $AIXme^+$. In contrast, the peptide $AXetN^{2+}$ does not show any response to $\Delta\psi$ under these conditions (Fig. 8). This is remarkable since both divalent peptides share a comparable affinity for negatively charged membranes (de Kroon et al., 1990, 1991). Since the divalent peptides are not expected to readily permeate across the vesicles membrane as argued above, the $\Delta\psi$-induced RET increase observed for $RXme^{2+}$ is attributed to an enhanced binding of this peptide to the outside of the vesicles due to the attraction by $\Delta\psi$. The difference in susceptibility to $\Delta\psi$ found for $RXme^{2+}$ and $AXetN^{2+}$ may be related to the different modes of membrane anchoring of these peptides (de Kroon et al., 1990). The difference between peptides carrying one and two charged groups with respect to their membrane translocation competence, has previously been inferred in a study where the lipid accessibility to the peptides was investigated by $^2H$-NMR (de Kroon et al., 1991*a*).

In conclusion, amphiphilic peptides with a single ionizable moiety were shown to permeate across phospholipid bilayers. This process is enhanced by a transmembrane pH gradient of the appropriate polarity resulting in an accumulation of the peptide inside the phospholipid vesicles. Although a direct stimulation of the uptake by $\Delta\psi$ cannot be excluded, this possibility is considered less likely in view of the very small effect exerted by $\Delta\psi$ on the divalent peptide. The putative "$\Delta\psi$ effect" on the uptake of $AIXme^+$ might as well be due to the unstirred layer phenomena discussed above.

The findings described in this study may be relevant for the absorption of small peptides that occurs in the intestine and in the kidney. It has been reported that ion gradients stimulate the uptake of these substrates into brush-border membrane vesicles isolated from these tissues (see Tiruppathi et al., 1990). The finding that a membrane potential is able to influence the membrane partitioning of charged peptides may contribute to the understanding of the role membrane potential plays in a number of biological processes e.g., in mitochondrial protein import (Hartl et al., 1989). The effect of a membrane potential on the interaction of biological active peptides with phospholipid vesicles has been addressed in a parallel study (de Kroon et al., 1991*b*).

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