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Biochimica et Biophysica Acta 1713 (2005) 73 – 82
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Interaction of a pseudosubstrate peptide of protein kinase C and its myristoylated form with lipid vesicles: Only the myristoylated form translocates into the lipid bilayer

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Received 4 September 2004; received in revised form 22 April 2005; accepted 6 May 2005
Available online 13 June 2005

Abstract

Lipopeptides derived from protein kinase C (PKC) pseudosubstrates have the ability to cross the plasma membrane in cells and modulate the activity of PKC in the cytoplasm. Myristoylation or palmitoylation appears to promote translocation across membranes, as the non-acylated peptides are membrane impermeant. We have investigated, by fluorescence spectroscopy, how myristoylation modulates the interaction of the PKC pseudosubstrate peptide KSIYRRGARRWRKL with lipid vesicles and translocation across the lipid bilayer. Our results indicate that myristoylated peptides are intimately associated with lipid vesicles and are not peripherally bound. When visualized under a microscope, myristoylation does appear to facilitate translocation across the lipid bilayer in multilamellar lipid vesicles. Translocation does not involve large-scale destabilization of the bilayer structure. Myristoylation promotes translocation into the hydrophobic interior of the lipid bilayer even when the non-acylated peptide has only weak affinity for membranes and is also only peripherally associated with lipid vesicles.

Keywords: Fatty acylated peptide; Lipid vesicle; Fluorescence spectroscopy; Translocation across membrane; Membrane binding; Membrane perturbation

1. Introduction

There has been considerable interest in developing methods to deliver peptides and proteins into cells in order to modulate metabolic activities, which could have potential therapeutic value [1–3]. The techniques that have been employed for delivery include microinjection, carrier-mediated transfer and permeabilization of plasma membranes [4,5]. However, there are limitations in these approaches, such as low yield of cargo delivery and degradation of targeted molecules. Recent approaches to increase the bioavailability of these peptides and nucleic acids involve conjugating them to various cell-penetrating peptides (CPP) [6,7]. CPP are a class of peptides that appear have the ability to translocate across the plasma membrane of cells and also, in the process, deliver into the cell any cargo covalently attached to them [8]. Though CPP have been used to target various molecules into the cell, the mechanisms involved in translocation across the plasma membrane are still unclear [9–13]. Studies directed towards understanding the mechanism by which CPP translocate across the lipid bilayer suggests that there is only minimal perturbation of the lipid bilayer and the process appears to be energy-independent [9–13]. However, there are also reports that the internalization of CPP involve endocytosis

Abbreviations: CD, circular dichroism; CF, 5-(and6-)-carboxyfluorescein; DNS, 5-(dimethylamino) naphthalene-1-sulfonyl); DNS PE, (5-(dimethylamino) naphthalene-1-sulfonyl)-L-α-phosphatidylethanolamine (Egg); GUV, giant unilamellar vesicles; FPLC, fast performance liquid chromatography; FRET, fluorescence resonance energy transfer; HEPES, *N*-(2-hydroxyethyl) piperazine- *N*′-(2-ethanesulfonic acid); LUV, large unilamellar vesicles; MLV, multilamellar vesicles; PC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; PG, 1-palmitoyl-2-oleoyl-phosphatidylglycerol; PKC, protein kinase C; SUV, small unilamellar vescles; TFA, trifluoroacetic acid

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0005-2736/$ - see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.bbamem.2005.05.008

and is inhibited at low temperatures and by the depletion of cellular ATP pool [14,15]. Another approach that is being explored to translocate peptides across cell membranes is by acylating the N-terminal end with fatty acids like myristic and palmitic acids [16–22]. Lipopeptides derived from interferon γ and pseudosubstrates of PKC, acylated with myristic or palmitic acids, when added externally to cells, have been able to modulate activities of their targets in the cytoplasm, whereas their non-acylated forms were unable to elicit any biological response. Hence, it appears that acylation with myristic or palmitic acid promotes translocation of these otherwise impermeant peptides across membranes.

Myristoylation and palmitoylation are known to target and anchor proteins with diverse biological functions to membranes [23]. Acylation appears to be essential for membrane attachment, especially in those proteins which do not have hydrophobic segments that facilitate membrane-anchorage. However, translocation of these proteins across membranes as a consequence of fatty acylation has not been observed.

In the present work, we have investigated by fluorescence spectroscopy how myristoylation modulates the interaction of the PKC pseudosubstrate KSIYRRGARRWRKL (Ppkc) with lipid vesicles and possible translocation across the lipid bilayer.

2. Materials and methods

2.1. Materials

Fluorenylmethyloxycarbonyl (Fmoc) amino acids used in peptide syntheses were purchased from Advanced ChemTech (Louisville, KY, USA). All phospholipids, including those labeled with fluorescent tags, were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Myristic acid was from Sigma-Aldrich (St. Louis, MO, USA). 5-(and 6-)-carboxyfluorescein was from Molecular Probes (Eugene, OR). All other chemicals were of the highest grade commercially available.

2.2. Synthesis of KSIYRRGARRWRKL (Ppkc) and derivatives

Peptides were synthesized and characterized as described earlier [24] by the Fmoc solid phase synthesis strategy. N-terminal myristoylation or labeling with CF was achieved using *N*-hydroxybenzotriazole (HOBT) and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and diisopropylethyl amine. The myristoylated peptide labeled with CF was prepared as follows: Fmoc-Lys (ϵ-NH_2)-OH was treated with HOBT active ester of myristic acid to obtain Fmoc-Lys (ϵ-Myr)-OH. The fatty acylated Fmoc amino acid was coupled to the resin-bound peptide. The free amino group generated after the removal of the Fmoc group was labeled with CF as described by Weber et al. [25]. The peptides, after modifications, were cleaved from the resin using reagent K [26] containing 82.5% TFA, 5% water, 5% phenol, 5% thioanisole, and 2.5% ethanedithiol. Peptides were purified using FPLC on a C18 (PEP RPC) reverse phase column (Pharmacia) and characterized by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The peptides were dissolved in dimethylsulfoxide, and concentration was estimated by measuring absorbance at 280 nm. The peptides thus synthesized were KSIYRRGARRWRKL(Ppkc), Myr-KSIYRRGARRWRKL (Ppkcm), CF-KSIYRRGARRWRKL (PpkcCF), and CF-K(ϵ-Myr) KSIYRRGARRWRKL (PpkcmCF).

2.3. Preparation of unilamellar vesicles

Lipids (PC and PC:PG, 1:1 molar ratio) were dissolved in chloroform and dried as a thin film, first under nitrogen followed by vacuum for 10 h. Lipid was hydrated in 5 mM HEPES buffer (pH 7.4) containing 150 mM sodium chloride for 3 h and then vortexed to obtain lipid suspensions. The suspension was sonicated at 4 °C for 30 min in a sonicator to get SUV. The titanium debris was removed by centrifugation. LUV were prepared by extrusion through polycarbonate filters with pore size of 100 nm.

2.4. Peptide binding

Peptide–lipid association was studied by monitoring changes in tryptophan fluorescence intensity of a 2 μM peptide solution in buffer (5 mM HEPES, pH 7.4, 150 mM NaCl) upon the addition of lipid vesicles. The emission spectra were recorded from 300 to 400 nm at 25 °C in Hitachi F4500 Fluorescence Spectrophotometer, 5 min after the addition of lipid vesicles. The excitation and emission band pass were set at 5 nm each. For each measurement, baseline spectra recorded in the absence of peptide were subtracted from the peptide spectra.

The fluorescence titration curves were analyzed as described by Christiaens et al. [27]:

$$F = (F_o + F_1 K_a[L_{tot}])/(1 + K_a[L_{tot}])$$

where F is the fluorescence intensity at a given added lipid concentration, F_0 is the fluorescence intensity at the beginning of the titration and F_1 is the limiting fluorescence at infinite lipid concentration. $[L_{tot}]$ denotes the total lipid concentration while K_a is the association constant. F_1 was obtained as the reciprocal of the Y intercept of a double reciprocal plot of $1/F$ and $1/[L]$. K_a was then the slope of a plot between $(F-F_0)/(F_1-F)$ and $[L]$. The reciprocal of K_a gave the dissociation constant K_d.

2.5. Fluorescence quenching

The influence of aqueous quencher iodide on the tryptophan fluorescence of the peptides was studied by the

addition of increasing amounts of 4M KI (containing 1 mM $Na_2S_2O_3$ to prevent I^{3-} formation) and monitoring the quenching of fluorescence. The data was analyzed according to the Stern–Volmer equation for collisional quenching:

$$I_o/I = 1 + K_{sv}[Q]$$

where I_o and I are fluorescence intensities in the absence and presence of quencher, respectively. $[Q]$ is the molar concentration of the quencher and K_{sv} is the Stern–Volmer quenching constant. Normalised accessibility factor [28] (NAF) values were obtained as the ratio of K_{sv} values in the presence and absence of lipid.

2.6. FRET measurements

To a solution of symmetrically labeled SUVs containing 2 mol% DNS PE in buffer (5 mM HEPES, pH 7.5, 150 mM NaCl), increasing concentration of peptides (P/L 1:50 to 1:11) were added and the energy transfer from tryptophan to the dansyl fluorophore upon excitation at 280 nm was monitored at 25 °C.

2.7. Gel filtration assay to assess peptide binding

The tryptophan fluorescence intensity at a given concentration of peptide in buffer (5 mM HEPES, pH 7.4, 150 mM NaCl) was measured before and after lipid addition. The peptide–vesicle complex was incubated for 30 min, by which time changes in the fluorescence intensity stabilized. The emission scan was recorded (λ_{ex}=280 nm; λ_{em}=300–400 nm). The peptide–vesicle complex was then passed through Sephadex G-75 gel filtration column (bed volume=3.5 ml) and 0.5 ml fractions were collected. Prior to running the peptide vesicle complex, a vesicle blank of the lipid alone at the same concentration was run and 0.5 ml fractions were collected. All the fractions were scanned for vesicle scatter (λ_{ex} and λ_{em}=350 nm). The fractions that showed 90° scatter were then scanned for tryptophan fluorescence. For each peptide vesicle fraction, the base line spectra of the corresponding fraction in vesicle blank was subtracted to yield the spectrum of the peptide that eluted bound to the vesicle.

2.8. Fluorescence microscopy of lipid vesicles

MLV were prepared from lipid which was dried, desiccated and hydrated in HEPES buffer (5 mM HEPES containing 150 mM NaCl) overnight. 15 μl of the solution was taken and spotted on a cover slip. The vesicles were imaged using a Zeiss Axioplan 2 microscope.

2.9. Membrane perturbation induced by peptides

LUVs were prepared in buffer containing 5 mM HEPES and 150 mM KCl. To a 1 ml buffer containing 5 mM HEPES and 150 mM NaCl, lipid vesicles (lipid concentration=25 μM) were added. The analysis involved the monitoring of the recovery of fluorescence of a potential sensitive indicator 3,3′-diethylthiocarbocyanine iodide diS_3-5, which is quenched due to negative diffusion potential inside the vesicles created by the addition of valinomycin. The fluorescent probe (1.5 μM) was added to the cuvette containing lipid, and the fluorescence was monitored with excitation at 620 nm and emission at 670 nm. After 30 s, 10 nM of valinomycin was added to create a negative diffusion potential inside the vesicles as a result of the selective efflux of K^+. This resulted in the quenching of fluorescence. After 5′, gramicidin D (0.5 μM) or peptides at various lipid-to-peptide ratios were added. An increase in fluorescence indicated that the diffusion potential was dissipated.

3. Results

3.1. Binding of peptides to lipid vesicles

The emission spectra of the Ppkc and Ppkcm in buffer and in the presence of SUV with varying lipid composition and variation of fluorescence intensities as a function of lipid concentration at a fixed peptide concentration are shown in Fig. 1A, B. In buffer, the peptides exhibit a λ_{max} of 355 nm, indicating that the tryptophan is exposed to aqueous environment. Blue shift of λ_{max} and enhancement in intensity is observed only in the presence of PC:PG vesicles for Ppkc, whereas changes are observed with both PC and PC:PG vesicles in the case of Ppkcm. However, the enhancement in intensities are considerably greater for Ppkcm, suggesting strong association with PC and PC:PG vesicles. The data also suggest that in the presence of lipid vesicles, the tryptophan is localized in a relatively more hydrophobic environment. The increase in F/F_0 as a function of lipid concentration (Fig. 1B) suggests that Ppkc associates with PC:PG vesicles but not with PC vesicles. However, on myristoylation, the increase in fluorescence is substantial both in the presence of PC and PC:PG vesicles. An initial rapid increase in F/F_0 up to a lipid concentration of 20 μM followed by a fall and subsequent increase is observed for Ppkcm in the presence of PC:PG vesicles. While the data shown in Fig. 1B represents a single experiment, this pattern was consistent in 5 independent experiments. Both the myristoylated and non-acylated peptides show emission λ_{max} of 355 nm in buffer, indicating that the tryptophan environment is similar in both the peptides, suggesting that acylation does not cause peptide aggregation or “micelle” formation. The dissociation constants calculated as described in Materials and methods was 86 μM for the binding of Ppkc to PC:PG vesicles. The values for the binding of Ppkcm to PC and PC:PG vesicles were 59 and 63 μM, respectively. The presence of a single myristic acid clearly favours binding of the acylated peptides to PC and PC:PG vesicles with similar binding affinities. The binding constant obtained from our data agrees with the

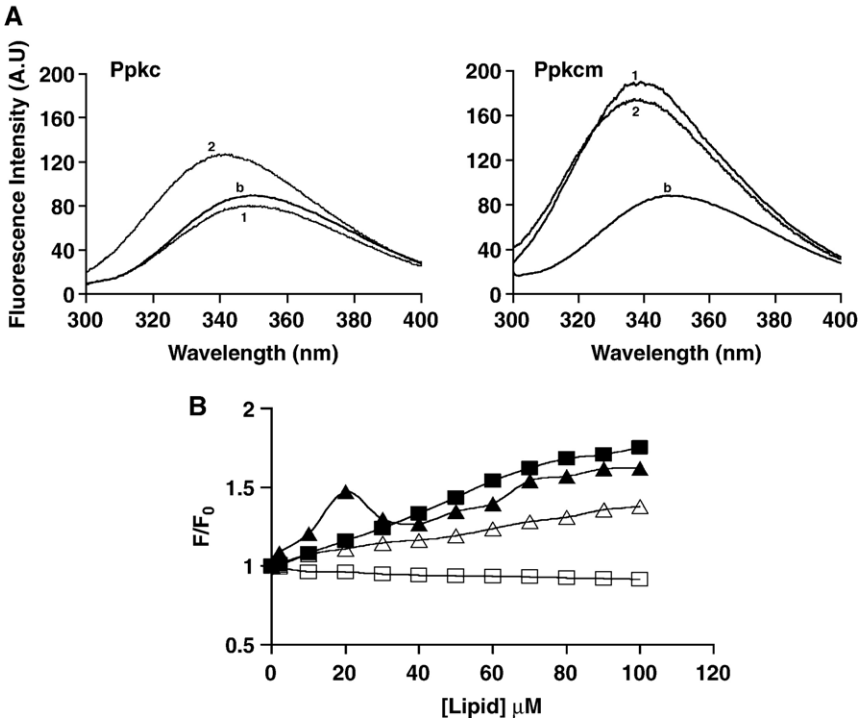

Fig. 1. Binding of peptides to lipid vesicles. (A) Fluorescence emission spectra of Ppkc and Ppkcm in buffer (indicated by b above the trace) and in the presence of small unilamellar vesicles composed of PC and PC:PG (1:1) (indicated by 1 and 2 above the traces respectively). Data presented are maximal changes observed. (B) Relative increase of tryptophan fluorescence intensity on titration with lipid vesicles (▲-▲), Ppkcm; PC:PG (1:1) vesicles (■-■), Ppkcm; PC vesicles (△-△), Ppkc; PC:PG (1:1) vesicles (□-□), Ppkc; PC vesicles. Peptide concentration=2 μM.

binding data observed for similarly charged acylated peptides/proteins (e.g., Src with +5 net charge) to zwitterionic vesicles using different assays [29]. These peptides were shown to bind to PC vesicles with a K_d of ~1 mM. However, the same peptides have been shown to bind to PC:PG vesicles with K_d in nM range. This appreciable increase in binding has been attributed to the 'synergistic' effect as a result of hydrophobic contributions from myristic acid and electro-

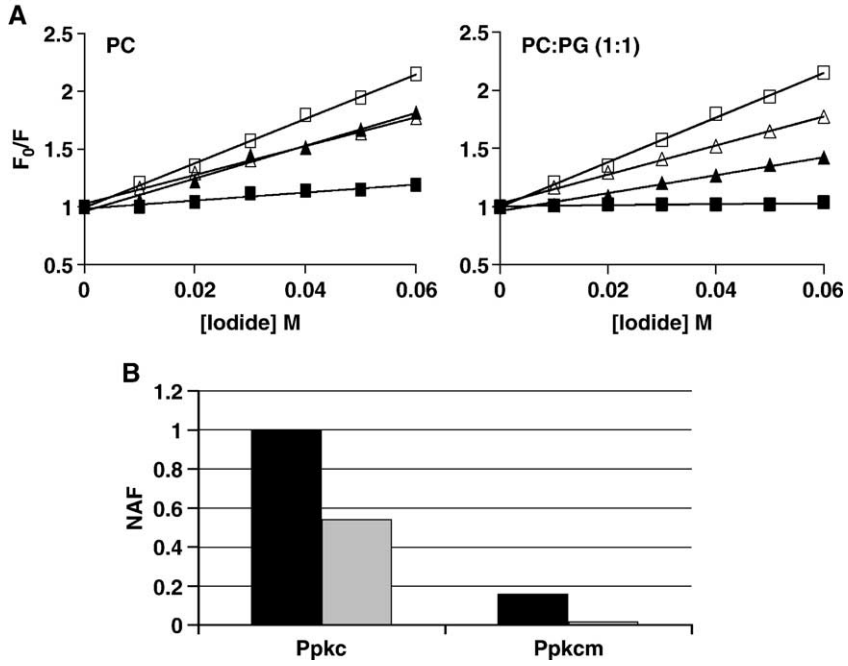

Fig. 2. Quenching of tryptophan fluorescence by iodide (A) Stern–Volmer plots in the presence (dark symbols) and absence (light symbols) of lipids. (△, ▲) Ppkc; (□, ■) Ppkcm. (B) Plots of NAF values: (black bar) PC vesicles; (gray bar) PC:PG vesicles. Peptide concentration was 2 μM and peptide–lipid ratios were 1:100.

static contributions from the cationic amino acids in the peptide. Our binding data for the interaction of Ppkcm with PC:PG vesicles do not suggest synergy.

3.2. Localization of tryptophan by iodide quenching

The accessibility of tryptophan to the aqueous quencher I^- was examined next in order to get an idea regarding the location of tryptophan when peptides were associated with lipid vesicles. The Stern–Volmer plots (Fig. 2A) indicate that the tryptophan is inaccessible to I^- in Ppkcm in the presence of PC and PC:PG vesicles. The accessibility of tryptophan in Ppkc suggests that the peptide does not bind to PC vesicles but is associated with PC:PG vesicles. The NAF plot (Fig. 2B) also indicates that myristoylation favours the association of Ppkcm with lipid vesicles so that the tryptophan residue is inaccessible to I^-. Considering that the tryptophan is located in the midst of cationic residues in the peptide, the results suggest that myristoylation "forces" the peptide chain into the bilayer. Although Ppkc does bind to PC:PG vesicles, it is evident that the tryptophan is considerably more accessible to I^- than in the myristoylated peptide.

3.3. Gel filtration assay to assess peptide binding

In order to determine whether the peptides were tightly associated with lipid vesicles, gel filtration was carried out after incubating lipid vesicles with peptides and fluorescence of the eluted vesicles recorded.(Fig. 3A–D). No fluorescence corresponding to the peptide is observed when Ppkc is incubated with PC:PG vesicles and the eluted vesicles analyzed after gel filtration (Fig. 3B). However, when Ppkcm is incubated with PC:PG vesicles, fluorescence was observed after gel filtration (Fig. 3D). The position of λ_{max} ~335nm suggests that the tryptophan residue is located in the hydrophobic interior of the lipid bilayer. The scatter profiles indicate that there is no vesicle lysis by Ppkcm.

These results indicate that though Ppkc binds to PC:PG vesicles, the peptide can actually be "stripped off" from the vesicle upon passing through a gel filtration column. The recovery of fluorescence when Ppkcm is associated with PC:PG vesicles indicates that the peptide is very tightly bound to the vesicle and cannot be "stripped off" even on gel filtration. The data shown in Fig. 3 are at a peptide–lipid ratio of 1:50. Similar results were obtained at ratios of 1:25 and 1:100.

3.4. Fluorescence resonance energy transfer (FRET) experiments

FRET experiments involving energy transfer from a donor to an acceptor have been used to study the spatial relationship between the donor and the acceptor in a given environment [30,31]. In order to assess the location of the

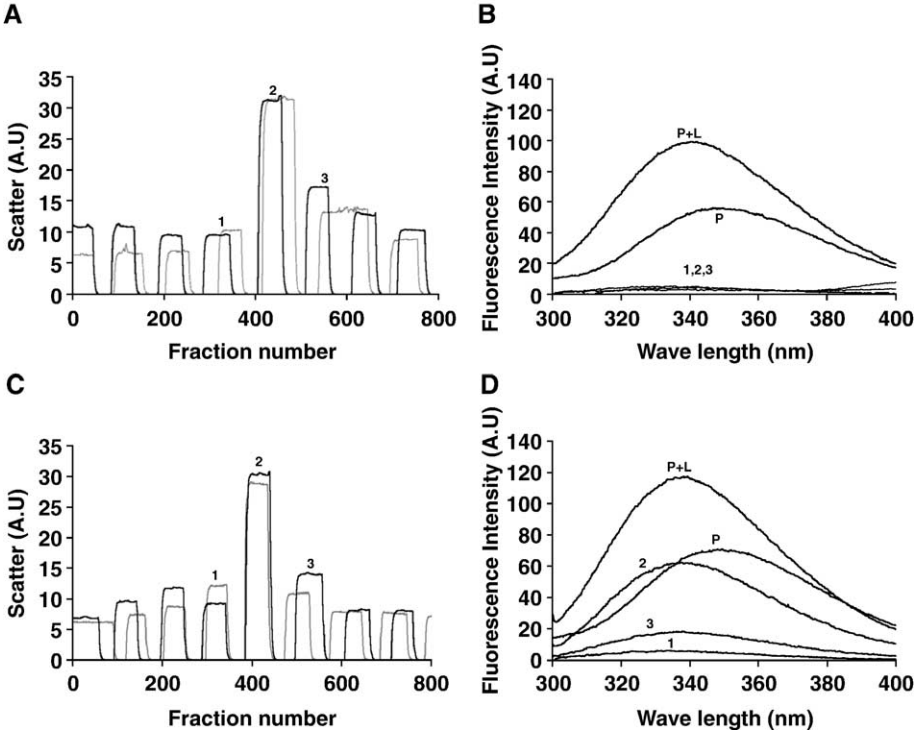

Fig. 3. Gel filtration assay to assess the strength of binding of peptides to lipid vesicles. Scatter (90°) profiles of lipid vesicles in the absence (thin lines) and after incubation with peptides (thick lines) after gel filtration. (A) Ppkc, (C) Ppkcm. Emission spectra of peptides in buffer in the presence of lipid vesicles prior to gel filtration and after gel filtration. (B) Ppkc, (D) Ppkcm. Spectra labeled 1, 2, and 3 correspond to fractions indicated in panels (A) and (C). The peptide–lipid ratio was 1:50. P and P+L denote spectra of peptides in buffer and in the presence of lipid vesicles before gel filtration.

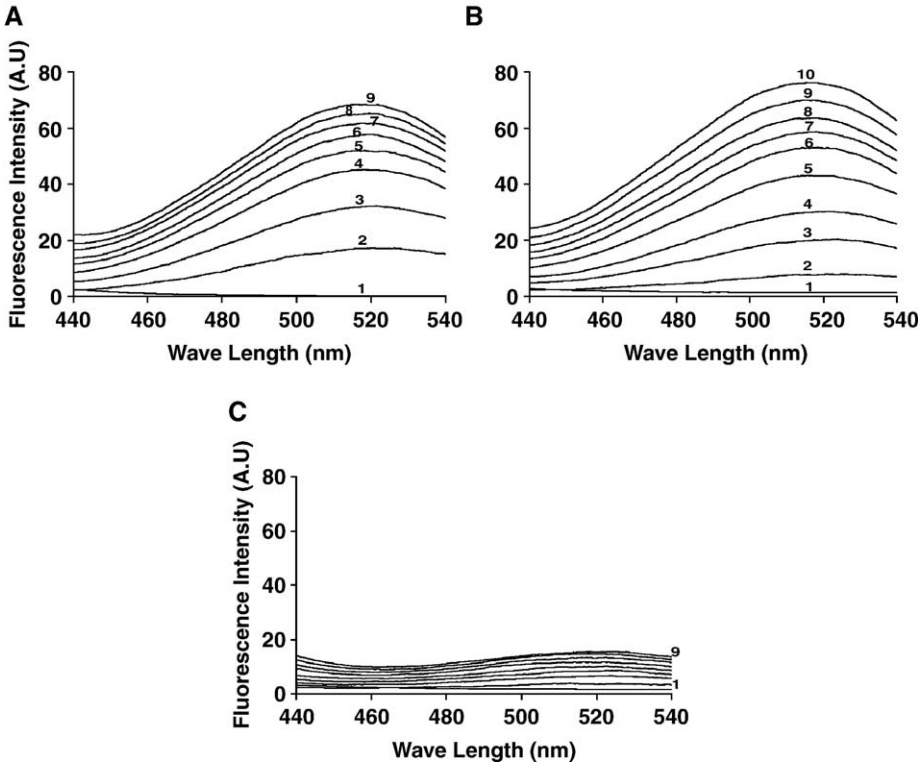

Fig. 4. Interaction of peptides with LUV doped with DNS PE. Increasing concentrations of peptides were added to lipid vesicles (lipid concentration=50 μM). Spectra were obtained with λ_{ex} at 280 nm. (A) Ppkcm and PC vesicles; (B) Ppkcm and PC:PG (1:1) vesicles; (C) Ppkc and PC:PG (1:1) vesicles. Numbers in the traces indicate peptide–lipid ratios. (1) Peptide blank; (2) 1:100; (3) 1:50; (4) 1:33; (5) 1:25; (6) 1:20; (7) 1:17; (8) 1:14; (9) 1:12; (10) 1:11.

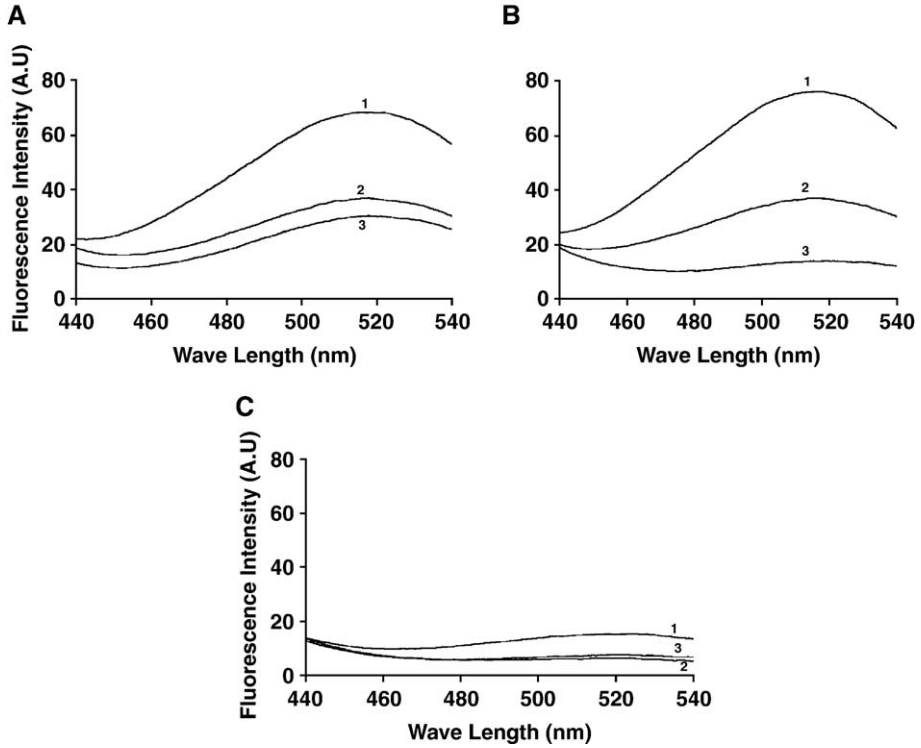

Fig. 5. Detection of translocation of peptides by FRET between tryptophan and DNS. (A) Ppkcm+PC vesicles; (B) Ppkcm+PC:PG (1:1) vesicles; (C) Ppkc+PC:PG (1:1) vesicles. Increasing concentrations of peptides were added to a fixed lipid concentration (50 μM) and spectra were recorded with λ_{ex} at 280 nm. Spectra labeled 1 were obtained at the highest peptide–lipid ratio. Spectra after unlabeled vesicles (400 μM) were added to peptides bound to DNS labeled vesicles are represented by 2. Spectra obtained when peptides were added to lipid vesicles containing both labeled and unlabeled vesicles are labeled 3.

tryptophan of the peptide in the lipid bilayer, we used FRET to monitor energy transfer to an acceptor dansyl group present in the membrane. The experiment would also help in detecting population of peptides that are intimately associated with lipid vesicles, as challenge with a large excess of unlabelled vesicles would result in the extraction of peripherally bound peptides. The FRET of peptide tryptophan to the dansyl group in the membrane of first population of vesicles allows the determination of unremovable fraction. We monitored FRET by adding increasing concentration of peptides to a fixed concentration 50 μM of 2 mol% DNS PE-labeled vesicles in buffer. The peptide–lipid ratios were varied from 1:100 to 1:12 or 1:11. The data show appreciable FRET only when Ppkcm is added to lipid vesicles (Fig. 4A,B). Considerably reduced FRET is observed when Ppkc is added to PC:PG vesicles (Fig. 4C). FRET was maximum when the peptide–lipid ratio was approximately 1:10 for both PC and PC:PG vesicles. At this ratio, a large excess (400 μM final concentration) of unlabeled vesicles was added to the vesicle population doped with DNS PE. A decrease in FRET (Trace 2, Fig. 5A–C) was observed. In a separate experiment, the same concentration of vesicles (400 μM containing similar ratios of labeled to unlabelled vesicles premixed) was added to the same concentration of peptide as in previous experiment (4 μM) and the amount of FRET was monitored after 10 min (Trace 3, Fig. 5A–C). The data indicate considerably reduced FRET when Ppkcm is added to a mixture of labeled and a large excess of unlabeled vesicles composed of PC:PG (1:1) (Fig. 5B). The difference in FRET is relatively less when Ppkcm is added to PC vesicles (labeled+unlabeled, Trace 3). The data suggest that myristoylation favours the translocation of Ppkcm to the hydrophobic core region of the lipid bilayers particularly in PC:PG (1:1) vesicles.

3.5. Localization of CF labeled peptides in MLV

In order to visualize the translocation/translocated peptide, the peptides were labeled at the N-terminus with CF and their localization in MLV was observed by fluorescence microscopy. The data are presented in Fig. 6. Fluorescence in the inner bilayers is observed only in the case of vesicles incubated with Ppkcm labeled with CF (Fig. 6, B1–B5). It was observed that dilution strips away only the non-acylated peptide but not the myristoylated peptide. Along with the results of the gel filtration assay, where only the non-acylated peptide can be easily stripped off the membranes, the localization experiments suggest that myristoylation promotes translocation across the lipid bilayer.

3.6. Perturbation of the lipid bilayer

Whether the association of myristoylated peptides with PC and PC:PG unilamellar vesicles leads to the destabilization of the lipid bilayer was examined next. The dissipation of diffusion potential induced by peptides was monitored (Fig. 7). As compared to gramicidin D, the dissipation is minimal particularly in PC:PG (1:1) vesicles. At a peptide–

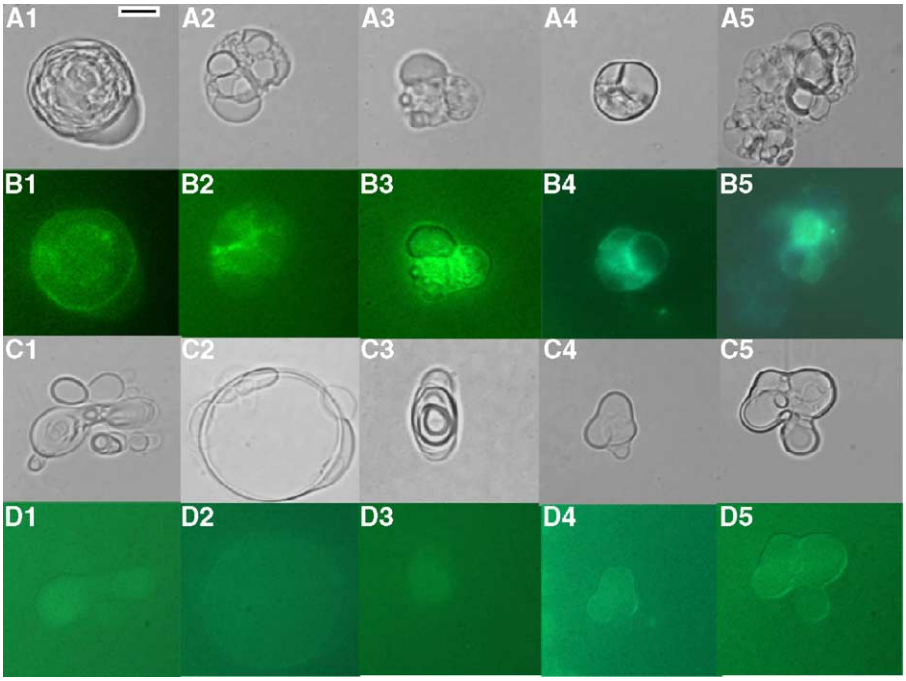

Fig. 6. Detection of translocation of CF labeled peptides across lipid bilayers composed of PC:PG (1:1) by fluorescence microscopy. Panels A and B, PpkcmCF; panels C and D, PpkcCF. (A, C) Imaged under bright field. (B, D) Fluorescent images. Panels 1–5 show different fields. The black line in A1 represents 10 μm. The magnification was same in all the other panels.

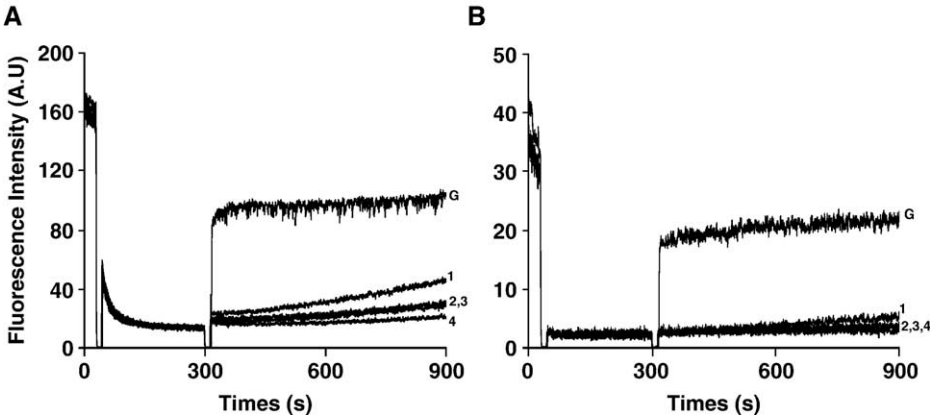

Fig. 7. Dissipation of diffusion potential in vesicles induced by peptides. (A) PC, (B) PC:PG (1:1). The emission of cyanine dye at 670 nm (λ_{ex} of 620 nm) was monitored over a period of time. At 30 s, to a solution of cyanine dye and lipid vesicles, valinomycin was added resulting in the setting up of a diffusion potential. At 5 min, the peptides are added and dissipation of diffusion potential was monitored. G, gramicidin D 0.5 μM; 1 and 2, Ppkcm at peptide: lipid ratios of 1:25 and 1:50 respectively; 3 and 4, Ppkc at peptide: lipid ratios of 1:25 and 1:50 respectively.

lipid ratio of 1:25, Ppkcm does appear to destabilize PC vesicles to a small extent.

4. Discussion

The PKC pseudosubstrate peptide, which has a net positive charge, does not associate with zwitterionic vesicles and associates with anionic vesicles only peripherally. On myristoylation, the peptide is intimately associated with lipid vesicles as evident from the gel-filtration experiments, where the eluted vesicles retain the myristoylated peptide. The similar K_d values obtained for Ppkcm and Ppkc for binding to PC:PG vesicles may be a reflection on the method of assay used for calculating the binding constant. The FRET experiments clearly indicate that the myristoylated peptides are strongly bound and not peripherally associated. The considerably decreased FRET observed when Ppkcm is added to a mixture of labeled and unlabelled vesicles (Fig. 5B, Trace 3) suggests translocation into the lipid bilayer. Difference in FRET (Fig. 5B, Traces 2 and 3) arises when membrane-active peptides are translocated into the inner leaflet of the lipid bilayer in lipid vesicles doped with dansyl lipid such as dansyl phosphatidyl ethanolamine [30]. It is unlikely that the myristoylated peptide co-elutes with the vesicles, as the peptide alone does not elute under the conditions employed and appears to bind to the gel matrix. When visualized under a microscope, myristoylation does appear to facilitate the translocation of the peptide across the lipid bilayer into the vesicles. The translocation of Ppkcm does not appear to involve large-scale destabilization of the lipid bilayer structure as indicated by the absence of pore formation. No membrane destabilization is seen even at a peptide–lipid ratio of 1:25, where a large fraction of the peptide is strongly membrane-bound as indicated by gel filtration and FRET data. Our results with model membranes are consistent with experiments with cultured cells incubated with acylated PKC peptides wherein no loss of viability is observed [18,22], as membrane damage would have led to cell death. Myristoylation appears to translocate a hydrophilic peptide into the hydrophobic interior of the lipid bilayer without perturbation of the bilayer structure very similar to CPPs [5,11,12]. The initial rise followed by a fall and subsequent increase when F/F_0 in Ppkc is monitored as a function of lipid concentration (Fig. 1B) could arise as a result of peptide translocation into the lipid bilayer. The fluorescence spectra for monitoring the binding of peptides to lipid vesicles were recorded after incubation for 5 min. Longer incubation times did not yield spectra different from the ones presented. It is likely that there is an ensemble of peptides in different locations in the lipid vesicles. While we are unclear about the exact mechanism of translocation, our results indicate that myristoylation can transfer a cationic peptide like Ppkc into the hydrophobic interior of the lipid bilayer, particularly in anionic lipid vesicles such as PC:PG, even when the non-acylated peptide is only peripherally associated with lipid vesicles. While myristoylation clearly facilitates transfer of the hydrophilic peptide chain into the hydrophobic interior of the lipid bilayer, it is not clear whether the myristoylated peptide has crossed the lipid bilayer. In fact, several reports indicate that CPPs bind to lipid vesicles and are located in the hydrophobic region of the bilayer in unilamellar lipid vesicles [37–44]. They translocate across the lipid bilayer only in GUV of diameter (1–300 μm) or MLVs [12]. In the present study too, the myristoylated peptide appears to translocate across the bilayer in MLVs but not in small unilamellar vesicles.

Based on our results, we suggest a new physiological function for fatty acylation. Covalently linked fatty acids could facilitate hydrophilic peptide segments to be associated with the hydrophobic interior of the lipid bilayers and possibly translocate across the membrane barrier. The turnover of fatty acid might be a way for trapping them in membrane compartments. Ghrelin, a hormone modified with an octanoyl fatty acid moiety, is known to exist in cells in both the fatty acylated and the des acylated form [32,33].

The exact role of this hormone is still being unraveled but it has been demonstrated that the acylated and non-acylated versions have different biological activities. The acylated hormone is known to bind very strongly to receptors and is also known to cross the blood–brain barrier by an unknown mechanism. Fatty acylation could conceivably play role in the ability of ghrelin to translocate the blood–brain barrier. Two other well-known proteins that are fatty acylated are morphogens, namely Wnt and Hedgehog [34–36]. These proteins are fatty acylated with palmitic acid and are known to be membrane anchored. They are also known to act as long range diffusible signals by an unknown mechanism. In case of human sonic hedgehog protein, it has been shown that N-terminal modification with myristic acid confers maximum activity to the protein in a cell-based signaling assay [36]. Surprisingly, fatty acylation did not seem to confer increased membrane binding of the protein to its receptor, and thus the other role of fatty acylation apart from membrane anchorage is not clear. Intriguingly, it has been shown that a major fraction of the patched protein which is the receptor for hedgehog is present in intracellular vesicles and not on the cell surface [45]. It is tempting to speculate that fatty acylation in hedgehog protein might also allow hedgehog to translocate the membrane and interact with its receptor. A recent report suggests that N-terminal myristoylation does indeed facilitate translocation of the peptide chain across membranes [46]. Hence, role of fatty acylation, especially when only one fatty acid is present, may not be restricted to membrane anchorage alone and may also facilitate the translocation of protein segments into the hydrophobic interior of the lipid bilayers.

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