Biochimica et Biophysica Acta 1828 (2013) 1198–1204

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Modeling the endosomal escape of cell-penetrating peptides using a transmembrane pH gradient

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ARTICLE INFO

Article history:
Received 27 September 2012
Received in revised form 26 November 2012
Accepted 10 December 2012
Available online 19 December 2012

Keywords:
Cell-penetrating peptide
Endosomal escape
Bacteriorhodopsin
Large unilamellar vesicle
Fluorescein-label
Membrane translocation

ABSTRACT

Cell-penetrating peptides (CPPs) can internalize into cells with covalently or non-covalently bound biologically active cargo molecules, which by themselves are not able to pass the cell membrane. Direct penetration and endocytosis are two main pathways suggested for the cellular uptake of CPPs. Cargo molecules which have entered the cell via an endocytotic pathway must be released from the endosome before degradation by enzymatic processes and endosomal acidification. Endosomal entrapment seems to be a major limitation in delivery of these molecules into the cytoplasm. Bacteriorhodopsin (BR) asymmetrically introduced into large unilamellar vesicles (LUVs) was used to induce a pH gradient across the lipid bilayer. By measuring pH outside the LUVs, we observed light-induced proton pumping mediated by BR from the outside to the inside of the LUVs, creating an acidic pH inside the LUVs, similar to the late endosomes in vivo. Here we studied the background mechanism(s) of endosomal escape. 20% negatively charged LUVs were used as model endosomes with incorporated BR into the membrane and fluorescein-labeled CPPs entrapped inside the LUVs, together with a fluorescence quencher. The translocation of different CPPs in the presence of a pH gradient across the membrane was studied. The results show that the light-induced pH gradient induced by BR facilitates vesicle membrane translocation, particularly for the intermediately hydrophobic CPPs, and much less for hydrophilic CPPs. The presence of chloroquine inside the LUVs or addition of pyrenebutyrate outside the LUVs destabilizes the vesicle membrane, resulting in significant changes of the pH gradient across the membrane.

1. Introduction

Cell-penetrating peptides (CPPs) were first introduced in the late 1980s by the discovery of the TAT peptide from the transactivator of transcription (TAT) of human immunodeficiency virus type one (HIV-1) [1–3]. Since then, many other peptides with similar cell-penetrating properties were found and characterized [4–6]. Generally, CPPs are defined as short, partly hydrophobic and/or polybasic peptides with a net positive charge at physiological pH [7,8]. The main characteristic is their ability to penetrate the cell membrane at low micromolar concentrations in vivo without using any chiral receptors and without causing significant membrane damage. Moreover, they are capable of internalizing electrostatically or covalently bound biologically active small or large cargoes, such as drugs and gene modulating agents, with high efficiency and low toxicity [7,9,10].

A variety of biological assays, biophysical methods and model systems have been utilized to study the cellular uptake and membrane translocation mechanisms of CPPs. The mechanism by which they enter cells has not been completely resolved. There is evidence for both direct cell membrane penetration and endocytotic pathways in internalization of CPPs [9,11,12]. Endocytosis and subsequent endosomal escape are believed to be a major uptake route for CPPs at low concentrations and in the presence of cargoes [13–18]. On the other hand, most CPPs may utilize two or more pathways depending on the experimental conditions [19–21].

Endosomal entrapment is a major challenge for CPPs and their cargoes that have entered the cells via an endocytotic pathway. For example, they could be digested by lysosomal hydrolysis before reaching a target. Increasing hydrophobicity of the complex is one way

Abbreviations: CPP, cell-penetrating peptide; BR, Bacteriorhodopsin; Pyrenebutyrate, 4-(1-pyrenyl)-butyric acid; CQ, chloroquine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3[phospho-rac-(1-glycerol)]; BMP,*sn*-(3-oleoyl-2-hydroxy)-glycerol-1-phospho-*sn*-1′-(3′-oleoyl-2′-hydroxy)-glycerol (ammonium salt); LUV, large unilamellar vesicle; R9, oligoarginine nonamer; TP10, transportan 10; pVEC, vascular endothelial cadherin peptide; TAT, transactivator of transcription peptide; ON, oligonucleotide; OG, n-octyl-β-D-glucopyranoside; FITC, fluorescein isothiocyanate; DLS, dynamic light scattering

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0005-2736/$ – see front matter © 2012 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.bbamem.2012.12.008

used to overcome the membrane barrier of the endosome [22]. Several studies have used the lysosomotropic agent, chloroquine (CQ) as an inhibitor of endosomal acidification and/or an agent for lysosomal membrane perturbation, in order to enhance the performance of a CPP [23]. In addition, CQ is also applied to provide evidence for an endocytotic pathway [24,25]. In other studies, the membrane interacting compound pyrenebutyrate (PB) has been applied as an agent that enhances the potency of a CPP [26–28], possibly by weakening the cell membrane.

In the present work, the aim is to obtain information about the background mechanism(s) of endosomal escape. For this purpose, 20% negatively charged large unilamellar vesicles (LUVs), encapsulating the CPP (labeled with the dye fluorescein) and a fluorescence quencher (KI) were prepared as a model endosome by the extrusion method. This method produces a relatively homogeneous solution of unilamellar vesicles of 100 nm diameter into which asymmetric insertion of the proton pumping protein bacteriorhodopsin (BR) could be achieved using the detergent mediated reconstitution method [29]. When BR absorbs light, it pumps protons in a direction that depends on the direction of protein insertion into the membrane, generating a pH gradient across the membrane. We have previously shown that in the presence of light, BR pumps proton from the outside to the inside of the LUVs, creating an acidic pH inside the vesicles [30]. This transmembrane pH gradient has been used to model the late endosomes similar to those existing in vivo.

Despite obvious similarities between CPPs, especially in their cationic nature, they have different physico-chemical characteristics based on their peptide sequence and membrane binding properties. According to these differences, they can be divided into three classes, including I) primary amphipathic CPPs, such as transportan [31] together with its shorter analog transportan 10 (TP10) [32], II) intermediately amphipathic CPPs, such as penetratin [33], pVEC [4] and M918 [34], and III) non-amphipathic CPPs, such as R9 [6] and TAT(48–60) [1,2,35]. Table 1 shows CPPs that have been included in this study together with some of their physical properties. CPPs from class I like TP10 could not be included in the pH gradient studies, since even at low concentrations they cause leakage from vesicles [26] and they cannot be entrapped inside the vesicles under the conditions employed here.

The CPPs studied here are non-amphipathic CPPs (R9 and TAT(48–60)) and intermediately amphipathic CPPs (M918 and pVEC). R9 is a synthetic homopolyarginine [6] and like TAT(48–60), it belongs to the non-amphipathic group of CPPs, but with less hydrophobicity (Table 1). In the report from Guterstam et al. [26], direct penetration is suggested for the hydrophilic, Arg-rich R9 and TAT(48–60) at high concentrations of the counteranion, PB. However at lower concentrations of PB, the R9 mediated oligonucleotide (ON) translocation mainly occurs by Arg-induced macropinocytosis. In several other studies, punctate cytoplasmic localization of labeled TAT(48–60) and R9 observed by live cell imaging shows the participation of endocytosis in the uptake mechanism [36–38].

pVEC is an intermediately amphipathic CPP, derived from the murine vascular endothelial-cadherin (VE-cadherin) protein [4]. Treatment of the cells with different endocytosis inhibitors, especially wortmannin, efficiently suppresses the cellular uptake of pVEC, indicating the activity of the clathrin dependent endocytotic pathway [39]. However, uptake at low temperatures confirms the presence of non-endocytotic pathways in the pVEC uptake mechanism [40]. M918 is a novel CPP with 22 residues, seven of them positively charged. The effects of endocytosis inhibitors on the cellular internalization confirmed the presence of endocytotic pathways in the uptake mechanism [34]. Penetratin, a fragment of the Antennapedia homeodomain with 16 residues is one of the most extensively studied CPPs and was used in the first study reporting on a pH gradient dependent transport of a CPP [41]. It may be classified as an intermediately amphipathic CPP [26]. The majority of reports on the uptake mechanism of penetratin suggest that endocytosis is the major route of uptake, both in the absence or presence of cargo molecules. On the other hand, like for Arg-rich CPPs, different types of endocytotic pathways for penetratin and its cargo conjugates have been reported [15,17,37,38,42–45].

Table 1
Investigated CPPs together with their physical properties at physiological pH.

Peptide	No. of Arg	Average hydrophobicity[a]	No. of residues	Sequence
R9	9	2.58	9	RRRRRRRRR
TAT (48–60)	6	2.37	13	GRKKRRQRRRPPQ
pVEC	4	1.10	18	LLIILRRRIRKQAHAHSK
M918	7	0.93	22	MVTVLFRRLRIRRACGPPRVRV

[a] Average hydrophobicity calculated according to the values from von Heijne's scale [54].

2. Materials and methods

2.1. Materials

Zwitterionic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3[phospho-rac-(1-glycerol)] with a negative head-group at neutral pH (POPG), as well as BMP, *sn*-(3-oleyl-2-hydroxy)-glycerol-1-phospho-*sn*-1'-(3'-oleyl-2'-hydroxy)-glycerol (ammonium salt) were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA) and were used without further purification. The detergent n-octyl-β-D-glucopyranoside (OG) was from Glycon Biochemicals (Luckenwalde, Germany). PD-10 desalting columns were purchased from GE Healthcare (Buckinghamshire, UK). Bio-Beads were from BIO-RAD (California, USA). FITC (fluorescein isothiocyanate) labeled R9 and TAT(48–60) were produced by Polypeptide Laboratories (Strasbourg, France). FITC (fluorescein isothiocyanate) labeled M918 and pVEC were chemically synthesized by Fmoc (9-fluorenylmethyloxycarbonyl) solid-phase peptide synthesis (SPPS) at the Department of Neurochemistry, Stockholm University. *Halobacterium salinarum* strain S9 was a generous gift from Professor Esteve Padrós (Universitat Autonoma de Barcelona, Spain). Bacteriorhodopsin (BR) was produced and purified essentially according to a published protocol [46].

2.2. LUV preparation: extrusion method

LUVs were prepared by dissolving the phospholipids (zwitterionic POPC and 20% negatively charged POPG) in chloroform to obtain a homogeneous solution, whereafter the solvent was removed by evaporation under high vacuum for 3 h. The resulting dried lipid film was resuspended by addition of 20 mM potassium phosphate and 100 mM KCl at pH 7.2. This solution was vortexed for 10 min and then subjected to five freeze–thaw cycles with liquid nitrogen to reduce the lamellarity. Next, an Avanti manual extruder was used to push the lipid solution 20 times through two polycarbonate filters (100 nm pore size). This method gave unilamellar vesicles with 100 nm diameter [47]. The size and stability of the LUVs were examined with a dynamic light scattering (DLS) instrument.

2.3. Preparation of fluorescein-labeled CPPs-entrapping LUVs

In the buffer used for LUV preparation, a 20 μM fluorescein-labeled CPP together with 100 mM potassium iodide (KI) as a fluorescence quencher was included. LUVs containing CPPs were made as described in the previous section. KI was used to quench the fluorescence of the entrapped peptide inside the vesicle and hence to minimize the background fluorescence intensity. Therefore any increase in fluorescence intensity should be due to the leakage of the labeled peptide from the inside of the LUVs.

2.4. Reconstitution of BR into the LUVs: detergent mediated reconstitution method

The preparation of BR-reconstituted LUVs (BR-LUVs) consists of three steps: vesicle solubilization, BR addition, and detergent removal. After 5 to 10 min of the vesicle solubilization by the addition of the detergent, n-octyl-β-D-glucopyranoside (OG), BR monomers resulting from detergent solubilization of purple membrane (BR 1 mg/mL, OG 100 mM) were added to the solubilized LUV suspension and incubated for another 5 to 10 min. The resulting suspension should be a mixture of BR/lipid/detergent vesicles and lipid/detergent micelles with the final concentrations of 2 μM, 4.3 mM, and 29 mM for BR, lipid, and detergent, respectively. At this stage, BR may be incorporated into the vesicles, which have been saturated and destabilized by the detergent. The BR/lipid/detergent mixtures were kept at room temperature for 5 min to 15 min, and the detergent was then removed. Detergent removal should best be performed in two steps: first slightly wet Bio-Beads (80 mg/mL) were directly added to the suspension. The mixture was lightly stirred (around 400 rpm) at room temperature. After 3 h of incubation at room temperature, a second portion of beads was added and mixed overnight with a small shaker and the rate of around 400 rpm to remove residual detergents. At the end, two PD-10 columns were used to remove Bio-Beads, residual detergents, quencher and labeled peptides from outside the LUVs. The final buffer outside contained 20 mM potassium phosphate and 100 mM KCl (pH 7.2). A detailed protocol describing this method is published in [30].

2.5. pH measurements

The buffer outside the BR-LUVs solution was replaced by 120 mM KCl pH 7.4, using a PD-10 column, like in our previous study [30] when outside pH was measured. The pH outside the BR-LUVs solution was measured in a cuvette equipped with a magnetic stirrer, using a pH meter (Thermo Scientific model 320). The pH outside the vesicles was recorded during the dark and under illumination by a 150 W xenon arc lamp.

2.6. Fluorescence spectroscopy and CPP leakage study

To study the effect of a pH gradient on the CPP translocation ability from the LUVs, fluorescence spectroscopy was used. Fluorescence intensity was measured in a Horiba Jobin Yvon Fluorolog-3 spectrometer using a 4∗10 mm quartz cuvette. The sample was excited at 494 nm, and its emission was scanned from 505 to 550 nm. All experiments were run at 20 °C.

3. Results

To evaluate the effect of the transmembrane pH gradient on the translocation abilities of different CPPs, the light sensitive proton pump BR was incorporated into the membrane of 20% negatively charged phospholipid LUVs composed of POPC and POPG. The vesicle properties and pH gradient that can be established over the membrane depend on vesicle composition in terms of phospholipid content. In this study POPC and POPG were selected since they have a low gel–liquid transition temperature and produce stable vesicles with a stable pH gradient under the conditions employed here. BMP is a major negatively charged component of the biological endosome membrane [48,49]. A lipid mixture approximately corresponding to the endosomal membrane (50% PC, 20% PE, 10% PI and 20% BMP) was also investigated in a vesicle model system for incorporating BR, as well as a pure zwitterionic POPC vesicle model system. We observed that a weaker pH gradient was induced in the latter systems (Fig. 1A, B and C). These results suggest that the BR proton pumping is less efficient with these vesicles or the vesicles form less of a membrane proton barrier compared to the POPG-containing ones.

Upon illumination of the POPC/POPG LUVs, the measured pH increased and reached a maximum for BR-LUVs without entrapped peptides, which is consistent with an increase in the pH on the outside of the LUVs (Fig. 1A). BR-mediated proton pumping from the outside to the inside of vesicles resulted in the establishment of an acidic pH inside the LUVs.

20 μM fluorescein-labeled CPP together with 100 mM KI as a fluorescence quencher was entrapped in the LUVs. In the dark, we observed no changes in the background fluorescence intensity for all examined CPPs, indicating that there is no leakage of the peptides out of the LUVs when there is no pH gradient across the membrane on the time scale of a few hours. The escape of the encapsulated fluorescein-labeled CPP from the LUVs during exposure to light was determined by measuring the fluorescence intensity of the sample after certain times of illumination and compared with the value of the background intensity (or the intensity of the sample kept in the dark). The time duration of illumination and darkness was selected according to the amount of fluorescence intensity changes for each peptide. Repeated measurements in identically prepared samples showed good reproducibility in the measurements of the relative fluorescence intensities measured with a particular peptide (Fig. S1).

Fluorescein free in solution or covalently attached to a peptide has a strong pH dependent fluorescence, showing decreased fluorescence towards lower pH (Fig. S2). Fluorescein was chosen as the peptide fluorescence label, since its pH dependence makes it suitable to distinguish between situations where the peptide remains inside the vesicles (lower pH, exposed to the quencher, thus lower fluorescence upon proton pumping) and where the peptide escapes from the interior of the vesicles to the outside (higher pH, absence of quencher, thus higher fluorescence upon proton pumping). Of course there may also be intermediate situations where the two effects more or less can cancel one another. Experiments with the less pH sensitive fluorophore label, carboxytetramethylrhodamine (TMR), were also attempted with some CPPs. However, the more hydrophobic rhodamine seems to interact with the bilayer independent of the nature of the peptide, giving rise to fluorescence quenching. This made the measurements of escaped peptides difficult to evaluate.

3.1. Non-amphipathic CPPs

In the presence of light, the fluorescence intensity decreased for POPC/POPG vesicle-entrapped non-amphipathic fluorescein-labeled CPPs, R9 and TAT(48–60) (Fig. 2A and B). The effect is explained by the decreased pH inside the LUVs after BR proton pumping, when no or only a minor fraction of peptides leak to the outside of the LUVs. KI (from 2 M stock solution) was added as a control to quench the fluorescence intensity of potential peptides outside the LUVs. Fig. S3 shows Stern–Volmer plots of the KI-quenching of fluorescence intensity of LUVs containing labeled R9, in the absence and presence of light, together with the free fluorescein-labeled R9 in buffer. The plot F_o/F versus concentration of KI (mM) showed an increase in the slope for the sample under illumination. The results from fluorescence quenching experiments show that there is a small amount of peptides outside the LUVs and thus available for the quencher, but the great majority of the peptides should remain inside where the pH-decrease effect was dominant. The small difference between R9 and TAT in fluorescence decrease kinetics during proton pumping may be associated with a somewhat higher tendency for TAT to translocate across the membrane.

3.2. Intermediately amphipathic CPPs

For the intermediately amphipathic CPPs M918 and pVEC, we observed that illumination causes an increase of the fluorescence intensity (Fig. 3A and B). This observation indicates that certain amounts of peptides are able to escape outside the LUVs in the presence

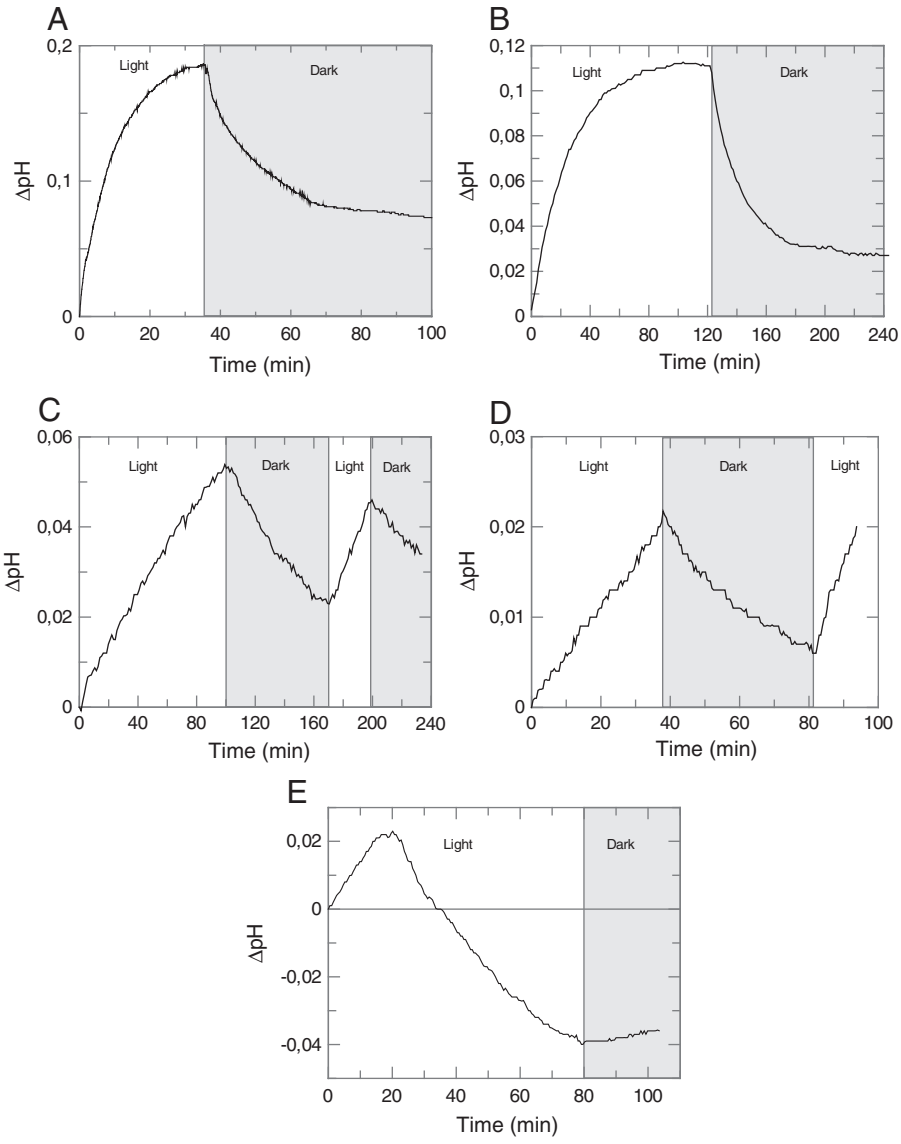

Fig. 1. Membrane pH gradient experiment. The pH changes outside the BR reconstituted LUVs as a function of illumination time. Conditions: 20 mM potassium phosphate and 100 mM KCl buffer, pH 7.2 inside the LUVs, 120 mM KCl outside the LUVs, pH 7.4, 25 °C. A) Buffer and quencher inside the 20% negatively charged BR-LUVs composed of POPC and POPG, B) buffer and quencher inside the zwitterionic LUVs composed of POPC, C) buffer and quencher inside the LUVs composed of 50% PC, 20% PE, 10% PI and 20% BMP D) 75 μM CQ and quencher inside the negatively charged BR-LUVs, and E) after addition of 50 μM PB from outside the negatively charged LUVs.

of the pH gradient. Longer period of illumination resulted in more leakage of the labeled peptide to the outside of the LUVs. However, the increase of fluorescence intensity is not permanent. It returned to almost its background value in the dark. This is due to the proton leakage to the outside of the LUVs which lowers the pH and decreases the fluorescence intensity. The behavior of M918 and pVEC is similar to that of penetratin in a previous light-driven proton pumping study [41]. In a separate experiment measuring the ΔpH outside the BR-LUVs, the hydrophobic CPP TP10 was applied to the outside of the LUVs. 0.01 μM TP10 had no obvious effect on the pH gradient whereas 0.5 μM TP10 did not allow a reproducible pH gradient over the membrane (data not shown).

3.3. Effects of membrane modification agents on the pH gradient in BR-LUVs

To investigate the effect of CQ on the pH gradient associated with BR proton pumping, we prepared CQ (75 μM) encapsulated in 20% negatively charged LUVs composed of POPC and POPG. BR was reconstituted into the 20% negatively charged LUVs through detergent-mediated reconstitution method. Resulting LUVs with and without BR were characterized using DLS instrument.

The increase of pH outside the LUVs was about 10 times less than the value observed with the LUVs in the absence of CQ (Fig. 1D). In the dark, pH again decreased, indicating that protons leaked out across the membrane, reaching an equilibrium state.

The effect of adding PB from the outside of the vesicles resulted in a short inward proton pumping of BR which continued by destabilization of the membrane (Fig. 1E).

4. Discussion

After the methodological artifacts arising from cell fixation processes had been eliminated from the studies, most CPPs have been found to use endocytotic pathways, probably together with other competing mechanisms, for their cellular internalization [50]. Endocytotic uptake route is composed of two steps: 1) endocytotic entry; and 2) endosomal escape. Apart from entry into the endosome, endosomal escape is important to obtain any bioactivity effects of the cargo molecules. In the present work, we aim to obtain information about the background

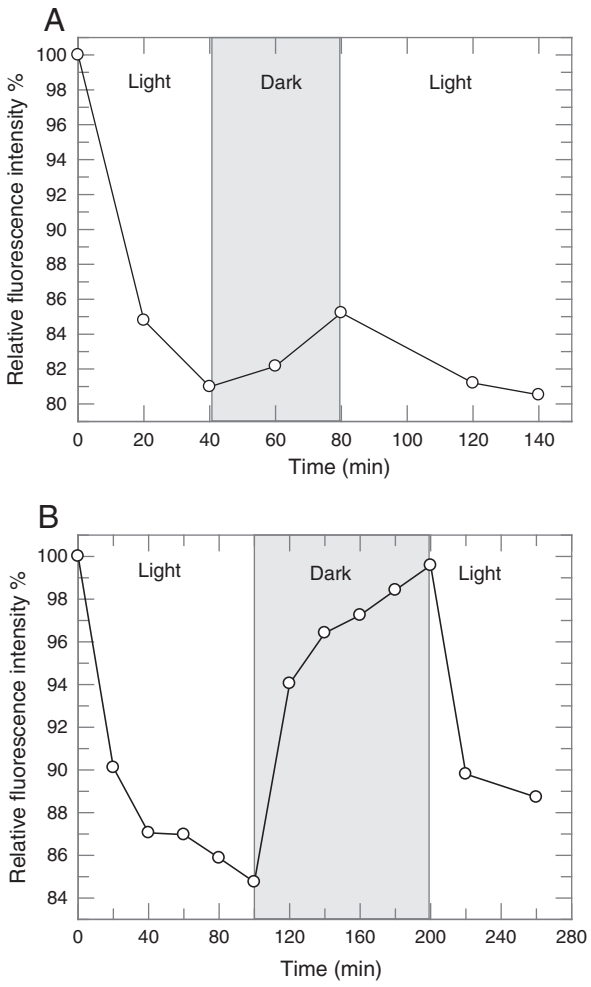

Fig. 2. Membrane translocation abilities of hydrophilic CPPs. Relative fluorescence intensity of the sample containing 20% negatively charged BR reconstituted LUVs with fluorescein-labeled A) R9 and B) TAT(48–60), inside the LUVs. Changes in fluorescence intensity at 520 nm (excitation wavelength 494 nm) were recorded in the absence of illumination (100%) and in the presence of light for indicated periods of time. Conditions: 20 mM potassium phosphate and 100 mM KCl buffer, 20 μM fluorescein-labeled R9 or TAT(48–60) and 100 mM KI inside the LUVs, total lipid concentration 2.3 mM, pH 7.4 and 20 °C. The line connecting the points is to guide the eyes.

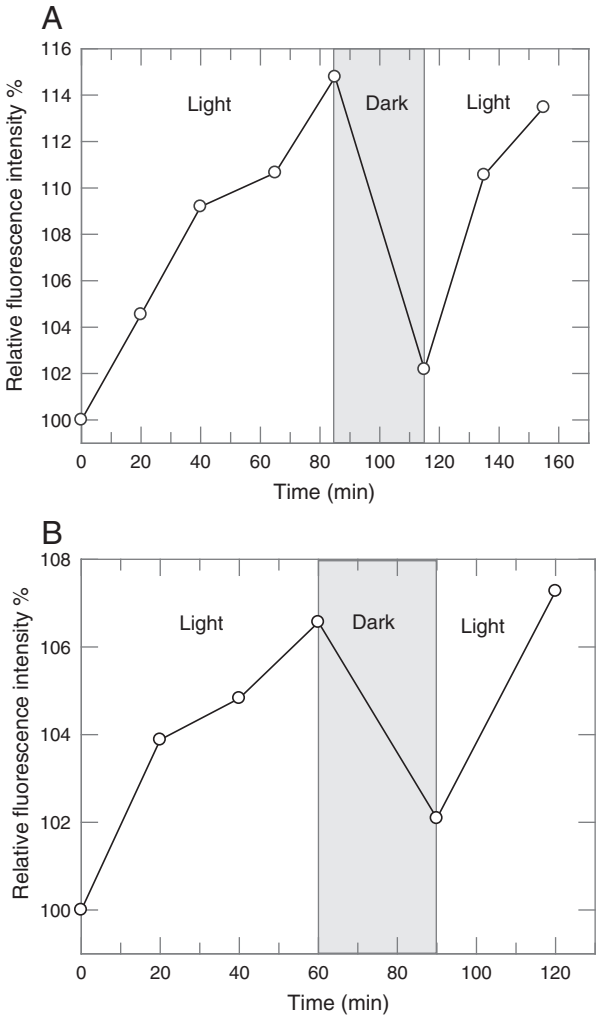

Fig. 3. Membrane translocation abilities of intermediately hydrophobic CPPs. Relative fluorescence intensity of the sample containing 20% negatively charged BR reconstituted LUVs with fluorescein-labeled A) pVEC and B) M918, inside the LUVs. Changes in fluorescence intensity at 524 nm (excitation wavelength 494 nm) were recorded in the absence of illumination (100%) and in the presence of light for indicated periods of time. Conditions: 20 mM potassium phosphate and 100 mM KCl buffer, 20 μM fluorescein-labeled M918 or pVEC and 100 mM KI inside the LUVs, total lipid concentration 2.3 mM, pH 7.4 and 20 °C. The line connecting the points is to guide the eyes.

mechanism(s) of endosomal escape. So-called late endosomes were modeled by LUVs with a 20% negative surface charge and integral BR as a proton pump. Fluorescein-labeled CPPs were entrapped together with a fluorescence quencher (KI) inside the LUVs. In the previous work we have shown that upon illumination, BR pumps protons into the LUVs, creating a pH gradient over the membrane and making inside the LUVs more acidic (about −2 pH units), similar to the late endosomes in vivo [30]. In these experiments, the rate of peptide translocation associated with vesicle acidification is on the order of 20 to 100 min comparable with the time course reflecting the endosomal escape process within cells [51].

This study shows that the non-amphipathic and intermediately amphipathic CPPs are not able to translocate across the POPC/POPG vesicular membrane without any promoting pH gradients. Moreover, in the presence of light, a pH gradient over the membrane has different effects for the examined fluorescein-labeled CPPs. For intermediately amphipathic peptides, M918 and pVEC, lowering the pH inside the LUVs results in escape of the peptides into the external solution. For non-amphipathic peptides, R9 and TAT(48–60), no or much less peptide translocation across the membrane was observed in the presence of the pH gradient. For zwitterionic POPC vesicles, both groups of peptides are unable to translocate across the membrane. This is probably related to the weaker generated pH gradient over the membrane compared with partly negatively charged vesicles (data not shown).

At least two competing processes are present in the BR-LUV model system during illumination: 1) BR proton pumping, which decreases the pH inside the LUVs and hence decreases the fluorescence intensity of fluorescein present inside the LUVs; and 2) possible translocation of the peptides across the membrane to the outside of the LUVs, which increases the fluorescence intensity. The results show that the proton gradient improves the vesicular escape efficiency of intermediately amphipathic CPPs, indicating a dominating effect of the second process. However, for non-amphipathic CPPs, the first process of pH drop inside the LUVs is more prominent.

The results indicate that the proton gradient applied in the present experiments is not sufficient to promote the escape across the vesicle membrane for non-amphipathic (Arg-rich) CPPs and that they may require more hydrophobicity to pass the membrane and escape from the endosome [52,53]. These observations are in good agreement with our previous mechanistic results observed in the presence of PB [26]. We have shown that electrostatic interactions between Arg-rich CPPs and PB enhanced their cellular uptake and cargo

delivery efficiency. A simple explanation is that the peptide–PB complex is more hydrophobic than the peptide itself. Also, based on our previous results from membrane model leakage studies, hydrophobicity is the essential parameter in model membrane interactions as seen for the hydrophobic and intermediately hydrophobic CPPs. Non-amphipathic or hydrophilic CPPs like TAT(48–60) induce no or less leakage compared with hydrophobic CPPs [26]. In the present study, the more hydrophobic peptides may have a tighter association with the membrane bilayer, and therefore be more sensitive towards the pH gradient across the membrane. Whereas the calcein leakage studies report on membrane perturbation by the peptides and the present results suggest membrane association and translocation of the peptide, both observations may have a similar molecular background in the peptide–membrane interaction.

CQ is a relatively hydrophobic weak base with two basic groups. It can absorb protons, resulting in reduction of acidification inside the LUVs, but it can also cause membrane destabilization due to vesicle swelling. Here we observed that in the presence of CQ inside the LUVs, the pH increase outside the vesicle membrane is significantly lower than the value with LUVs in the absence of CQ (Fig. 1D). Since we recorded the pH outside of the LUVs, the effect observed here should be due to leakage of the protons to the outside of the LUVs as a result of transient membrane destabilization by CQ. This membrane destabilization effect is however relatively minor compared to that of PB added to the vesicles from outside. It should be mentioned that although PB has a strong effect on the pH gradient stabilization, it does not cause leakage of entrapped calcein from similar LUVs [26].

In summary, this study gives further emphasis on hydrophobicity as an important property to direct CPP mechanisms, since hydrophobicity has been shown to promote both general membrane leakage [26] and now also pH gradient promoted vesicular escape for intermediately amphipathic CPPs. An additional insight from the study is that the endosomal model system (BR-reconstituted LUVs) used here may also be useful for studies of the membrane proton transport barrier and how it is affected by the presence of various barrier modulating agents, such as CQ or PB.

Acknowledgements

This study was supported by the Swedish Research Council (to A.G. and Ü.L.), the Swedish Foundation for Strategic Research (project no. MDB09-0015, to Ü.L. and A.G.) and Strategic Japanese–Swedish Cooperative Programme on 'Multidisciplinary BIO' from Japan Science and Technology Agency (JST) and VINNOVA (to S.F.). Hisaaki Hirose is grateful for Research Fellowships from the Japan Society for the Promotion of Science (JSPS) for Young Scientists.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2012.12.008.

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