Elucidating cell-penetrating peptide mechanisms of action for membrane interaction, cellular uptake, and translocation utilizing the hydrophobic counter-anion pyrenebutyrate

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A R T I C L E   I N F O

Article history:
Received 10 June 2009
Received in revised form 14 September 2009
Accepted 22 September 2009
Available online 29 September 2009

Keywords:
Cell-penetrating peptide
Oligonucleotide delivery
Pyrenebutyrate
Cellular translocation
Locked nucleic acid
Splice switching

A B S T R A C T

Cell-penetrating peptides (CPPs) are membrane permeable vectors recognized for their intrinsic ability to gain access to the cell interior. The hydrophobic counter-anion, pyrenebutyrate, enhances cellular uptake of oligoarginine CPPs. To elucidate CPP uptake mechanisms, the effect of pyrenebutyrate on well-recognized CPPs with varying hydrophobicity and arginine content is investigated. The cellular CPP uptake and CPP-mediated oligonucleotide delivery is analyzed by fluorescence activated cell sorting, confocal microscopy, and a cell-based splice-switching assay. The splice-switching oligonucleotide is a mixture of 2′-O-methyl RNA and locked nucleic acids delivered as a non-covalent complex with 10-fold molar CPP excess. CPP-induced membrane perturbation on large unilamellar vesicles is investigated in calcein release experiments. We observed that pyrenebutyrate facilitates cellular uptake and translocation of oligonucleotide mediated by oligoarginine nonamer while limited effect of pyrenebutyrate on more hydrophobic CPPs was observed. By combining the different experimental results we conclude that the pathway for cellular uptake of oligoarginine is dominated by direct membrane translocation, whereas the pathway for oligoarginine-mediated oligonucleotide translocation is dominated by endocytosis. Both mechanisms are promoted by pyrenebutyrate and we suggest that pyrenebutyrate has different sites of action for the two uptake and translocation mechanisms.

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1. Introduction

Cell-penetrating peptides (CPPs) are relatively short peptides, mainly cationic, with the ability to penetrate through biological membranes and gain access to the intracellular environment. These peptides share a distinguishing characteristic to also deliver various cargo molecules, such as proteins and oligonucleotides (ONs), into cells, making them highly interesting for drug delivery [1]. Direct energy-independent penetration has been suggested as uptake mechanism for some CPPs [2,3] while endocytosis is the proposed dominating uptake mechanism for most CPPs, implying that endosomal release influences translocation [4,5]. Several CPPs are rich in arginine residues and the number of arginine residues seems to be an important factor for cellular uptake [6,7]. This is substantiated by the fact that oligoarginines have proven to be efficient CPPs [8,9]. However, the efficient CPP transportan 10 (TP10) has no arginine residues in the sequence [10].

For oligoarginines, the ability to cross lipid bilayers and gain access to the cell interior is enhanced in the presence of the hydrophobic counter-anion 4-(1-pyrenyl)-butyric acid (pyrenebutyrate) [3,11,12]. It has been proposed that electrostatic interactions between peptide and pyrenebutyrate increase the hydrophobicity of the hydrophobic oligoarginine, facilitating cellular uptake and translocation [3,11]. Here, cellular uptake is defined as the accumulation of the CPP and potentially associated cargo within the cell, irrespectively of its intracellular localization and translocation is defined as direct access to non-vesicular compartments, e.g., cytosol and/or nucleus [13].

The guanidinium head group of arginine enables bidentate hydrogen bonding, which most likely is the explanation for the higher octanol/water partition coefficient registered for oligoarginine than for corresponding ornithine oligomers in the presence of hydrophobic counter-anchors [14] as well as oligoarginine partition in chloroform in the presence of phosphatidylglycerol [15]. It has been proposed that the ability of guanidinium groups to form bidentate

Abbreviations: CPP, cell-penetrating peptide; pyrenebutyrate, 4-(1-pyrenyl)-butyric acid; FACS, fluorescence assisted cell sorting; POPC, palmitoyl-2-oleoyl-phosphatidylcholine; POPG, palmitoyl-2-oleoyl-phosphatidylglycerol; ON, oligonucleotide; LNA, locked nucleic acid; 2OMe, 2′-O-methyl RNA; LUV, large unilamellar vesicle; R9, oligoarginine nonamer; Pen, penetratin; TP10, transportan 10; pVEC, vascular endothelial cadherin peptide

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0005-2736/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2009.09.014
hydrogen bonds is important for formation of ion pair complexes with, for example, the carboxyl of pyrenebutyrate or with phospholipids [12,14,15]. Complexes of pyrenebutyrate and oligoarginines can result in addition of hydrophobicity to the hydrophilic oligoarginine peptide, and thereby increase the potential of the peptide to interact with lipid bilayers [16]. Pre-treating cells with pyrenebutyrate before oligoarginine exposure increases cellular uptake as compared to non-pre-treated cells. This observation indicates the positive effect of pyrenebutyrate on membrane permeation for oligoarginine CPPs and could, possibly, facilitate translocation of oligoarginine associated cargo molecules [3,11].

The effect of the hydrophobic counter-anion pyrenebutyrate on cellular CPP uptake has, so far, mainly been studied for oligoarginine and not, in detail, for other CPPs [3]. To delineate the mechanisms for cellular uptake and translocation of CPPs we have in this study selected a number of previously characterized CPPs in addition to oligoarginine. These peptides have distinctly different hydrophobic properties and number of arginine residues. We assessed the impact of pyrenebutyrate on their capacity to gain intracellular access and by an ON cargo to give rise to biological activity. We investigate the effect of pyrenebutyrate on live cells with fluorescently labeled peptides utilizing fluorescence activated cell sorting (FACS) and confocal microscopy to quantify cellular uptake of the peptides. A cell-based splice-switching reporter assay was used to examine the effect of pyrenebutyrate on CPP-mediated translocation of bioactive phosphorothioate 12-mer ON with 2'-O-methyl RNA (2OMe) and locked nucleic acid (LNA) monomers, so called mixmer. The ONs were delivered to cells as non-covalent complexes with CPPs at a CPP:ON molar ratio of 10:1. By using bioactive cargoes, the cargo delivery to intracellular targets can be assessed [16]. To gain further insight into the effects of pyrenebutyrate on phospholipid membranes, we determined CPP- and pyrenebutyrate-induced membrane perturbation, by studies of calcein release from uncharged 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) and partly charged POPC/1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) (70/30) large unilamellar vesicles (LUVs). The LUV experiments show that cellular uptake of CPPs differs from the ability for interaction with LUV membranes, and indicate that specific cellular structures and/or biological mechanisms are involved in the uptake of CPPs into live cells. However, the number of arginine residues and the overall hydrophobicity of a CPP do influence the impact that pyrenebutyrate has on its uptake and translocation into cells. This gives insights regarding the crucial properties needed for cellular delivery and opens for design of further optimized CPPs.

2. Materials and methods

2.1. Oligonucleotides and peptides

Phosphorothioate 2OMe ONs, with and without 5'-linked Cy5 label, were synthesized on an AKTAm oligopilot™ plus 10 synthesizer (GE Healthcare) as previously described [17,18]. The 2OMe/LNA mixmers were obtained from RiboTask.

FITC (fluorescein isothiocyanate) labeled peptides used for FACS and confocal microscopy were chemically synthesized by Fmoc (9-fluorenylmethyloxycarbonyl) solid-phase peptide synthesis (SPPS) to have a γ-aminobutyric acid (GABA) residue as linker to N-terminally attached FITC to the peptides as previously reported [19]. Non-labeled peptides used for the splice-switching experiments were synthesized using t-Boc (di-tert-butyl dicarbonate) SPPS and purified by reversed phase HPLC as previously described [17]. Average hydrophobicity, for peptides without label or cargo, is calculated utilizing the von Heijne scale for biological hydrophobicity (Table 1) [20].
2.2. Confocal microscopy

HeLa cells (200,000) were plated into 35-mm glass-bottomed dishes (Iwaki) and cultured for 48 h. After removing the medium, the cells were washed twice with phosphate buffered saline (PBS) supplemented with 100 mg/ml CaCl₂ and 100 mg/ml MgCl₂ × 6H₂O (PBS(+)). In the cellular CPP uptake experiments (Fig. 1), the cells were first incubated with pyrenebutyrate in PBS(+) for 5 min, and then the peptide solution in PBS(+) (40 μM) was added to yield the final concentration of pyrenebutyrate and peptides (50 and 10 μM, respectively). After incubation for 15 min, the cells were washed with PBS(+). The localization of the fluorescently labeled peptides was analyzed without fixing using a confocal scanning laser microscope (Olympus FV300) equipped with a 40× objective lens (UplanApo, NA 0.85).

In the cellular CPP-mediated ON uptake experiments (Fig. 8), HeLa cells were washed twice with PBS(+), treated with 50 μM pyrenebutyrate in PBS(+) for 1 min, and thereafter the cells were washed twice with PBS(+). The localization of the fluorescently labeled peptides was analyzed without fixing using a confocal scanning laser microscope (Olympus FV300) equipped with a 40× objective lens (UplanApo, NA 0.85).

2.3. Quantification of peptide uptake

HeLa cells were pre-incubated at 37 °C with pyrenebutyrate (50 μM) in PBS(+) for 5 min prior to the addition of FITC-labeled CPPs (10 μM) in PBS(+). After 15 min incubation, the cells were washed twice with PBS containing 0.5 mg/ml heparin, and then treated with 0.01% trypsin in PBS at 37 °C for 10 min. The cells were centrifuged at 3000 rpm (600 g) for 5 min. The supernatant was removed; the cells were washed with PBS and centrifuged at 3000 rpm for 5 min. After this washing cycle was repeated, the cells were suspended in PBS (400 μl) and subjected to fluorescence analysis on a FACSCalibur flow cytometer (BD Bioscience) using a 488 nm laser excitation and a 515–545 nm emission filter. Each sample was analyzed for 10,000 events.

2.4. Preparation of large unilamellar vesicles

Large unilamellar vesicles with POPC (Avanti Polar Lipids) and optionally 30% POPG (Avanti Polar Lipids) were prepared by dissolving the lipid, without further purification, at the desired concentration in chloroform to obtain a homogeneous solution of the lipid and then the solvent was removed by evaporating it under high vacuum for 3 h. The resulting dried lipid film was re-suspended by adding 50 mM potassium phosphate buffer solution (pH 7.4). This solution was then vortexed for 10 min followed by five freeze-thaw cycles to reduce the lamellarity. After the freezing and thawing cycles, the lipid solution was pushed through two polycarbonate filters (100 nm pore size) 20 times by using an Avanti manual extruder. This method gave unilamellar vesicles with 100 nm diameter.

2.5. Calcein release from large unilamellar vesicles

LUVs with entrapped calcein (C30H26N2O13, 622.5 Da, Molecular Probes) were prepared by using a buffer solution containing 55 mM calcein with the above mentioned method. The final pH was adjusted to 7.4. Free calcein outside the vesicles was removed by passing through Sephadex-G25 (GE Healthcare) columns two times. The fluorescence intensity of calcein was recorded on a Horiba Jobin Yvon Fluorolog-3 spectrofluorometer using a 490 nm laser excitation and a 510 nm emission filter. The fluorescence intensity in the presence of 55 mM calcein is low due to self-quenching, but increases upon dilution. Pyrenebutyrate with the final concentration of 50 μM was added to the vesicle solution composed of 100 μM POPC or POPC/POPG (70/30). Each CPP was first titrated to the LUVs to define a suitable treatment concentration that induces observable calcein release without the presence of pyrenebutyrate. It was found that a concentration of 5 μM was suitable for all investigated CPPs, except TP10 where it was 0.01 μM. The CPPs were applied to LUVs after 10 min pyrenebutyrate pre-treatment at 20 °C. The release of calcein was monitored as an increase in the fluorescence intensity at various peptide incubation time points. One hundred percent calcein release was induced by the addition of 10% (w/v) Triton X-100 (Sigma-Aldrich). Using this as a reference and by subtracting the background

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**Fig. 1.** Observations of cellular uptake by confocal microscopy. Uptake of FITC-labeled CPPs in live HeLa cells as analyzed by confocal microscopy. HeLa cells were exposed for 10 μM of peptide for 15 min with or without 5-min pre-treatment of 50 μM pyrenebutyrate (PB) before addition of peptide.
fluorescence, the degree of calcein release induced by different peptides was calculated using the following equation: % calcein release = [(F1 – F0)/(F2 – F0)] × 100, where F0 and F1 are the initial fluorescence intensities observed without peptide and after treatment with Triton X-100, respectively. F is the fluorescence intensity in the presence of peptides. The standard deviation in the endpoints (10 min) was less than ±4 in units of % calcein release (Fig. S1).

2.6. Splice-switching assay

The day before experiments, 80,000 pLuc705 HeLa cells were seeded in 24-well plates (Sigma-Aldrich), and grown overnight in Dulbecco’s modified Eagle medium (DMEM) supplemented with 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 10% (v/v) fetal bovine serum (full media). Cells were washed with PBS (Invitrogen), treated with 50 μM pyrenebutyrate (Sigma-Aldrich) in PBS for 1 min, and thereafter the cells were washed two times with PBS. Then 180 μl PBS was added to cells followed by immediate addition of 20 μl of CPP and ON complexes with chloroquine diphosphate (Sigma-Aldrich) giving rise to a final treatment concentration of 5 μM peptide, 500 nM mixmer, and 75 μM chloroquine. The transfection mixture was prepared by mixing CPP and ON in PBS at room temperature 30 min prior to addition of chloroquine. The cells were then incubated at 37°C for 1 h, washed with PBS, and allowed to grow for 16 h in DMEM supplemented with 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum (full growth media).

For transfection of oligonucleotides utilizing Lipofectamine™ 2000, the cells were washed with PBS (Invitrogen), and treated for 16 h in 200 μl of transfection mixture. The transfection mixture consisted of 100 μl of full growth media and 100 μl of DMEM, oligonucleotide, and Lipofectamine™ 2000 (2.5 μl/μg ON).

The cells were then washed with PBS and lysed using 100 μl of cell culture lysis buffer (Promega) for 15 min at room temperature. Luciferase activity in the cell lysate was measured using Glomax™ 96 plate luminometer (Promega). Protein content in the cell lysate was determined by Lowry-assay (Bio-Rad) and mitochondrial activity was determined with wst-1 assay (Roche Applied Science) according to the manufacturer’s protocol.

2.7. Statistical analysis

Results are presented as means ± standard error of the mean in all figures and standard deviation is shown within parenthesis in Table 1. Each splice-switching experiment was performed in triplicate while each confocal microscopy and cellular uptake experiment was performed in duplicate. Each POPC and POPC/POPG LUV experiments were performed as single treatments after initial optimization of peptide treatment concentration. GraphPad Prism (4.00) was used for graphs and associated calculations.

3. Results and discussion

3.1. Cell-penetrating peptides

Most CPPs are cationic and arginine residues or, more specifically, guanidinium groups are often important for activity. Guidelines for CPP design have been proposed but universally applicable rules are lacking [21]. The use of pyrenebutyrate as counter-anion to CPPs should add hydrophobic properties to hydrophilic peptides and thereby it increases the ability for insertion into lipid bilayers. Here, CPPs with distinctly dissimilar structures in terms of amino acid residue composition, sequence length, and hydrophobic properties have been selected for investigation of the influence of pyrenebutyrate on cellular uptake and translocation capacity (Table 1). The CPPs selected are the hydrophilic oligoarginine nonamer, R9 [8], and the HIV-1 transactivator of transcription derived peptide, pTat48–60 [22,23]. Intermediately hydrophobic CPPs [24] selected are penetratin (Pen) derived from the Antennapedia protein [2], M918 derived from the tumor suppressor protein p14ARF [25], and pVEC derived from the cell adhesion molecule vascular endothelial cadherin [26]. The most hydrophobic peptide examined, TP10, has no arginine residues and it is the only CPP in this selection that has been assigned to be taken up by clathrin-mediated endocytosis [10,27]. To reveal the impact of arginine residues for ON delivery and lipid bilayer interaction we have also included a penetratin analogus peptide, PenArg, in which all lysine residues are replaced by arginine residues [7]. The CPPs included in this study can be divided into three subgroups: hydrophilic (R9 and pTat48–60), intermediately hydrophobic (Pen, PenArg, pVEC, and M918), and hydrophobic (TP10) (Table 1). The CPPs also display different arginine content. The CPPs have been studied for two biological assay endpoints, namely, the cellular uptake of FITC-labeled CPPs and the splice-switching assay, and one chemical assay endpoint, calcein release from LUVs, and the effect of pyrenebutyrate on these three endpoints.

3.2. Cellular uptake of fluorescently labeled CPPs

The FITC-labeled CPPs were capable of crossing the cellular membrane of HeLa cells as seen by confocal microscopy and FACS (Figs. 1 and 2 and Table 1). The peptides with high hydrophobicity were virtually unaffected by the presence of the pyrenebutyrate counter-anion while the uptake of more hydrophilic peptides, R9 and possibly pTat48–60, increased in the presence of and after pre-treatment with pyrenebutyrate (Fig. 2 and Table 1). The CPP treatments were performed at 10 μM concentration for 15 min after 5-min pre-treatment with only pyrenebutyrate (50 μM). In agreement with an earlier report, the utilized treatment, including pyrenebutyrate, does not induce neither acute leakage nor long-term alterations in cell viability for R9 [3]. Despite the relatively short treatment time the hydrophilic peptide TP10 gave rise to cytotoxicity as observed by microscopy while cells treated with the other CPPs included in the study induced no or minor toxic effects.

In the presence of 50 μM pyrenebutyrate the cellular uptake was most prominently enhanced for R9. A most likely explanation is that pyrenebutyrate inserts into the plasma membrane as previously reported [11] and thereafter interacts electrostatically by bidentate hydrogen bonding with the arginine residues during the CPP treatment, inducing direct translocation. The pTat48–60 CPP has an overall low cellular uptake but the uptake tends to increase in the presence of pyrenebutyrate (Fig. 2 and Table 1). Analysis with

![Fig. 2. Quantification of cellular uptake by fluorescence assisted cell sorting.](Image 329 to 526)
confocal microscopy reveals that the intracellular CPP-distribution is diffuse for the hydrophilic peptides and to some extent also for the intermediately hydrophobic CPPs in the presence of 50 μM pyrenebutyrate (Fig. 1). Diffuse intracellular distribution is a characteristic indicative for non-endosomal uptake. In contrast, the hydrophobic peptide, TP10 without arginine residues, shows a more punctate distribution, a phenomenon characteristic for endosomal uptake [19,28] (Fig. 1). The diffuse cellular distribution of the other CPPs does not prove that direct translocation is the exclusive uptake route, although it has been confirmed for R9 that pyrenebutyrate assisted uptake occurs at 4 °C [3]. Rapid endosomal uptake followed by endosomal release may also result in diffuse cytosolic distribution of the fluorescently labeled CPPs. However, direct penetration aided by presence of pyrenebutyrate (50 μM) appears to dominate the cellular uptake of the arginine-containing CPPs with a fluorescein label as a covalently attached cargo but a contribution of endosomal uptake of such CPPs cannot be excluded.

3.3. CPP interaction with large unilamellar vesicles

To investigate whether the effect of pyrenebutyrate is directly correlated with lipid bilayer interaction, peptide and pyrenebutyrate-induced efflux of calcine from uncharged POPC and partially negatively charged POPC/POPG (70/30) LUVs was investigated [29]. In the LUV experiments one can assess the CPP-induced translocation of calcine or membrane perturbation by observing calcine release from the vesicles [12]. The CPPs were applied to 100 μM of calcine-loaded LUVs after pyrenebutyrate (50 μM) pre-treatment for 10 min and the induced calcine release was recorded at various time points. A peptide treatment concentration of 5 μM was used for all CPPs except TP10 which had to be applied at 500 times lower concentration due to its potent membrane interaction.

A clear increase in calcine release was observed in the presence of pyrenebutyrate for both neutral and partially negatively charged LUVs treated with the intermediately hydrophobic CPPs M918, pVEC, and Pen Arg (Fig. 3). For the hydrophilic peptides, calcine release generated by R9 was clearly enhanced by pyrenebutyrate in the zwitterionic LUVs (Fig. 3 and Table 1). The hydrophobic counter-anion had a minor effect on calcine release caused by pTat48–60 (Fig. 3). The intermediately hydrophobic CPP, Pen, behaved together with R9 in a different manner for charged and uncharged vesicles. As shown in Fig. 3, we observed significant calcine release in the presence of pyrenebutyrate for the zwitterionic POPC vesicles but not for the partly charged POPC/POPG vesicles. One possible explanation is that the partly negatively charged vesicles (POPC/POPG) may compete with pyrenebutyrate, which diminishes its interaction with the peptides. There is obviously a very delicate balance between hydrophobic residues, number of arginine residues, and lipid bilayer interaction that determines the outcome of the LUV experiments with zwitterionic POPC and partly charged POPC/POPG (70/30) vesicles. The observation that the intermediately hydrophobic CPPs induce a dramatic release of calcine in the presence of pyrenebutyrate may have potential implication in the development of novel antibiotics.

3.4. Delivery of bioactive oligonucleotide

It is assumed that CPP interaction with pyrenebutyrate facilitates the internalization of R9 [11]. To assess the impact of pyrenebutyrate for translocation of a bioactive ON cargo, a splice-switching reporter assay was utilized [30]. The splicing reporter system is based on a plasmid (pLuc705) carrying a luciferase-coding sequence that is interrupted by an insertion of intron 2 from β-globin pre-mRNA, which includes an aberrant splice-site that activates a cryptic splice-site. Unless the aberrant splice-site is masked by a complementary ON, the pre-mRNA of luciferase will give rise to expression of non-functional luciferase. By using HeLa cells which are stably transfected with pLuc705, it is possible to evaluate cellular delivery of splice-
Fig. 4. Truncated LNA/2OMe mixmer equals activity for full-length 2OMe SSO. Luciferase activity, normalized to activity induced by the 18-mer 2OMe ON, after treatment at 250 nM utilizing the Lipofectamine™ 2000 transfection reagent. The truncated 12-mer LNA/2OMe mixmer displays similar splice-switching activity and specificity as the corresponding 18-mer 2OMe SSO. Capitals in the sequence list refer to 2OMe monomers and small letters are LNA monomers. Letters in bold refer to mismatches to target pre-mRNA.

Fig. 5. Influence of pyrenebutyrate on CPP-mediated delivery of splice-switching oligonucleotide. Luciferase activity induced by successful delivery of splice-switching oligonucleotide into pLuc705 HeLa cells exposed to CPP and LNA/2OMe mixmer complexes. The luciferase activity is presented as normalized luciferase activity for pLuc705 HeLa cells.

Fig. 6. Influence of chloroquine and pyrenebutyrate on R9-mediated delivery of splice-switching oligonucleotide. Luciferase activity induced by successful delivery of splice-switching oligonucleotide into pLuc705 HeLa cells exposed to CPP and LNA/2OMe mixmer complexes. Chloroquine (CQ) buffers endosomes and delays the lysosomal pathway and is needed for delivery of the bioactive mixmer. Pyrenebutyrate (PB) may enhance the endosomal release but CQ is needed for mixmer delivery indicating that an endosomal pathway is exploited in R9-mediated ON delivery. The mixmer is included in all treatments except ‘untreated.’
factor for facilitating endosomal release after initial endocytotic entry, since endocytosis relies on initial invagination of the plasma membrane [36].

Cellular pre-treatment with pyrenebutyrate clearly ameliorates both uptake of R9 and R9-mediated translocation of mixmer inducing splice-switching and subsequent increase in luciferase activity (Figs. 1, 2, and 5 and Table 1). The mechanism of action for pyrenebutyrate may have similarity in the two experimental set-ups but the site of action for the hydrophobic counter-anion seems to differ. For the arginine-rich CPPs the proposed endocytotic pathway in the splice-switching studies is supported by earlier observations that arginine residues have a capacity to stimulate endocytosis, or more specifically macropinocytosis [37]. This would also facilitate endocytotic uptake of ONs that are in non-covalent complexes with arginine-rich CPPs. The low presence of pyrenebutyrate in the splice-switching experiments, as compared to the cellular uptake experiments, probably alters the uptake pathway away from direct penetration. The high impact of pyrenebutyrate presence on the R9-mediated ON delivery is possibly explained by promotion of the translocation through the endosomal membranes, which seems to be the rate-limiting step for R9-mediated ON delivery in this experimental set-up in combination with the delay of endosomes entering the lysosomal degradation pathway created by the presence of chloroquine. The impact of pyrenebutyrate on calcein release observed in the POPC LUV experiments (Fig. 3) is probably sufficient to facilitate endosomal translocation or release of ON, in complex with R9, explaining the enhanced splice-switching activity in the presence of pyrenebutyrate (Figs. 5 and 8). Similar, but less prominent, enhancement of splice-switching activity was also observed for pTat48–60 and PenArg, and this was presumably because of smaller arginine content in these sequences influencing the capability for endocytotic entry (Fig. 5 and Table 1).

For the other intermediately hydrophobic CPPs, Pen, pVEC, and M918, the uptake is also mainly endocytotic since treatment time had to be extended and chloroquine had to be included in the CPP–ON transfection mixture to be able to record any splice-switching activity (data not shown). However, the hydrophobicity and the relatively low proportion of arginine residues in these peptides may limit the endocytotic entry and thereby the potential effect of pyrenebutyrate as an endosomal releaser. Together this disfavors the ability of pyrenebutyrate to ameliorate the rate of ON translocation.

The mechanism of uptake for the most hydrophobic CPP TP10 is outside of the reasoning used for the hydrophilic and the intermediately hydrophobic CPPs investigated here. The TP10 peptide does not have any arginine residue and its uptake route is clathrin-mediated endocytosis [27]. Therefore, TP10 neither stimulates macropinocytosis nor complex formation with pyrenebutyrate, and this probably explains the very little enhancement of splice-switching activity by the addition of pyrenebutyrate (Fig. 5 and Table 1). The result of clathrin-mediated endocytosis is also endocytotic vesicles and the high hydrophobicity of the peptide per se may outrule the potential contribution of a hydrophobic counter-anion for endosomal release.

Fig. 8. Cellular peptide and oligonucleotide distribution after R9-mediated delivery. Confocal microscopy analysis of HeLa cells treated with non-covalent complexes of FITC-labeled R9 and Cy5-labeled 2OMe ON in accordance with the protocol for the splice-switching assay shows that the ON is taken up by the cells. The same cells have been detected for FITC-labeled R9, Cy5-labeled ON, and by differential interference contrast. The cells have grown for 16 h in full growth media after transfection. The vesicular distribution of the intracellular ONs indicates endosomal uptake. The endosomal release of ON for the pyrenebutyrate (PB) pre-treated cells is by no means complete.
4. Conclusions

From the present observations we can draw some mechanistic conclusions. We first conclude that the hydrophobic TP10 induces toxicity in the cellular uptake studies. TP10 exploits a non-arginine induced endocytotic pathway (clathrin-mediated endocytosis) [19,27] that is unaffected by pyrenebutyrate (Fig. 5) and TP10 gives rise to membrane perturbation and associated calcine release from LUVs at very low peptide concentrations (Fig. 3). This peptide is an interesting CPP with many potential applications but it displays deviating properties as compared to the other CPPs in the selection and it will therefore be excluded from the remaining mechanistic discussion.

The results suggest distinct patterns of cellular uptake and translocation mechanisms, varying between the different peptides. Overall, two major cell entry pathways are considered: the endosomal pathway composed of two steps, namely, endocytotic entry followed by endosomal escape, and direct cell membrane penetration. First we observe that the two biological endpoints in general go together (Figs. 2 and 5 and Table 1), even though the effective pyrenebutyrate concentration employed in the cellular uptake experiments is considerably higher than in the splice-switching assay.

The LUV calcine release experiments are indicators of the interaction and perturbation of the lipid membrane caused by the different CPPs. Release from self-quenching of vesicle enclosed calcine is commonly used as a measure of bilayer perturbation and interpreted as “transient pore formation” [38,39]. The present results on calcine release from LUVs can be used as qualitative indicators of CPP-related calcine translocation or bilayer perturbation, with relevance for the direct penetration mode of cell entry, or for the steps of endosomal membrane translocation or endosomal escape. Chloroquine acts to some extent in parallel with pyrenebutyrate, in that it promotes the endosomal escape by prolonging the time it takes for the endosome to enter the lysosomal pathway, i.e., chloroquine facilitates the endosomal escape step during the endosomal pathway [34]. However, there is no evidence that chloroquine promotes the direct penetration mode of entry.

For the biological endpoints, pyrenebutyrate clearly promotes the cellular uptake of R9 and R9-mediated ON delivery (Figs. 2 and 5). The other CPPs are less affected by pyrenebutyrate in both biological assays. For R9, chloroquine is needed to give any splice-switching activity at all, independent of the absence or presence of pyrenebutyrate (Fig. 6) indicating that an endosomal pathway is employed for ON delivery as also observed by confocal microscopy (Fig. 8). The calcine release from POPC LUVs was to some extent promoted by pyrenebutyrate for R9 but less extensively than for the intermediately hydrophobic CPPs Pen, PenArg, pVEC, and M918 (Fig. 3 and Table 1). This suggests also that very little, if any, stable pore formation or significant membrane perturbation may occur for the translocation of R9.

The suggested interpretation for the hydrophilic and arginine-rich R9 is that direct penetration is the major pathway for FITC-labeled R9 peptide itself in the presence of high pyrenebutyrate concentration (50 μM) [1] [3], while the endocytotic pathway, presumably arginine-induced macropinoscytosis, dominates in the splice-switching assay, i.e., ON translocation mediated by R9 as a non-covalent complex, where minor amounts of pyrenebutyrate are present (Fig. 5 and Table 1). In the splice-switching assay the endosomal escape, from macropinosomes, is rate limiting and this vesicular release is promoted by pyrenebutyrate (Fig. 5). The enhanced luciferase activity observed for R9-mediated ON translocation is not reflected in analysis with confocal microscopy (Fig. 8). This can be explained by the fact that the amount of bioactive ON needed to induce splice-switching activity in the nucleus is very low [4] and therefore the improved ON-release from endocytotic vesicles observed as enhanced luciferase activity does not have to be clearly distinguishable by microscopy.

Similar to R9, but less prominent effect of pyrenebutyrate was observed for the hydrophilic pTat48–60, which also is arginine-rich but inferior to R9. For pTat48–60, very low cellular uptake was observed but pyrenebutyrate has a positive effect on the uptake and gives diffuse cytosolic distribution of the peptide, indicating direct penetration (Fig. 1). In the splice-switching assay, pyrenebutyrate yields a smaller enhancement of ON delivery even though the bioactive mixmer ON is being rather efficiently delivered (Fig. 5 and Table 1). The shift to an endocytotic pathway may be due to the longer treatment time or due to treatment with a non-covalent CPP:ON complex instead of free peptide. The rate-limiting step in this experimental set-up is probably the translocation of ON through the endosomal membranes. The ON is taken up by the cells in non-covalent complex with R9 into endosomes (Fig. 8) and pyrenebutyrate may accelerate the ON translocation into the cytosolic environment utilizing mechanisms that do not result in extensive, but minor, membrane perturbation (Fig. 3).

For the intermediately hydrophobic CPPs, Pen, pVEC, and M918, the biological endpoints are only moderately affected by pyrenebutyrate but calcine release from LUVs is significantly enhanced by pyrenebutyrate (Fig. 3 and Table 1). The rate-limiting step for both biological endpoints should therefore, in these cases, not be linked to direct membrane perturbations, neither on the plasma membrane nor on endosomes. Since interaction of arginine-rich peptides with membrane associated proteoglycans has been reported to be critical for the induction of macropinoscytosis [36], increase in hydrophobicity in arginine-rich peptides might increase in their interaction with membranes while decreasing its affinity to proteoglycans and this may eventually result in the decreased endocytotic entry of these peptides and the accompanied ON. However, further study is necessary to establish its validity. Delivery of the bioactive ON mediated by PenArg (Fig. 5 and Table 1), having a higher arginine density compared to Pen, is more enhanced by pyrenebutyrate, and this supports the importance of arginine residues in CPPs for effective cellular uptake and translocation of accompanying ON over endosomal membranes in the presence of pyrenebutyrate.

In summary, the cellular delivery of hydrophilic arginine-rich CPPs, particularly R9, becomes more effective when the hydrophobic counter-anion pyrenebutyrate is present in both experimental set-ups investigated here. This is seen both in the cellular uptake experiment where the uptake mechanism is shifted towards direct cellular penetration and in the splice-switching experiment where the uptake pathway for R9 and translocation of associated ON is shifted towards endosomal uptake. The combined experiments yield information that we have used to delineate potential mechanistic pathways for CPP-mediated cellular delivery under varying conditions. These findings may also have implication on the development of new covalently modified guanidinium-rich CPPs, that will have favorable properties under in vivo conditions where the opportunity to use hydrophobic counter-ions is restricted. Such peptides or peptoids may have applications for drug delivery, in particular for oligonucleotide-based therapeutics.

Acknowledgements

pLuc705 HEp2 cells were kindly provided by R. Kole and B. Lebleu. Suzy Lena (RiboTask) is thanked for synthesis of mixmers. This work was supported by the Swedish Science Foundation (VR-NT and VR-MED), the Knut and Alice Wallenberg Foundation, the Swedish Governmental Agency for Innovation Systems (VINNOVA-SAMBIO 2006), the Swedish Center for Biomembrane Research, Strategic Japanese-Swedish Cooperative Programme on ‘Multidisciplinary BIO’ from Japan Science and Technology Agency (JST) and VINNOVA, and Grant-in-Aid for Scientific Research on Priority Areas “Life Surveyor”
from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbamem.2009.09.014.

References