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Biochimica et Biophysica Acta 1768 (2007) 1769–1776

Biochimica et Biophysica Acta BBA

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Peptide degradation is a critical determinant for cell-penetrating peptide uptake

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Received 14 November 2006; received in revised form 8 March 2007; accepted 30 March 2007
Available online 19 April 2007

Abstract

Cell-penetrating peptide mediated uptake of labels appears to follow an equilibrium-like process. However, this assumption is only valid if the peptides are stabile. Hence, in this study we investigate intracellular and extracellular peptide degradation kinetics of two fluorescein labeled cell-penetrating peptides, namely MAP and penetratin, in Chinese hamster ovarian cells. The degradation and uptake kinetics were assessed by RP-HPLC equipped with a fluorescence detector. We show that MAP and penetratin are rapidly degraded both extracellularly and intracellularly giving rise to several degradation products. Kinetics indicates that intracellularly, the peptides exist in (at least) two distinct pools: one that is immediately degraded and one that is stabile. Moreover, the degradation could be decreased by treating the peptides with BSA and phenanthroline and the uptake was significantly reduced by cytochalasin B, chloroquine and energy depletion. The results indicate that the extracellular degradation determines the intracellular peptide concentration in this system and therefore the stability of cell-penetrating peptides needs to be evaluated.

Keywords: Cell-penetrating peptide; Protein transduction domains; Uptake; Degradation; Endocytosis inhibitor

1. Introduction

Cell-penetrating peptides (CPPs) or protein transduction domains (PTDs) are peptides that translocate across the plasma membrane of mammalian cells both in vitro [1,2] and in vivo [3,4]. CPPs are capable of delivering functional cargos into cells, such as oligonucleotides [5], peptide nucleic acids [6], plasmids [7] and liposomes [8]. The mechanism by which CPPs enter cells is not clear although many recent papers have indicated different forms of endocytosis.

A few studies have investigated the metabolic stability or the pattern of degradation of these peptides. In a paper by Elmquist and colleagues the enzymatic degradation of pVEC and its all-D analog was investigated in buffer containing physiological concentration of trypsin or carboxypeptidase A and B and the half-lives were found to be 10.5 and 44.6 min in respective buffer sample [9]. This was followed by another paper by Lindgren and colleagues who investigated transportan, TP10 and penetratin in contact with Caco-2 cells and found the stability of the peptides was in the order of transportan > TP10 > penetratin [10]. Moreover, Tréhin and colleagues investigated Tat, penetratin and several calcitonin analogs in MDCK, Calu-3 and TR146 cells and found that the levels of proteolytic activity varied highly among the cell lines tested [11].

Recently, we showed that the main mechanism by which CPPs, or rather the cargo attached to the CPPs, accumulate in the cellular interior is by the proteolytic processing of the CPP-cargo conjugate into membrane impermeable products [12]. Hence, it is of interest to characterize the degradation kinetics and products of CPPs in order to investigate how they relate to cargo-delivery efficiency. The understanding of the internalization and degradation kinetics of CPPs is important for the practical aspects of cargo delivery. In uptake experiments, with incubation time points up to 1 h or longer, the peptide needs to be stabile outside the membrane

Abbreviations: CPP, cell-penetrating peptide; PTD, protein transduction domain; fl-HPLC, fluorescence-HPLC; MAP, model amphipathic peptide; PBS, phosphate buffered saline; IC, intracellular; EC, extracellular; CHO, Chinese hamster ovarian

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doi:10.1016/j.bbamem.2007.03.029

Table 1
Names and sequences of the cell-penetrating peptides used in this work

Peptide	Sequence
MAP(KLAL) [16]	Fluo-KLALKLALKALKAALKLA-amide
Penetratin [19]	Fluo-RQIKIWFQNRRMKWKK-amide
Perforin 1–16 [15]	Fluo-PCHTAARSECKRSHKF-amide
Perforin 1–34 [15]	Fluo-PCHTAARSECKRSHKFVPGAWLAGEGVDVTSLRR-amide

and not degraded within that time, hence, evaluation of peptide stability is an important parameter. These studies are also essential in order to achieve a better understanding of the mechanism by which CPPs pass through membranes and enter cells.

In this study we investigate intracellular (IC) and extracellular (EC) peptide degradation products and kinetics of two structurally different CPPs, namely MAP and penetratin. The degradation products and kinetics of fluorescently labeled MAP and penetratin after incubation with Chinese hamster ovarian (CHO) cells were assessed by RP-HPLC equipped with a fluorescence detector. Data obtained from the experiments were used to generate a schematic of different pathways in uptake of the peptides. An attempt to inhibit degradation of the peptides was made by using several common protease inhibitors and the toxicity of these protease inhibitors and substrates was investigated in an LDH leakage assay. Wortmannin, cytochalasin B and nocodazole, which have previously been shown to inhibit different forms of endocytosis [13], were analyzed for their effect on uptake of MAP and penetratin. In addition, the uptake and degradation was investigated in cells treated with chloroquine and in energy depleted cells.

2. Materials and methods

2.1. Materials

5(6)-Carboxyfluorescein was purchased from Molecular Probes. All amino acids were purchased from Bachem, Switzerland. Cell culture medium DMEM-F12, Foetal Bovine Serum (FBS), Bovine Serum Albumin (BSA), Penicillin–Streptomycin (PEST) and Trypsin were purchased from GIBCO, USA. Other chemicals and reagents, when not specified were purchased from Sigma-Aldrich. Data evaluation was performed using the software GraphPad Prism 4.0 from GraphPad soft Inc.

2.2. Peptide synthesis

Peptides (Table 1) were synthesized in a stepwise manner in a 0.1 mmol scale on a peptide synthesizer (Applied Biosystems model 431A, USA) using *t*-Boc strategy of solid-phase peptide synthesis. *t*-Boc amino acids were coupled as hydroxybenzotriazole (HOBt) esters to a *p*-methylbenzylhydrylamine (MBHA) resin to obtain C-terminally amidated peptides. The peptides were labeled with 5-(and-6)-carboxyfluorescein as HOBt ester at the N-terminus as described in Fischer et al. [14]. The peptides were finally cleaved from the solid phase with liquid HF at 0 °C for 1 h in the presence of *p*-cresol (and thiocresol 1:1 for penetratin). Purification of peptides with reverse-phase (RP) HPLC was carried out with a Supelcosil LC-18 preparative column 5 μm (250×21.2 mm) (Sigma Aldrich Chemie, Steinheim, Germany) using acetonitrile–water, both containing 0.1% trifluoroacetic acid, gradient from 20 to 100% acetonitrile. Correct masses of purified peptides were obtained using a Perkin Elmer prOTOF™ 2000 MALDI O-TOF mass spectrometer.

2.3. Cell culture

CHO-K1 cells were cultured in Dulbecco's Modified Eagle's Media (DMEM) F-12 with Glutamax-I (GIBCO, USA) supplemented with 10% foetal calf serum, 100 μg/ml streptomycin, 100 U/ml penicillin.

2.4. Cellular uptake experiments

CHO cells seeded in 24-well plates in 500 μl medium (200,000 cells/well) were used for experiments performed in triplicates 1 day after seeding. Cells for uptake experiments were washed 2 times with ice-cold PBS and incubated with 200 μl, 1 μM penetratin or MAP in PBS buffer supplemented with 1 g/l D-glucose at 37 °C for 5, 15, 30 or 60 min. As negative controls we used two sequences originating from the pore forming perforin protein, namely, amino acids 1–16 and 1–34 [15]. After each time point, the incubation buffer containing the peptides was removed, diluted with 0.1% Triton X-100 containing 0.1% trifluoroacetic acid and injected to HPLC where quantification was performed by fluorescence measurement at 524 nm after excitation at 445 nm using calibration values obtained with 10 pmol of the parent peptide analyzed under identical conditions. The cells were washed 2 times with ice-cold PBS, incubated with 500 μl of ice-cold PBS and treated with diazotized 2-nitroaniline as described previously [16] in order to modify any remaining extracellularly located peptide. Briefly: to 400 μl ethanol/water 1/1 v/v containing 2-nitroaniline (0.06 M) and HCl (0.125 M), 50 μl 0.6 M $NaNO_2$, were added. After standing for 5 min at ambient temperature, 10 μl of this reagent was added to the ice-cold PBS covering the cell layer and allowed to react for 10 min at 0 °C. After aspiration of the diazo reagent the cells were washed 2 times with ice-cold PBS and finally lysed with 200 μl 0.1% Triton X-100 containing 0.1% trifluoroacetic acid for 2 h at 0 °C. The resulting lysates were used for HPLC-analysis. The effects of the protease inhibitors, bacitracin (5 mg/ml), leupeptin (50 μM), phenanthroline (2 mM), PMSF (2 mM) were analyzed by incubating cells with MAP or penetratin together with each inhibitor or BSA (1%) at 37 °C for 1 h. In addition BSA and bacitracin were pre-incubated with the peptides 40 min prior to incubation with cells. In order to investigate the endocytosis inhibitors, the cells were pre-incubated with wortmannin (5 nM), nocodazole (5 μM) or cytochalasin B (5 μM) for 30 min, followed by incubation with peptides in the presence of inhibitors for 1 h at 37 °C. The same procedure was performed with chloroquine (50 μM) and sodium azide (60 mM) together with deoxyglucose (20 mM). Samples were taken and analyzed by RP-HPLC.

2.5. LDH leakage assay

The assay was performed using the In Vitro Toxicology Assay Kit, Lactic Dehydrogenase Based (Sigma). In the assay, CHO cells were seeded in 24-well plates in 500 μl medium (100,000 cells/well) and used for experiments performed in triplicates 1 day after seeding. Prior to the incubation with 100 μl peptide and 100 μl inhibitor at 37 °C, cells were washed with PBS buffer supplemented with 1 g/l D-glucose two times. After 1 h incubation, samples of 100 μl were added to 200 μl of an equal mixture of LDH Assay Substrate, Cofactor and Dye solution in a 96-well-plate and incubated at 21 °C. After 30 min the reaction was quenched with 1/10 of 1M HCl. The absorbance was measured at 490 nm and the background absorbance was measured at 690 nm and subtracted. Untreated cells and cells treated with LDH Assay Lysis Solution, were used as controls.

2.6. HPLC-analysis

HPLC was performed using a Gynkotek-HPLC-gradient system (Dionex, USA) equipped with a 5 μm (150×4.6 mm,) C_{18} column, a precolumn containing the same adsorbent and a fluorescence detector. The elution was carried out with water containing 0.1% TFA (A) and acetonitrile containing 0.1% TFA (B) at a flow rate of 1.0 ml/min with gradients from 100% A (5 min) 20% B (5–10 min) and 20–100% B (10–50 min). Quantification was performed by fluorescence measurement at 524 nm after excitation at 445 nm using calibration values obtained with 10 pmol of the parent peptide analyzed under identical conditions.

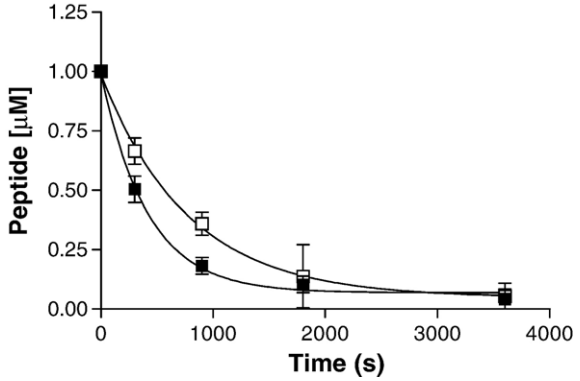

Fig. 1. EC degradation of MAP (□) and penetratin (■). The graph illustrates how intact MAP and penetratin decreases over time after cells were incubated with 1 μM of each peptide at different time points. Each time point represents the mean of three independent experiments±S.E.M.

2.7. Data fitting

EC degradation was fitted to Eq. (1) (A→D) and the resulting constant was used in fitting the kinetic data to Eq. (2) (A→D A→Z, Z→B) where A corresponds to intact EC peptide which can be degraded to D, extracellularly. Z is peptide bound to the membrane and B is total IC label. Finally, the constants obtained from Eq. (1) and Eq. (2) was used to fit the data to Eq. (3): (A→D A→Z, Z→B, Z→C, B→C) where A corresponds to intact EC peptide which can be degraded to D, extracellularly. Z is peptide bound to the membrane internalized to either intact IC peptide, B or degraded IC peptide, C. In addition, intact IC peptide B can be degraded to C, intracellularly. For the fitting, the Gepasi 3.0 program package with the evolutionary programming method run for 500 generations, for global optimisation followed by Hook and Jeeves local optimisation was used [17,18]. Fitting constrains were set to 0<constants<100 and the IC volume to 0.5 μl. The membrane volume was set to 1% of cell volume.

3. Results

In this study detailed fluorescence-HPLC (fl-HPLC) examination was used to obtain quantitative data on uptake and degradation of CPPs. In all studies relating to peptide uptake it is critically important to separate membrane attached peptide from internalized peptide. To achieve this, cells were treated prior to cell lysis with diazotized 2-nitroaniline, which is a chemical agent that binds to primary amino groups [16]. After the modification the peptide will be more hydrophobic and have a longer retention time in the HPLC. Internalized and membrane bound peptides are separated since the reagent cannot cross the plasma membrane and modify internalized peptides.

The uptake and degradation time-course of fluorescently labeled MAP and penetratin was investigated in CHO cells using fl-HPLC. The cells were incubated with various concentrations of peptide at 37 °C at different time points. The curves in Fig. 1 illustrate the EC degradation of MAP and penetratin at 1 μM. Half-lives of EC degradation were calculated with first-order kinetics and corresponded to about 5 min ($k = 0.0024$) and 10 min ($k = 0.0012$) for penetratin and MAP, respectively. The disappearance of the intact peptide peak coincided with an increase in peaks corresponding to peptide fragments. In order to ascertain that indeed the disappearance of the intact peptide peak was due to degradation, not precipitation or plastic binding, parallel treatments in cell-free conditions were applied. Here no change in peptide levels after 1 h was seen (data not shown). This also rules out that residual trypsin from the cell-culturing process were responsible for the degradation. Peptide stability in buffer conditioned for 1 h on cells was analyzed, and again no degradation could be seen after 1 h incubation at 37 °C.

The uptake of MAP and penetratin is illustrated in Fig. 2 where the IC degradation for both peptides is similar, whereas the curves corresponding to intact peptide differ. Studying the curve corresponding to MAP (Fig. 2A), intact peptide increases for 1 h whereas intact penetratin (Fig. 2B) starts to decrease after 15 min, which correlate well with the half-lives of the peptides. After 15 min almost all EC penetratin should be degraded, hence; IC intact peptide should decrease whereas MAP, which has a longer half-life, continues to have an increase in IC intact peptide. When comparing the uptake of MAP (Fig. 3A) and penetratin (Fig. 3B) to the formation of EC degradation products, both curves correlate quite well and indicate that EC degradation, not equilibrium, could be determining the IC concentration. In accordance with earlier studies [12], the main IC degradation product, regardless of peptide, corresponded to the fluorescently labeled N-terminal amino acid (data not shown). Moreover, several shorter peptides could be identified extracellularly. However, the EC degradation products could not be found intracellularly nor could the IC degradation products be found extracellularly.

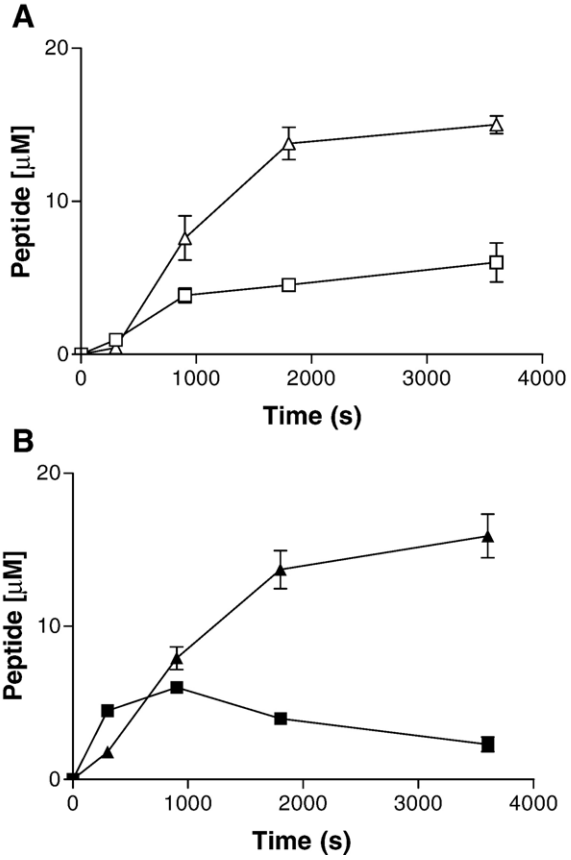

Fig. 2. Uptake and IC degradation of MAP (A) and penetratin (B). The curves correspond to IC intact (■) and degraded (▲) peptide after incubation with 1 μM of each peptide at different time points. Each time point represents the mean of three independent experiments±S.E.M.

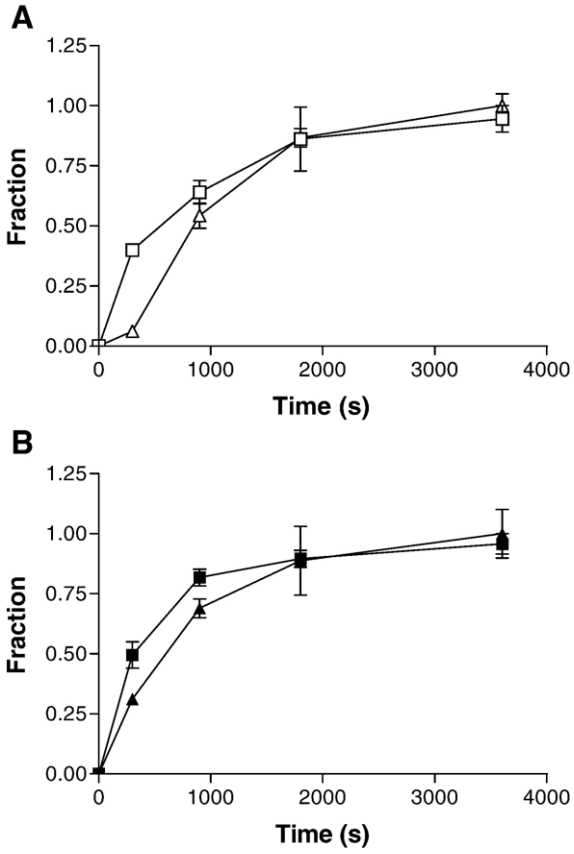

Fig. 3. A comparison between total uptake and EC degradation of MAP (A) and penetratin (B). The curves correspond to the formation of EC degradation products (■) and the total uptake, intact and degraded, IC peptide concentration (▲). For ease of comparison both data sets are shown as fraction of maximum, where the maximum corresponds to 0.91 ± 0.09 μM and 0.84 ± 0.11 μM for MAP and penetratin, respectively. Each time point represents the mean of three independent experiments ± S.E.M.

An attempt was made to inhibit degradation using several protease inhibitors covering the most common proteases namely, leupeptin, which is an inhibitor of serine and cysteine proteases, phenanthroline, which inhibits matrix metalloproteases (MMPs), phenylmethylsulfonyl fluoride (PMSF), an inhibitor of serine proteases and bacitracin, a non-specific protease inhibitor. In addition, BSA was analyzed in order to investigate if it could saturate proteases resulting in an increase in intact EC peptide. Cells were incubated with inhibitors or BSA at different concentrations together with MAP or penetratin (Fig. 4) for 1 h at 37 °C. Leupeptin and PMSF did not increase the intact EC peptide significantly and the IC and EC peptide concentration of bacitracin could not be determined properly due to a new broad peak found in the background of the bacitracin treated samples (Fig. 4A). To determine the origin of the peak, bacitracin was analyzed alone, together with diazotized 2-nitroaniline or peptides. The results indicated that bacitracin alone could cause this broad peak but that it was even stronger together with peptides (data not shown). Treatment with 1% BSA increased the EC intact MAP with more than 50%, whereas, penetratin was unaffected by the BSA treatment. Unfortunately, the increment of EC intact MAP due to BSA treatment did not affect the uptake (Fig. 4B). To investigate if this was due to complex formation between the peptides and BSA we incubated each peptide with BSA or bacitracin prior to incubation with cells. The pre-incubation led in both cases to a 50% decrease of MAP whereas penetratin was unaffected. Phenanthroline increased EC intact MAP and penetratin by more than 2-fold (Fig. 4A) and the degradation products disappeared in both EC and IC samples (data not shown). Here, the uptake of penetratin was increased almost 2-fold whereas the uptake of MAP decreased with both phenanthroline and PMSF (Fig. 4B). To investigate if the protease inhibitors affected the uptake or degradation due to toxicity, cells were incubated with peptides and/or inhibitors, and subjected to a LDH leakage test (Fig. 5). No toxicity was found for the peptides alone or with leupeptin. However, MAP together with PMSF and phenanthroline caused 25% of maximal leakage.

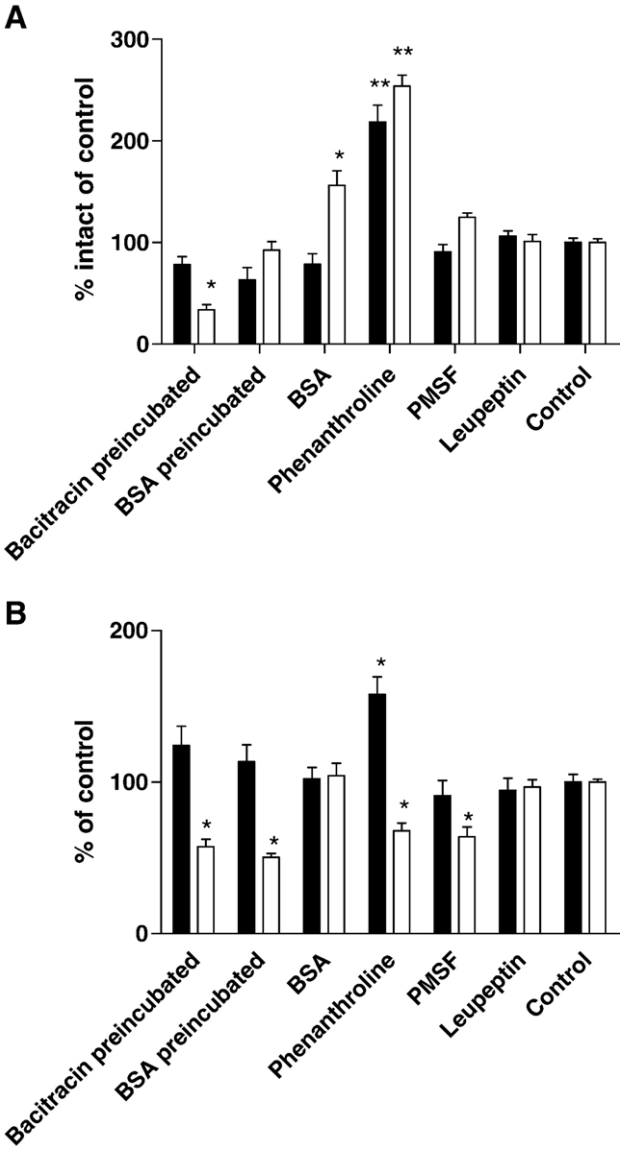

Fig. 4. Effects of protease inhibitors and BSA on EC peptide degradation (A) and uptake (B). The protease inhibitors, bacitracin (5 mg/ml), leupeptin (100 μM), phenanthroline (2 mM), PMSF (2 mM) or BSA (1%) were incubated with MAP (white) or penetratin (black) for 1 h at 37 °C followed by analysis with fl-HPLC. The data represent the mean of three independent experiments ± S.E.M. Statistical analysis was made by ANOVA followed by Dunnett's multiple comparison test (*$P < 0.05$, **$P < 0.01$).

Penetratin caused 20% leakage together with PMSF but less than 10% with phenanthroline.

Moreover, three inhibitors of endocytosis were studied for their effect on uptake namely, nocodazole, which blocks IC transport by depolymerizing microtubules, cytochalasin B which inhibits the assembly of actin filaments and thereby blocks IC transport and wortmannin, a phosphoinositide 3-kinase inhibitor which inhibits the fusion of early endosomes with late endosomes (Fig. 6). In addition, the uptake was investigated in energy depleted cells and cells treated with chloroquine, an inhibitor of maturation and formation of transport vesicles into late endosomes. Cytochalasin B reduced the uptake of both peptides with almost 30% and chloroquine inhibited the uptake with more than 50 and 40% for MAP and penetratin, respectively. In energy depleted cells the uptake of MAP was reduced more than 60%. Wortmannin, energy depletion and nocodazole decreased the uptake of penetratin with 25%, however, it could not be statistically ascertained.

Finally, the data were analyzed using Gepasi 3.0, a program used for modeling the kinetics of biochemical systems and pathways [17,18]. The data were fitted to the equations seen in Fig. 7 where A corresponds to intact EC peptide which can be degraded to D, extracellularly. Z is peptide bound to the membrane internalized to either intact IC peptide B, or degraded IC peptide C. In addition, intact IC peptide B can be degraded to C, intracellularly. In summary, the fitting indicated two uptake processes; one resulting in degraded peptides (Z→C) possibly through direct delivery to the cytoplasm, where it can be degraded within minutes, and another process delivering intact peptides (Z→B), possibly by endosomal encapsulation, shielding the peptides from proteases in the cytoplasm. The degradation of B→C could illustrate the escape of peptides from endosomes. The half-lives of the uptake and degradation processes, derived from Gepasi, are displayed in Table 2 and correlate well with the uptake and degradation of MAP and penetratin in Figs. 1 and 2.

4. Discussion

The translocation of CPPs into cells was first thought to be independent of metabolic energy [19,20]. Today, several well-characterized CPPs have been shown to enter cells by different types of endocytosis [13,21,22]. However, none of the endocytosis inhibitors could inhibit the uptake totally. Nevertheless, the capacity of CPPs to deliver cargo molecules to the cell interior, as shown by the presence of many different biological responses of treated cells, proves that the cargo is successfully delivered in a functional state [23] and has opened up new possibilities in drug delivery research [24,25]. A crucial aspect of drug delivery is, of course, efficient transport of the pharmaceuticals to the site of action, but also the following clearance of the transporting moiety from the cell. The CPP needs to translocate its cargo before it is extracellularly degraded, although, well inside the cell it has to be eliminated in order not to cause toxicity. In addition, if the CPPs remain intact in the cellular environment, there is a possibility that the peptide-cargo conjugate could leak back to the EC environment. Hence, IC degradation of CPPs is important for cargo delivery, and degradation kinetics is an important parameter to include when choosing a peptide to act as a delivery vector.

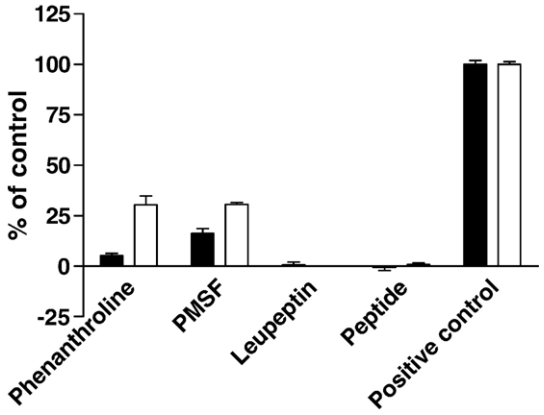

Fig. 5. Cytotoxicity of different protease inhibitors and peptides. Cells were incubated for 1 h at 37 °C with phenanthroline (2 mM), PMSF (2 mM) or leupeptin (100 μM) together with MAP (white) or penetratin (black) followed by an LDH leakage test. Leakage is shown as percent of maximum. The data represent the mean of at least three independent experiments ± S.E.M.

Kinetic measurements of CPP degradation are so far sparse. Tréhin and colleagues followed the degradation of Tat, penetratin and several calcitonin analogues in three different epithelial cell lines [11]. This study concluded that the EC half-life of the individual peptides depended on cell type and cell density in the experiment. The shortest half-life measured was 50 min for penetratin exposed to Calu-3 cells. However, the stability of the peptides varied widely, and the longest half-life was 1444 min for Tat(48–60) exposed to MDCK cells. In another paper by Rennert and colleagues, the proteolytic resistance of hCT(9–32) was increased in human blood plasma and HEK 293T cell culture supernatants, by replacing the amino acids at position 12 and 16 with either *N*-methylphenylalanine or D-phenylalanine [26]. However, these studies did not include intracellular degradation and how it affects the uptake.

In this study MAP and penetratin were incubated with CHO cells and the EC half-lives corresponded to 10 and 5 min (Table 2), respectively. In contrast, in conditioned buffer no degradation could be seen after 1 h, indicating that the proteases responsible for EC degradation are membrane bound rather than soluble. The EC degradation of MAP but not penetratin could be reduced by the addition of BSA (Fig. 4A), however, no corresponding increase in IC peptide concentration could be seen (Fig. 4B). On the contrary, pre-incubation of MAP with BSA or bacitracin reduced its uptake whereas penetratin was unaffected. This increase in stability without a corresponding increase in uptake probably is due to complex formation between the peptides and BSA or bacitracin, shielding them from proteases but preventing them from translocating the cell. This is supported by the appearance of the new broad peak in the HPLC spectrums (data not shown). The structural differences between MAP and penetratin could explain the different effects of BSA on EC degradation of MAP and penetratin, indicating that BSA is able to bind and shield MAP from proteases but not penetratin. Phenanthroline, which inhibits MMPs, increased the EC stability of both MAP and penetratin, with a corresponding increase in uptake for penetratin. However, both phenanthroline and PMSF decreased the uptake of MAP. This was probably due to toxicity, which was supported by the LDH

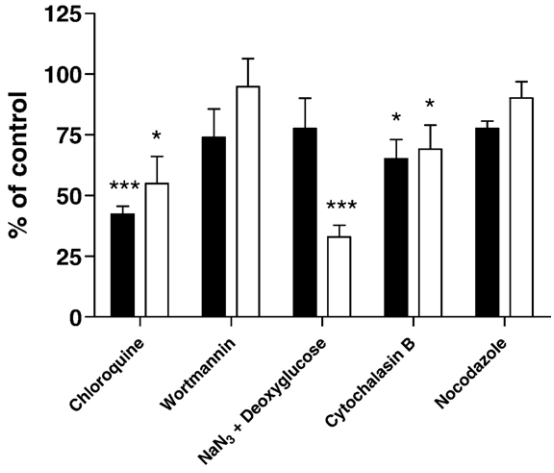

Fig. 6. Effects of different inhibitors on uptake. Cells were preincubated for 30 min with chloroquine (50 μM), wortmannin (5 nM), sodium azide (60 mM) and deoxyglucose (20 mM), cytochalasin B (5 μM) or nocodazole (5 μM) followed by incubation with MAP (white) or penetratin (black) for 1 h at 37 °C. The data represent the mean of three independent experiments±S.E.M. Statistical analysis was made by ANOVA followed by Dunnett's multiple comparison test (*$P < 0.05$, ***$P < 0.001$).

leakage test where a 25% leakage was found in both cases for MAP but not penetratin (Fig. 5).

Studying the IC concentrations, intact penetratin increased rapidly the first 5 min whereas intact MAP continuously increased after 15 min. This correlates with the uptake half-lives (Table 2) where MAP had a 3 times longer half-life than penetratin (Fig. 2). Moreover, IC intact penetratin started to decrease after 15 min most likely due to the short EC half-life, indicating that the EC degradation could be determining the IC concentration, which is corroborated by the comparison of uptake and formation of EC degradation products (Fig. 3). Even though it may appear that the peptides are in equilibrium over the plasma membrane, this is unlikely since the IC degradation is rapid and does not result in the same degradation products as the EC degradation.

In accordance with earlier studies [13,27], cytochalasin B decreased the uptake of both peptides with approximately 30% (Fig. 6) indicating that the uptake is partly dependent on the

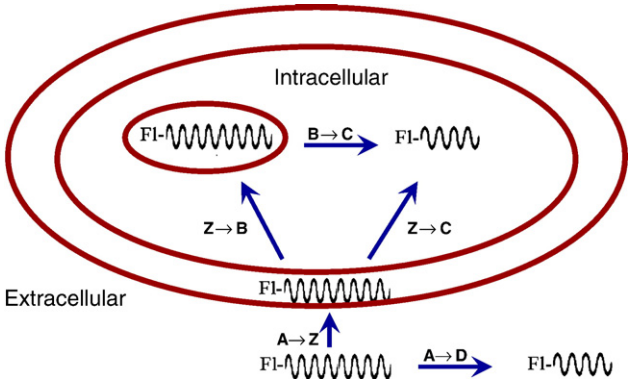

Fig. 7. Schematic of the proposed mechanisms in uptake. The equations were derived from analyzing the data with the Gepasi 3.0 program package, illustrating EC intact peptide (A), EC degraded peptide (D), membrane bound peptide (Z), IC intact peptide (B) and IC degraded peptide (C).

Table 2
Half-lives of internalization and degradation for MAP and penetratin

Half-lives	Penetratin (min)	MAP (min)
A→D(1)	5.7	10
A→Z(2)	123	110
Z→B(3)	2.3	6.2
Z→C(4)	4.2	2.7
B→C(5)	20	86

assembly of actin filaments for IC transport and confirms that some form of endocytosis is involved. Chloroquine, a membrane disrupting agent that increases the lysosomal pH and prevents the maturation and formation of transport vesicles into late endosomes, decreased the uptake of both peptides with 40–50%. Wortmannin and nocodazole did not have a significant effect on the uptake at these concentrations; however, the uptake of penetratin was decreased by 25% augmenting the involvement of endocytosis. In addition, the uptake of MAP was found to be energy dependent since the IC concentration of MAP decreased with 60% in energy depleted cells. Surprisingly the uptake of penetratin did not decrease in energy depleted cells significantly, which was expected since the uptake of penetratin decreased with cytochalasin B, which inhibits an energy dependent mechanism. One explanation could be that the variation in uptake was not statistically significant and what appeared to be a decrease in uptake of penetratin could not be ascertained. These results indicate that the uptake of MAP is partly energy dependent and that endocytosis, requiring the assembly of actin filaments for transport, is involved in the uptake of both peptides in this system.

It is important to note that the total label content in the cells appear to reach an equilibrium (Fig. 3) [28–31]. In this work we show that this is in fact determined not by the export/import rate constants but mainly by the EC degradation/import rate constants. This was corroborated by comparing the curve for total IC label (intact and degraded) to the curve corresponding to the formation of EC degradation products (Fig. 3). Both curves correlate well indicating that EC degradation and not equilibrium, determines the IC concentration. It is conceivable that peptide fragments could pass the plasma membrane in both directions. We consider this as less likely as the EC degradation products are not the same as the IC degradation products (data not shown). In addition, it has previously been shown that C-terminally truncated forms of both MAP [32] and penetratin [33] are not translocated into cells, or retained, as efficiently as the parent peptides. From this we can presume that what appears to be transport equilibrium, in fact is not and that saturation like effects are probably due to EC degradation. This would explain the discrepancy reported by our group previously [34]. However, it is possible that equilibrium can be reached if the peptide stability would be substantially better.

Moreover, from transport kinetics we know that the concentration found in cells should be dependent on the ratio between inside and outside volumes [35], which was experimentally verified for CPPs by our group recently [12]. However, this is now complicated by the fact that degradation is dependent on the cell amount, (and hence protease concentration). Thus, at the same time as the IC volume increases, leading to a decrease of IC concentration, also the EC degradation rate will increase, further decreasing uptake.

As the degraded peptide increases faster than the intact peptide decreases (Fig. 2), we propose that there could be two mechanisms involved in the uptake of CPPs. A representation of this hypothesis is shown in Fig. 7 and the half-lives of the processes are shown in Table 2. From our fitting we can see that the first mechanism, resulting in IC degraded peptides (Z→C in Fig. 7), could be translocation of the peptides directly to the cytoplasm where they can be rapidly degraded. It has been shown previously that peptides in the cytoplasm are degraded within seconds [36], however, in this study the peptides were injected directly to the cytoplasm and transport across the plasma membrane was not taken into consideration. Alternatively, a rapid endocytosis followed by an immediate escape from endosomes to the cytoplasm could be responsible. The other uptake mechanism, resulting in IC intact peptides (Z→B in Fig. 7), could be some form of vesicle formation shielding the peptides from proteases in the cytoplasm. This is supported by the fact that we could detect intact peptides intracellularly after 1 h in spite of rapid IC degradation. Another explanation could be that this stabile pool of peptide is deposited in the nucleus or other proteolytically privileged sites. Moreover, it should be noted that the half-life for several processes are in the order of 2 times longer or shorter than the longest or shortest time measured, which is slower or faster than we can measure. From the fitting we conclude that MAP predominantly uses the first mechanism, while penetratin mostly uses the second, which is in accordance with Fig. 2. This model is attractive as it would possibly include various forms of endocytosis (Z→B), endosomal escape (B→C) and direct cell-penetration (Z→C).

We have shown that MAP and penetratin are degraded extracellularly with half-lives of 10 and 5 min, respectively, and that decreasing EC degradation can increase the uptake. This is important since at least penetratin is a popular peptide used in many uptake studies where incubation time points sometimes are several hours and even though MAP is not as common as penetratin, there are amphiphilic peptides with structures similar to MAP. In conclusion, EC degradation is a critical determinant for delivery efficiency as all peptide is degraded before transport is saturated, thus, the stability of cell-penetrating peptides needs to be evaluated.

Acknowledgment

This work was supported by the Jeansson foundation.

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