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Cellular uptake of an α-helical amphipathic model peptide with the potential to deliver polar compounds into the cell interior non-endocytically

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Abstract

Evidence that multiple, probably non-endocytic mechanisms are involved in the uptake into mammalian cells of the α-helical amphipathic model peptide FLUOS-KLALKLALKALKAALKLA-NH_2 (I) is presented. Extensive cellular uptake of N-terminally GC-elongated derivatives of I, conjugated by disufide bridges to differently charged peptides, indicated that I-like model peptides might serve as vectors for intracellular delivery of polar bioactive compounds. The mode of the cellular internalization of I comprising energy-, temperature-, pH- and ion-dependent as well as -independent processes suggests analogy to that displayed by small unstructured peptides reported previously (Oehlke et al., Biochim. Biophys. Acta 1330 (1997) 50–60). The uptake behavior of I also showed analogy to that of several protein-derived helical peptide sequences, recently found to be capable of efficiently carrying tagged oligonucleotides and peptides directly into the cytosol of mammalian cells (Derossi et al., J. Biol. Chem. 269 (1994) 10444–10450; Lin et al., J. Biol. Chem. 270 (1995) 14255–14258; Fawell et al., Proc. Natl. Acad. Sci. USA 91 (1994) 664–668; Chaloin et al., Biochemistry 36 (1997) 11179–11187; Vives et al., J. Biol. Chem., 272 (1997) 16010–16017).

Keywords: Peptide transport; Amphipathic peptides; Cellular uptake

1. Introduction

Polar, biologically active compounds, such as antisense oligonucleotides and peptides capable of blocking associations between signal transducing proteins, are becoming increasingly interesting as pharmacological probes and potential drugs. The applicability of such compounds, however, is severely impaired by inefficient delivery to their sites of action within the cell. Most approaches currently applied to translocate such compounds across plasma membranes rely on endocytic mechanisms [1]. An endocytic mode of uptake, however, poses problems in that the quantity of transported substrates and particularly the rate of their liberation from endosomes

Abbreviations: AEC, bovine aortic endothelial cells; CLSM, confocal laser scanning microscopy; DNP-SG, *S*-(2,4-dinitrophenyl)glutathione; DOG, 2-deoxy-D-glucose; DPBSG, Dulbecco's phosphate-buffered saline supplemented with 1 g/l D-glucose; FLUOS, 5(6)-carboxyfluoresceinyl; NEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline

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are limited; sufficient concentrations at the target sites in cytosol or nucleus are, therefore, difficult to achieve [1,2].

As promising alternatives, non-endocytic translocation approaches have been presented recently by several authors, based on the use of protein derived vector peptides [3–7]. These natural peptides proved capable of translocating covalently tagged, membrane-impermeable peptides and oligonucleotides across the plasma membrane directly into the cytosol. The actual mechanism of entry of these peptides and their conjugates remains unclear. A non-endocytic mode of the cellular uptake is indicated by the observation of efficient translocation across the plasma membrane even at 0°C [3].

Recently, we reported on the internalization into endothelial cells of a simple helical amphipathic model peptide, FLUOS-KLALKLALKALKAAL-KLA-NH_2 (I) via a non-endocytic, amphipathicity-dependent mechanism, similar to that of the aforementioned natural peptides [8]. In the present work, we further investigate the mode of uptake of this model peptide. Our results indicate that multiple non-specific, energy-dependent and -independent processes are involved in the translocation of I across the plasma membrane. Analogy is suggested to the cellular uptake of natural membrane permeable peptide sequences [8] as well as to that of small unstructured enkephalin-derived peptides described previously [9].

2. Materials and methods

2.1. Materials

5(6)-Carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS) was purchased from Boehringer (Mannheim, Germany). 2,4-Dinitrophenyl-*S*-glutathione (DNP-SG) was synthesized according to Hinchman et al. [10]. For dialysis Spectra/Por DispoDialyzers (cellulose ester, 5 mm diameter, 2 ml sample volume) with molecular weight cut-offs of 10 and 50 kDa (Sigma, Deisenhofen, Germany) were used. Release of lactate dehydrogenase was assessed by means of LDH-L reagent from Sigma. Other chemicals and reagents, when not specified, were purchased from Sigma or Bachem.

2.1.1. Peptide synthesis

The peptides were synthesized automatically by solid phase methods using standard Fmoc chemistry as described previously [11]. To introduce the fluorescent label, the peptides were N-terminally conjugated with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS; Boehringer, Mannheim) (two equivalents in DMF), and the final cleavage was performed with 95% TFA/5% water for 3 h.

For connecting cysteine tagged vector- and cargo-peptides by disulfide bridges, either the vector peptide (in the case of acidic or neutral cargo peptides) or the cargo peptide (when basic) was dissolved in water (to 1 mM) and reacted with a 100-fold excess of a 100 mM aqueous solution di-Na-5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) for 5 h at 60°C. In the case of activated vector peptides, the reaction product was virtually quantitatively centrifuged off, washed four times with water and dissolved in 1 M NaCl in ethanol/water 1:1 to a concentration of about 100 μM. For the activated basic cargo peptides, the reaction solution was acidified to about pH 2 with 10% trifluoroacetic acid and extracted four times with ethyl acetate. Subsequently, an equal amount of ethanol was added.

To generate the disulfide bonds, a 5-fold excess of 5 mM aqueous solutions of the SH-group containing counterparts were added to the peptides activated with Ellman's reagent, allowed to react for 2 h at 60°C, and HPLC purified. To avoid aggregation of the reaction products the reaction mixture was supplemented with NaCl and ethanol to 1 M and 50%, respectively. The HPLC fractions containing the reaction products were also supplemented with NaCl up to about 0.1 M and concentrated almost to dryness by means of a vacuum centrifugal evaporator.

2.2. Cell culture

Calf aortic endothelial cells (AEC), 12.–20. subculture of a cell line (LKB Ez 7), established and characterized by Halle et al. [12], were seeded at an initial density 5×10^4 cells/cm^2 into 24-well culture plates. The cells were cultured at 37°C in a humidified 5% CO_2 containing air environment in minimal essential medium (MEM; Sigma, Deisenhofen) supplemented with additional 290 mg/l glutamine and 10% fetal calf serum (FCS; Sigma, Deisenhofen).

After 4 days without replacing the medium, the cells were used for the uptake experiments.

Porcine endothelial cells (SEZ; MEM/5% FCS) [13], human umbilical vein endothelial cells (ECV 304, ATCC no. CRL 1998; M199/10% heat inactivated FCS), human neuroblastoma cells (SK-N-SH; MEM Eagle/10% FCS supplemented with 1% non-essential amino acids, 1% Na-pyruvate and 1% glutamine (Biochrom, Berlin, Germany)), and human hepatoma cells (Hep G2; MEM Eagle/10% FBS supplemented with 1% non-essential amino acids, 1% Na-pyruvate and 1% glutamine) were cultured similarly.

2.3. Uptake experiments

After removal of the medium, the cell layers were rinsed two times at 37°C with Dulbecco's phosphate-buffered saline (DPBS; Biochrom, Berlin) supplemented with 1 g/l D-glucose (DPBSG) and subsequently exposed, unless indicated otherwise, at 37°C for 30 min to 500 μl of the peptide solutions in DPBSG. Thereafter, the incubation solutions were removed, the cells were washed two times with ice-cold PBS, incubated with 500 μl of ice-cold PBS and treated with diazotized 2-nitroaniline as described previously [14] in order to modify any surface bound peptide. In brief, 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 two times with ice-cold PBS and finally lysed with 0.2 ml 0.1% Triton X-100 containing 10 mmol/l trifluoroacetic acid for 2 h at 0°C. The resulting lysate was used for HPLC analysis and for protein determination according to Bradford [15]. The average protein content of 10^6 cells assayed by this method was 110 μg. The average volume of the cells was determined to be 1.4 pl by means of a Coulter-ZM counter (Coulter Electronics, Luton, UK).

In each experiment, cells in triplicate were exposed in parallel for 30 min at 37°C to 1.8 μM I to obtain reference values for normalization. The basis values for normalization, determined in eight independent uptake experiments were 50 ± 31, 56 ± 29 and 155 ± 37 pmol/mg protein for the metabolized, intact and membrane inserted peptide fractions, respectively.

To measure the pore-forming propensity of the peptides, the cells were pre-exposed to a 12 μM solution of fluorescein diacetate in DPBSG (containing 0.5% DMSO) for 15 min at 37°C. After aspiration of the fluorescein diacetate solution, the cells were rinsed four times with DPBSG 37°C and then exposed to the peptides. Since for the control cells a noticeable export of the fluorescein generated within the cells was also observed (half-life time at 37°C, 9 min) an exact time regime was required for the wash steps to warrant reliability.

2.3.1. HPLC analysis

HPLC was performed using a Bischoff HPLC-gradient system (Leonberg, Germany) with a Polyencap A 300, 5 μm column (250×4 mm i.d.), precolumns containing the same adsorbent and a Fluorescence HPLC Monitor RF-551 (Shimadzu).

Up to 200 μl of the cell lysates was passed through a precolumn containing 60 mg of Polyencap (A_{300}, 5 μm), which was subsequently connected to the HPLC system. The elution was carried out with 0.01 M TFA (A) and acetonitrile/water 9:1 (B) at a flow rate of 1.0 ml/min with gradients of 30–45% B (0–10 min) and 45–80% B (15–20 min) for peptides and 30–60% B (0–15 min) and 60–80% B (15–20 min) for peptide conjugates. Quantitation was performed by fluorescence measurement at 520 nm after excitation at 445 nm by means of calibration lines obtained with the parent peptides under identical conditions. For unknown metabolites a 2-fold molar fluorescence intensity as found during HPLC for I was used for quantitation, based on the generally 2–3-fold higher molar fluorescence intensities observed for HPLC peaks of characterized derivatives of I, with lower structure forming propensity.

2.3.2. Confocal laser scanning microscopy (CLSM)

10^4 cells were plated on 22×22 mm coverslips glued (with silicones RTV 615A/615B, Hellermann, Pinneberg) above the hole (15 mm diameter) of punched plastic Costar culture dishes (35 mm diameter; Tecnomara Deutschland, Frankfurt/Main) and cultured, exposed to peptides and washed as described above. After subsequent overlaying of the

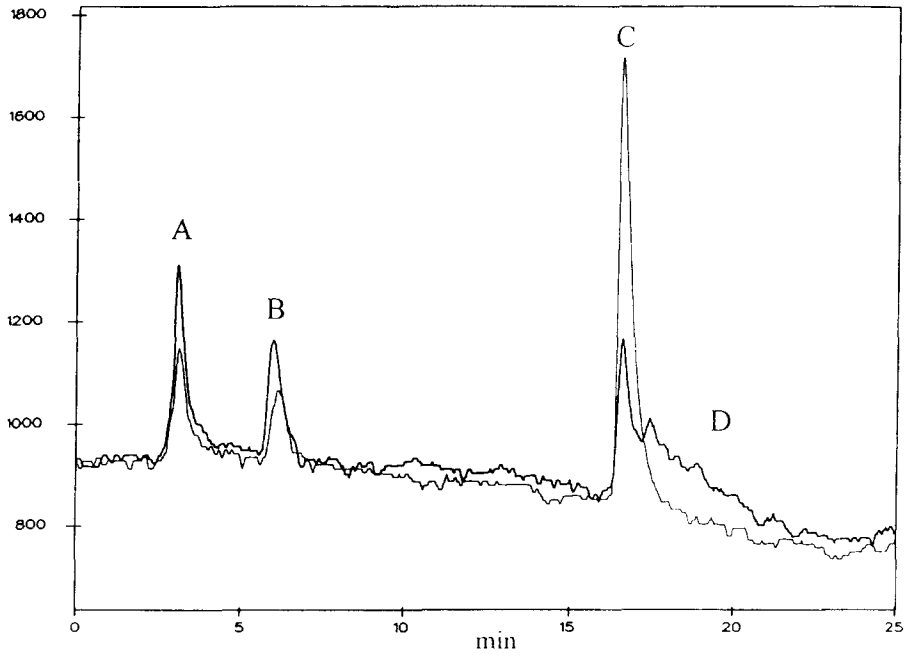

Fig. 1. HPLC chromatogram of the Triton X-100 lysate of AEC exposed for 30 min at 37°C to 1.8 μM I, without (thin line; injection of 100 μl cell lysate containing 25 μg cell protein) and with treatment with diazotized 2-nitroaniline (thick line; to facilitate comparison with the former experiment, in this case 200 μl cell lysate containing 46 μg cell protein were injected). Peaks correspond to: A, intrinsic cell fluorescence; B, metabolite (FLUOS-lysine), generated intracellularly; C, intact I; D, peptide portion modified partially by diazotized 2-nitroaniline.

cells with 200 μl PBS, microscopy was performed within 10 min at room temperature using an LSM 410 invert confocal laser scanning microscope (Zeiss, Jena, Germany). Excitation was performed at 488 (FLUOS) or 364 nm (dansyl) by means of an argon–krypton or an argon–ion laser and a dichroitic mirror FT 510 or FT 395 for wavelength selection, respectively. Emission was measured upon 515 or 420 nm with cut-off filters LP 515 and LP 420, respectively, in front of the detector. For optical sectioning in z-direction, 16 frames with a thickness of 1 μm were made.

3. Results

3.1. Discrimination of cell associated I into surface bound, membrane inserted and internalized portions

After exposure of AEC to the fluorescein labeled α-helical amphipathic 18-mer peptide FLUOS-KLALKLALKALKAALKLA-NH_2 (I) high amounts of intact peptide accompanied by small quantities of fluorescent peptide fragments were detected in the lysates of the washed cells (Fig. 1).

To obtain quantitative information about the portions of peptide associated with the plasma membrane and that actually internalized, we treated the peptide exposed, washed cells with diazotized 2-nitroaniline, a procedure shown previously to modify surface bound primary amino compounds, while leaving those within the cell intact [14]. This reagent has the crucial advantage of being highly reactive even at 0°C, so that bias of the results by counteracting efflux processes, strongly attenuated or suppressed at this temperature, is minimized.

About 20% of the cell associated peptide proved to be inaccessible to the diazo reagent and therefore appeared to be internalized (Fig. 1). Related to the peptide concentration in the incubation solution this amount represents a 4–5-fold enrichment of the peptide within the cell, taking into account the 100 pmol/mg protein internalized peptide obtained after exposing AEC to 1.8 μM I for 30 min at 37°C (see below) in conjunction with the ratio of

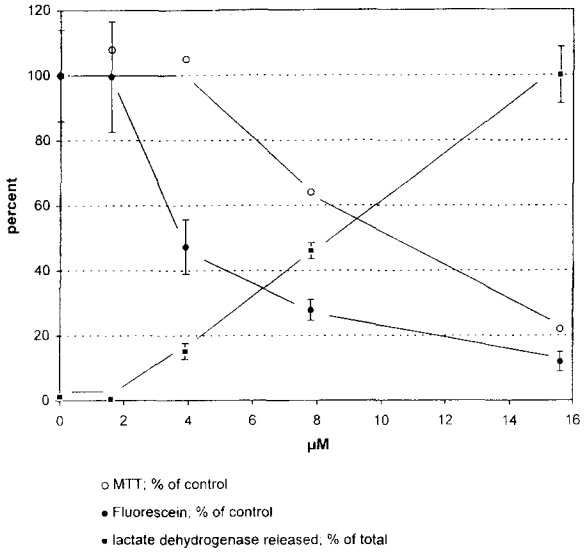

Fig. 2. MTT reactivity, fluorescein content (generated from internalized fluorescein diacetate) and liberation of lactate dehydrogenase from AEC after exposure for 30 min at 37°C to different concentrations of I. Each point represents the mean of three samples ± S.D. (S.D. of LDH release in all cases remained below 10% of the mean.)

110 μg protein/10^6 cells and the cell volume of 1.4 pl (see Section 2.

About 50% of the cell associated peptide disappeared from the HPLC chromatogram after treatment with diazotized 2-nitroaniline. Since treatment of I with diazotized 2-nitroaniline in control experiments (aqueous solutions of comparable concentration or I adsorbed onto the surface of empty cell culture plates, which is resistant to washing with PBS and desorbable with 0.1% Triton X-100) rendered the peptide completely undetectable; this portion was attributed to surface bound peptide. The inability to detect any modified peptide derivatives is very likely accounted for by the strong retention of highly hydrophobic deamination derivatives on the stationary phase of the HPLC column or on the plastic of the wells.

About 30% of the cell associated peptide was transformed by the diazo reagent into moderately more hydrophobic modification products, appearing in the HPLC chromatogram immediately after the intact peptide (Fig. 1). In contrast to the complete modification observed in the control experiments, in this case, only a part of the five side-chain amino-groups of I appeared to have been modified, suggesting partial protection against the attack of the reagent. Most likely this protection effect appears to be interpretable by an insertion of this peptide fraction into the plasma membrane.

Apart from the intact peptide, a further fluorescent fragment, assigned by electrospray-mass spectroscopy to FLUOS-lysine, remained unaffected by diazotized 2-nitroaniline (Fig. 1). This derivative represents an additional portion of the internalized peptide after metabolization in the cell throughout the incubation period. Generation by surface bound peptidases followed by internalization can be discounted as the cause of its appearance in the cell lysate by its absence in the incubation solution. Generation of this metabolite after the incubation period by peptidases liberated during cell lysis can be excluded because exposure of the intact peptide to cell lysates after Triton X-100/0.1% TFA lysis (see Section 2 did not give rise to this product.

After analogous exposure of the cells to FLUOS-KALKLKLALALLAKLKLA-NH_2 (II) an analog of I with virtually identical amino acid composition and comparable high helicity, but lacking amphipathicity [16], the subsequent treatment with diazotized 2-nitroaniline rendered the cell associated peptide completely undetectable (not shown). In the absence of the diazo reagent, II was found in the cell lysate to about 30% of the amount observed with I [8], indi-

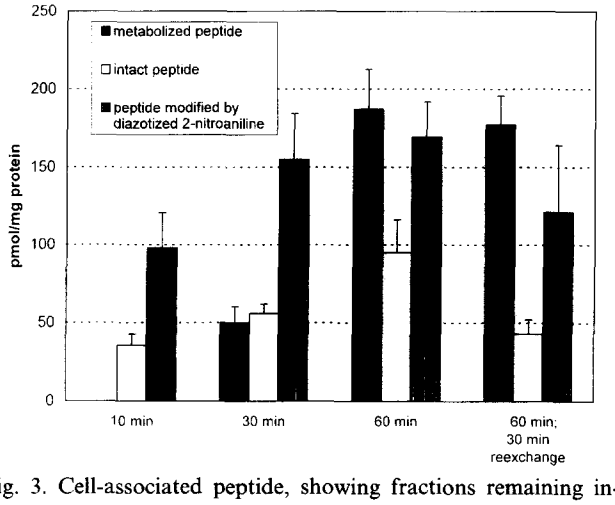

Fig. 3. Cell-associated peptide, showing fractions remaining intact, metabolized within the cell interior and partially modified by diazotized 2-nitroaniline respectively, after exposing AEC for different times at 37°C to 1.8 μM I, and after re-exchange for 30 min at 37°C of cells preloaded for 60 min as described above. Each bar represents the mean of three samples ± S.D.

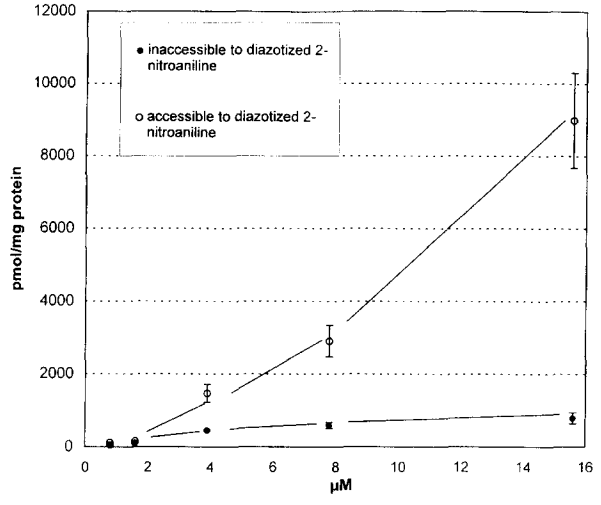

Fig. 4. Cell-associated peptide, showing fraction inaccessible to diazotized 2-nitroaniline (sum of intact and metabolized peptide) and fraction modified partially by this reagent, respectively, after exposing AEC to different concentrations of I for 30 min at 37°C. Each point represents the mean of three samples ± S.D.

cating comparable surface binding for I and II, while also confirming the efficiency of the diazo regent.

In contrast to the above described results, acid wash of the peptide loaded cells (5 min at 0°C with HAc/NaCl, 0.2 M/0.05 M), a procedure commonly used to strip surface bound peptides [17], failed to remove I from the cell surface (loss $< 5\%$; not shown).

3.2. Membrane toxicity of I

To minimize possible additional influences we omitted BSA and thiorphan additives from the incubation solution in contrast to our previous experiments [8]. Initial indications of perforation of the plasma membrane were detected at 4 µM peptide by impaired Trypan blue exclusion (not shown) as well as by liberation of both LDH and fluorescein (generated from pre-incorporated fluorescein diacetate) (Fig. 2). Concomitantly, the viability estimated by the MTT method [18] became impaired (Fig. 2).

3.3. Concentration- and time-dependence of the internalization of I

The internalization of I, represented by the peptide fractions inaccessible to the diazo reagent proceeded roughly linearly throughout a period of 60 min (Fig. 3). Longer incubation periods were precluded because of increasing interference due to enzymatic cleavage of I within the incubation solution. Distinct from the internalization, the insertion of I into the plasma membrane, measured as the fraction prone to modification by diazotized 2-nitroaniline, showed no significant time dependence within 10–60 min and approached an equilibrium at least at about 30 min (Fig. 3), suggesting that the two processes are independent of one another.

Generation of metabolites, represented by the predominant FLUOS-lysine (see above), exhibited a delayed onset, suggesting that the peptide reached the

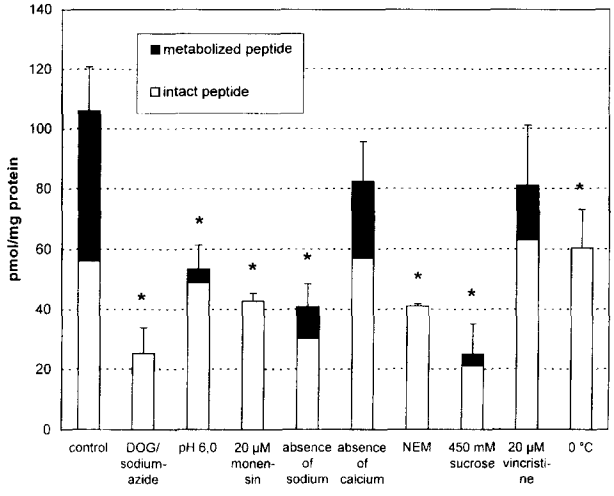

Fig. 5. Sum of the fractions of cell-associated peptide and peptide fragments inaccessible to diazotized 2-nitroaniline after exposing AEC for 30 min at 37°C to 1.8 µM I dissolved in DPBSG (control and NEM, respectively), in DPBSG adjusted with HCl to pH 6.0 (pH 6.0), in sodium-free buffer (DPBSG with NaCl and phosphates replaced by 137 mM choline chloride and 10 mM HEPES, respectively), in DPBSG without calcium (absence of calcium), in DPBS containing 25 mM 2-deoxyglucose/10 mM sodium azide (DOG/sodium azide) or 20 µM monensin (monensin) or 20 µM vincristine (vincristine), respectively, and in DPBSG at 0°C. Before exposure to the peptide, the cells used for the NEM, DOG/sodium azide and 0°C experiments were incubated at 37°C for 5 min in DPBSG containing 1 mM *N*-ethylmaleimide and subsequently rinsed three times with DPBSG (NEM) or incubated for 60 min at 37°C in DPBS containing 25 mM 2-deoxyglucose/10 mM sodium azide (DOG/sodium azide) or in DPBSG for 60 min at 0°C respectively. Each bar represents the mean of three samples ± S.D. (calculated from the S.D. values of both components). The differences between the respective controls and the asterisk labeled bars are statistically significant at $P \leq 0.05$ (Student's *t*-test).

metabolizing compartment only after > 10 min. Together with a perinuclear enrichment of fluorescence observed by CLSM [8], and the absence of any effect of raising the lysosomal pH by the use of chloroquine (see below), our data implicate the endoplasmic reticulum as being the metabolizing compartment.

Within the concentration range 1–5 μM the uptake of I rose nearly linearly (Fig. 4). At higher concentrations the amount of cell associated peptide and also the ratio of the peptide modified by diazotized 2-nitroaniline to that remaining unmodified increased overproportionally (Fig. 4), indicating increased access to the cell interior for both peptide and diazo reagent due to perforation of the plasma membrane. Peptide binding to internal cell structures proved sufficiently avid to resist the wash process, as observed for the peptide portion inserted into the plasma membrane. Such avid binding to plasma membrane and intracellular structures might explain the relatively small loss of both the internalized (intact and metabolized) and membrane inserted peptide fractions found in efflux experiments with peptide loaded intact cells (Fig. 3). Addition of proteins (albumin, FCS), to facilitate the release of the peptide from the plasma membrane had no significant effect (not shown).

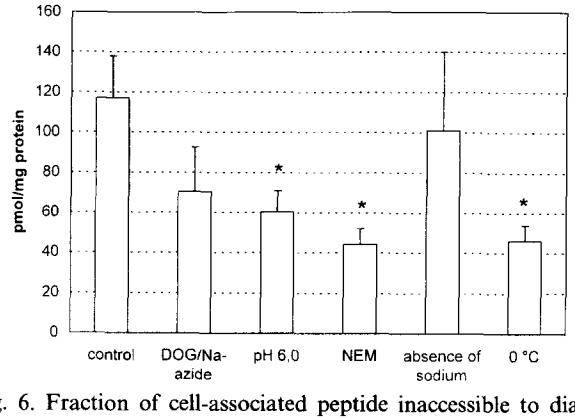

Fig. 6. Fraction of cell-associated peptide inaccessible to diazotized 2-nitroaniline after exposing AEC for 30 min at 37°C to 1.8 μM III (all-D enantiomer of I) dissolved in DPBSG (control and NEM, respectively), in DPBSG adjusted with HCl to pH 6.0 (pH 6.0), in sodium-free buffer, in DPBS containing 25 mM 2-deoxyglucose/10 mM sodium azide (DOG/sodium azide), and in DPBSG at 0°C (conditions for pretreatment as outlined in the legend of Fig. 5). Each bar represents the mean of three samples ± S.D. The differences between the respective controls and the asterisk labeled bars are statistically significant at $P \leq 0.05$ (Student's t-test).

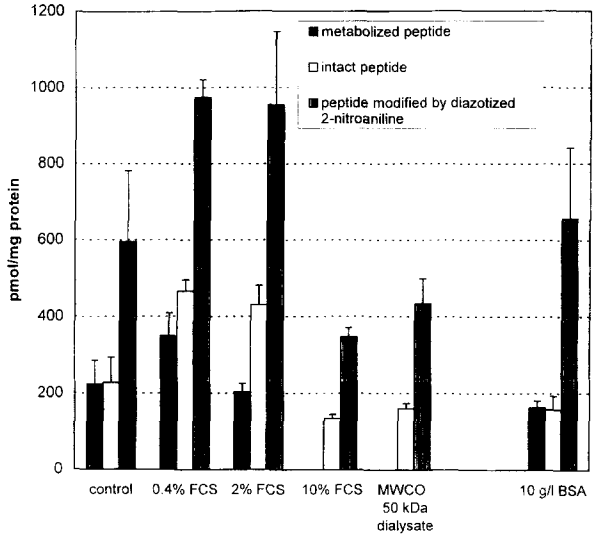

Fig. 7. Cell-associated peptide, showing fractions remaining intact, metabolized within the cell interior and partially modified by diazotized 2-nitroaniline respectively, after exposing AEC for 30 min at 37°C to 4.5 μM I in DPBS containing, 0.4, 2, 10% fetal calf serum (FCS), 10% FCS dialyzed at a molecular weight cut-off of 50 kDa (MWCO 50 000) and 1% bovine serum albumin (BSA), respectively. Each bar represents the mean of three samples ± S.D.

3.4. *Effects of factors known to affect endocytosis or transport protein function on the cellular uptake of I*

To gain insights into the mode of the cellular uptake of I, we varied temperature, energy supply, pH and ionic composition of the incubation buffer and compared the uptake behavior of I with that of its all-D-analog III. Furthermore, to assess the possible contributions of endocytic processes and mdrp- [19] and MRP-like [20] and large anion transporters [21] which are thought to mediate peptide transport [22–24], the influence of additives known to affect endocytosis or to inhibit mdrp or MRP and the large anion transporter respectively, was examined.

Fig. 5 shows the effects of energy depletion, lowered temperature and the above-mentioned reagents upon internalization of I into the cell. The respective membrane inserted portions (partially modified by the diazo reagent) did not differ significantly and are not shown. Different effects are apparent on

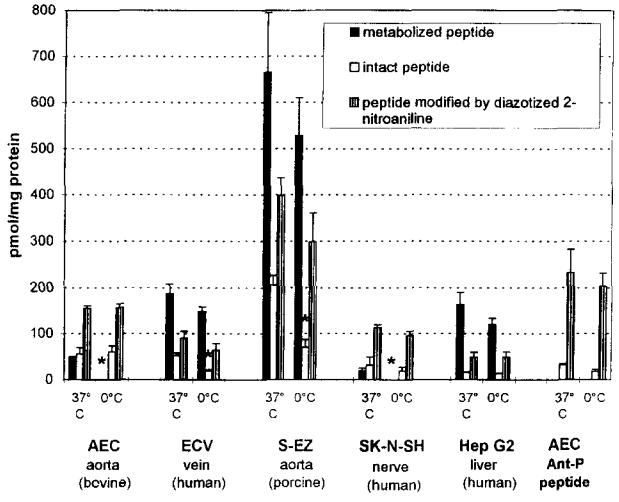

Fig. 8. Cell-associated peptide, showing fractions remaining intact, metabolized within the cell interior and partially modified by diazotized 2-nitroaniline, respectively, after exposing various cell types from different species and organs to 1.8 μM I for 30 min at 37 or at 0°C, respectively. Before exposure to the peptide, the cells were incubated for 60 min at 37 or at 0°C in DPBSG. Additionally, the results of exposing AEC to FLUOS-labeled Ant-P-peptide [3], a natural plasma membrane permeable sequence, using the same conditions are shown. Each bar represents the mean of three samples ± S.D. The differences between the respective controls and the asterisk-marked bars are statistically significant at $P \leq 0.05$ (Student's *t*-test).

both the total quantity of internalized peptide (sum of intact and metabolized peptide portions) and on the metabolized portion, indicating distinct modes for crossing the plasma membrane and entering the metabolizing compartment (Fig. 5). With the exception of calcium depletion and vincristine treatment, the total quantity of internalized I was affected significantly in all cases. Most of the incubation variations applied in Fig. 5 (energy depletion, reduced temperature, lowering of both the external and, after monensin treatment, the internal pH, hyperosmolar sucrose, NEM) are commonly regarded as inhibiting either clathrin-dependent [25–27] or cavaeolae-mediated endocytosis [28,29]. Potassium depletion, known to inhibit clathrin-dependent endocytosis [26,30], elevation of lysosomal pH by 100 μM chloroquine [31], disturbance of endosomal transport by 5 μM Brefeldin A [32], addition of DNP-*S*-glutathione (100 μM), an inhibitor of MRP and the large anion transporter MOAT [20,21] and depletion of magnesium had no effect upon internalization and metabolization of I (not shown).

With the all-D derivative of I (designated III), comparable cellular uptake to that of the parent peptide was achieved (Fig. 6), ruling out sterically specific interactions to account for internalization. Moderate differences between the uptake behavior of both enantiomers became apparent after alteration of the incubation conditions (Figs. 5 and 6), suggesting that the involved mechanisms participate to different degrees in their translocation. As expected, only intact peptide, i.e. no cleavage product, was found in either incubation solution or cell lysate in the case of III.

3.5. *Influence of serum proteins on the cellular uptake of I*

Since binding to serum proteins might alter the cellular uptake, we exposed AEC to I in the presence of different concentrations of FCS and BSA (Fig. 7). Surprisingly, at low concentrations, FCS seems to elevate the quantity of cell associated peptide in all fractions. At higher concentrations, the metabolized peptide fraction was more strongly depleted than were the other two fractions (Fig. 7). Both observations indicate that simple adsorption of peptide to serum proteins is unlikely. The presence of 1% BSA, expected to exert at least comparable adsorption effects had no significant influence (Fig. 7; undetectable at BSA $< 0.5\%$) supporting the latter notion and suggesting that activation and inhibition, respectively, should be taken into consideration in any interpretation.

To obtain information about the molecular size of the FCS components which might influence the cellular uptake of I we performed dialysis using cellulose ester membranes with molecular weight cut-offs (MWCO) of 10 and 50 kDa. After dialysis with the MWCO 10 kDa membrane, the inhibitory effect of FCS remained unchanged, excluding a role for components of less than 10 kDa. A comparable inhibitory effect was observed for FCS dialyzed with a MWCO 50 kDa membrane (Fig. 7), suggesting that the predominant portion of the putative inhibitory serum components are protein(s) of > 50 kDa.

3.6. Comparison of the cellular internalization of I with that of the Ant-P peptide and uptake of I into other cell types

For comparison, AEC were exposed to the Ant-P peptide, a successfully established natural vector peptide [3,33], using the same conditions. A principally similar uptake behavior was found for I and this natural peptide at 37°C. As a noticeable difference, the ratio between the peptide fraction being accessible to the membrane impermeable diazo reagent and the internalized peptide was enhanced in the latter case (Fig. 8).

To rule out the possibility that the observed uptake behavior might primarily reflect peculiarities of the cell line used, we exposed endothelial cells from other species, as well as other cell types to I. In all cases, we found an extensive uptake of I, with significant variability regarding both uptake and metabolizing activity (Fig. 8). A considerable portion of the uptake, however, was retained at 0°C in all cases (Fig. 8), indicating that non-endocytic mechanisms were generally operative. Except in the case of AEC and human nerve cells, metabolization of the peptide also was observed at 0°C (Fig. 8), suggesting the transport into the metabolizing compartment to be non-endocytic in these cells. In distinction to AEC, in the lysates of the other cell types, fluorescent metabolites exhibiting retention times between that of FLUOS-lysine and intact I were observed. The metabolites found within the cell lysates were not detected in the incubation solution and therefore must have been generated within the cell. Trypan blue exclusion revealed no sign of membrane damage under the conditions used.

3.7. Cellular uptake of I tagged by disulfide bridges to negatively and positively charged short peptides

To assess the ability of I to serve as vector for introducing polar compounds of biological interest into the cell interior, we examined the cellular uptake of I tagged by disulfide bridges to negatively and positively charged short peptides by CLSM (Fig. 9). The cargo peptides of potential biological interest used were pYEEWE, whose isoleucine in place of a tryptophan containing analog exhibits affinity to the SH2 domains of various signal proteins [34,35] and

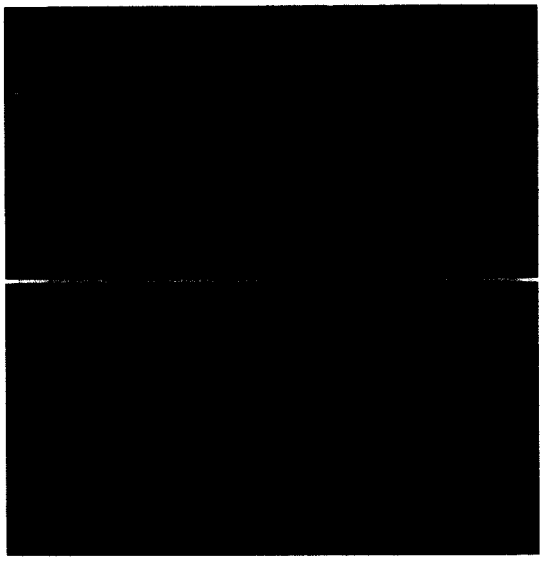

Fig. 9. CLSM image of a 1-μm-thick central horizontal optical section trough AEC pre-exposed for 30 min at 37°C to 1 μM of FLUOS-GCKLALKLALKALKAALKLA-NH_2 tagged by disulfide bridges to AcCpYEEWE-NH_2 (upper panel, ×1000, FLUOS mode) or Dansyl-CPKKKRKV-NH_2 (lower panel, ×1600, dansyl mode).

PKKKRKV, the nuclear localization sequence of the SV40 large T antigen [36]. For generating the conjugates, both components were extended N-terminally with cysteine or a G-C elongation (I) and a disulfide bridge was formed using Ellman's reagent (see Section 2). Analogous attempts to generate a disulfide bridged conjugate with a 15-mer oligonucleotide failed, since we were unable to isolate a water soluble reaction product, probably due to aggregation.

As CLSM revealed, the peptide conjugates were taken up into the cytosol similarly to I; the conjugate bearing the SV40 nuclear localization sequence reached into the nucleus (Fig. 9). No enzymatic cleavage was detectable by subsequent HPLC examination of both incubation solution and cell lysate, indicating that the peptide conjugates had been internalized in the intact form.

4. Discussion

In a previous study, we described an extensive, amphipathicity-dependent uptake into bovine aortic endothelial cells (AEC) of the α-helical amphipathic

model peptide I (FLUOS-KLALKLALKALKAAL-KLA-NH_2) [8]. The observation that the quantity of cell associated I was only moderately affected by lowering the incubation temperature to 0°C and energy depletion suggested a non-endocytic mechanism and an analogy with the recently reported cellular uptake of natural structure forming peptides proceeding by a non-endocytic, but still unidentified pathway [3–5].

Detailed HPLC examination into the mode of uptake of I in the present study, avoiding interference between plasma membrane bound and internalized peptide fractions, indicated participation of multiple, energy-dependent and -independent mechanisms. A comparable uptake behavior was observed for the all-D analog of I (III), indicating non-specificity of the involved transport processes. A similar uptake behavior has been reported by Derossi et al. [37] for the Ant-P-peptide and its inverso and retro-inverso derivatives. Analogy between the uptake behavior of I and the Ant-P peptide became apparent also after direct comparison of the internalization of both peptides into endothelial cells performed in the present study.

About 50% of the uptake of I was found to be retained at 0°C and after energy depletion, indicating that this peptide fraction, at least, had been internalized in a non-endocytic manner. Since the aforementioned natural peptides have been exploited successfully for delivering covalently tagged oligonucleotides and peptides directly into the cytosol with the crucial advantage of bypassing the endosomal compartment [3–5,33,37], this at least partially non-endocytic cellular uptake of I is of particular interest.

The mechanism of this non-endocytic entry into the cell interior remains elusive. Any pore-forming activity of I could be eliminated below concentrations of 2 μM as shown by Trypan blue exclusion, LDH release and capture within the cell of fluorescein generated from internalized fluorescein diacetate. The most attractive alternative interpretations are either direct penetration into or across lipidic areas of the plasma membrane or facilitated diffusion. Crossing of lipid bilayers has been repeatedly reported for helical amphipathic peptides e.g. mitochondrial presequences [38], so this possibility cannot be ruled out. The repeatedly reported transfer across the plasma membrane of conjugates between such peptides and relatively high molecular weight, negatively charged polar entities, e.g. antisense oligonucleotides [4,33], however, appear inexplicable by such a mechanism. That facilitated diffusion plays a significant role, on the other hand, must remain hypothetical, since an interaction of amphipathic peptides with proteinaceous membrane transporters would be required; such interactions have, as yet, not been demonstrated.

Interpretation of the energy-dependent portion of the uptake of I would normally, according to the established knowledge, imply an endocytic mechanism. Our results, however, provide arguments against as well as in favor of an endocytic mode. In particular, the complete lack of cellular uptake of II, the non-amphipathic analog of I exhibiting identical positive charges, argues strongly against a significant contribution from endocytosis. If adsorptive endocytosis were to be the predominant mechanism for the uptake of I, then peptide II also should be internalized to a clearly measurable degree, since this derivative has also been found to be adsorbed extensively to the cell surface (to about 30% to that of cell associated I) [8]. Moreover, the high quantity of internalized I contraindicates an endocytic mechanism, considering that this amount corresponds to an enrichment within the cell interior of up to a 10-fold of the externally provided peptide and that the volume of the endocytic compartment amounts to maximally 10% of the total cell volume [25,39]. Further arguments against the significant involvement of endocytosis are the absence of any effect of potassium depletion, previously reported to characteristically affect clathrin-dependent endocytosis [30], and the significant inhibitive effect of sodium depletion.

On the other hand, the strong effects of energy depletion, lowered temperature, alteration of both the internal and external pH, the presence of hyperosmolar sucrose and pretreatment with NEM are commonly considered to be characteristic of clathrin-dependent [25–27] or cavaeolae-mediated endocytosis [28,29], respectively. It should be noted, however, that the effects above might also be explainable by translocation events mediated by carrier proteins or proceeding across protein channels, which have often been found to exhibit pH- and energy-dependence [40–42]. In the same way, the effect of hyperosmolar sucrose might be interpreted as the effect of

membrane shrinking due to the hyperosmolar stress [43].

Alternatively to an endocytic uptake, a translocation by proteinaceous import machineries might be taken into consideration. Such a possibility is supported by structural similarities between I and helical amphipathic leader sequences initiating an energy-dependent transport of various proteins across the membranes of ER, mitochondria and nuclei of eukaryotic cells [44–46] and the cell membrane of prokaryotes [47]. The inhibitive effects observed on the internalization of I of proteinaceous FCS components appear reconcilable with the action of such putative protein import machineries. However, the existence of such transport facilities has not yet been demonstrated for plasma membranes of mammalian cells, mainly for technical reasons as e.g. Isenman et al. have suggested [48].

A non-endocytic mode of uptake for I is further implied by the close analogy between the internalization of I and that of smaller peptides (tetra- to undecamer); these have been found previously to cross mammalian plasma membranes extensively and unspecifically by energy-dependent and -independent unknown mechanisms [9]. This analogy tempts to assume a common mode for the uptake of both types of peptide. In this case, however, helical properties would not be decisive for the actual transport step, as the small peptides are unable to adopt helical structures. Support for such an interpretation is provided by the recent study of Vives et al. [7], revealing that non-helical short basic sequences are responsible for the membrane permeability of the TAT-peptide. That helicity is not an essential structural requirement for peptides to cross biological membranes is also suggested by the recently reported cellular internalization of various β-sheet forming peptides [6,49].

Such interpretation, however, would mean that the here found extensive uptake of I only resembles an avid binding to intracellular structures subsequent to internalization rather than increased membrane passage.

Based on the data currently available, an unequivocal decision for either of the mechanisms discussed appears impossible. Additional studies to address these questions are under way.

The ability of I to cross the plasma membrane was retained after an N-terminal glycine–cysteine elongation and subsequent coupling of acidic or basic 5–7 mer peptide sequences, demonstrating the distinct possibility of using I-like model peptides as vectors for mediating the passage of polar bioactive compounds across the plasma membrane of mammalian cells generally. Moreover, differences with respect to efficiency of uptake and rate of metabolic breakdown observed with various cell types promise cell and tissue selectivity utilizing such vector peptides.

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