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Evidence for extensive and non-specific translocation of oligopeptides across plasma membranes of mammalian cells

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Abstract

After exposure of bovine aortic endothelial cells to various small peptides (tetra- to undeca-mer), extensive transport of the peptides across the plasma membrane was observed in the concentration range 10^{-7} to 10^{-2} M. The observed transport events, which contradict the generally anticipated poor permeability of peptides across plasma membranes, exhibited high complexity and showed no saturability up to a concentration of 10^{-2} M. Evidence was found for the involvement of mdrp-like transporters as well as of energy-independent facilitated diffusion events. The peptide levels within the cells approximated those of the incubation solution within 30 min, indicating high capacity and velocity for the involved transport processes. Correspondingly, preloaded cells exported about 80% of the internalized peptide within 5 min at 37°C. Analogous results were found after peptide exposure to several other mammalian cell types, indicating a more general importance of the transport phenomena described here. Our findings contradict the prevailing opinion that the often observed lack of activity of externally administered peptides against their targets within intact cells is accounted for primarily by poor cellular uptake and point to export processes counteracting the uptake to be more important in this context. © 1997 Elsevier Science B.V.

Keywords: Peptide transport; Cellular uptake; Mammalian cell

1. Introduction

The value of small peptides as biological agents is compromised according to the prevailing opinion, apart from metabolic instability, primarily by poor permeability across biological membranes [1–3]. Metabolic degradation appears to be of lesser importance in this context, since there exist multiple approaches to improve the enzymatic stability of peptides. To overcome the poor propensity of peptides to cross lipidic membranes, however, provides principal

Abbreviations: AEC, bovine aortic endothelial cells; BEC, porcine brain microvessel endothelial cells; BSP, sulfobromophthalein; 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 1g/l D-glucose; FLUOS-, 5(6)-carboxyfluoresceinyl; NBD-, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; PBS, phosphate-buffered saline

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problems. Besides insufficient lipophilicity, this low permeability is accounted for primarily by the high hydrogen-bonding potential of the peptide backbone's amide groups [4], which often cannot be reduced by structural alterations without affecting the affinity for the biological target. Numerous attempts to improve the permeability by simply increasing the lipophilicity of the molecule have not met with success [5].

The existence of peptide carriers in the plasma membrane of mammalian cells, which could circumvent this principal obstacle, has been demonstrated to date only for di- and tripeptides [6,7]. Additionally, evidence has been provided for the presence of peptide transporters at the blood–brain barrier, exhibiting, however, high structural specificity for a limited number of centrally active peptides [8,9].

In this study, we found evidence that, in contrast to prevailing opinion, oligopeptides (tetra- to at least undeca-mers) can extensively and non-specifically cross the plasma membranes of mammalian cells. Our findings point to the presence of multiple transport facilities for oligopeptides in the plasma membrane of mammalian cells which, if considering counteracting export processes, appear exploitable for non-specifically translocating oligopeptides into living cells. Such a possibility seems to be of particular interest with respect to an introduction into intact cells of small peptides that have appropriate sequences for blocking associations between signal-transducing proteins [10]. So far, competition experiments of this kind have only been carried out in cell free systems, permeabilized cells or by means of microinjection techniques.

2. Materials and methods

2.1. Materials

^{3}H-DAMGO and 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS) were purchased from Amersham and Boehringer, Mannheim, respectively. DNP-SG was synthesized according to Hinchman et al. [11]. Buserelin (HOE 766) was a generous gift from Dr. J. Sandow (Hoechst). Other chemicals and reagents, if not specified, were purchased from Sigma or Bachem.

2.2. Peptide synthesis

The peptides were synthesized manually by the solid phase method using standard Fmoc chemistry. Boc-Lys(Fmoc)-OH was coupled to *o*-chloro-trityl resin in the presence of two equivalents of ethyl-diisopropylamine. After Fmoc-deprotection by 20% piperidine/dimethylformamide (DMF), subsequent couplings of Fmoc-Leu, Fmoc-Phe, Fmoc-Gly and Fmoc-Tyr(tBu) were carried out by means of TBPipU [12]. The crude peptide was obtained from the final cleavage in acetic acid–trifluoroethanol–methylene dichloride (2:2:6, v/v/v) for 1 h and purified using preparative reversed-phase high-performance liquid chromatography (RP-HPLC). The peptide purity (> 98%) was determined by analytical RP-HPLC (220 nm) and the identity was confirmed by electrospray mass spectrometry.

Nitrobenzoxadiazole (NBD)- and FLUOS-derivatives were obtained by reacting the Fmoc-deprotected resin-bound peptides with two equivalents of 7-chloro-4-nitrobenz-2-oxa-1,3-diazole or 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester, respectively, for about 48 h at pH 8–9 and ambient temperature.

2.3. Tritiated peptides

With the exception of [^{3}H]DAMGO, which was purchased from Amersham, the tritiated peptides were synthesized as described previously [13–15]. Before use, the labelled peptides, all exhibiting specific radioactivities of about 1 Tbq/mmol, were cleaned up by HPLC to a radioactive purity > 95% and were mixed with the respective inactive parent compound to attain the required peptide concentration.

2.4. Cell culture

Calf aortic endothelial cells (BkEz-7; AEC), a 12.–20. subculture of a cell line that was established and characterized by Halle et al. [16], were cultured to almost confluency (four days) in 24-well plates (10^{5} cells/well) at 37°C in a humidified 3% CO_2 in air environment in Dulbecco's modified Eagle's medium (DMEM) (Biochrom, Berlin, Germany) containing 4.5 mg/ml of glucose, 100 U/ml of peni-

cillin, 100 μg/ml streptomycin and supplemented with 290 mg/l glutamine and 10% fetal bovine serum (FBS; Sigma, Deisenhofen, Germany).

Porcine endothelial cells (SEZ; MEM/5% FBS), human endothelial cells (ECV 304; M199/10% heat inactivated FBS), murine corticotrophic pituitary tumour cells (AtT-20; DMEM/10% FBS), human neuroblastoma cells (SK-N-SH; MEM Eagle's/10% FBS supplemented with 1% non-essential amino acids, 1% sodium pyruvate and 1% glutamine (Biochrom), human embryonic kidney tumour cells (HEK; MEM Earle's/10% FBS supplemented with 1% non-essential amino acids) and human hepatoma cells (Hep G2; MEM Eagle's/10% FBS supplemented with 1% non-essential amino acids, 1% sodium pyruvate and 1% glutamine) were cultured analogously.

Brain microvessel endothelial cells (BEC) were isolated from fresh porcine brains, as described by Mertsch et al. [17]. The cells were plated into 24-well plates at an initial density of $5 \cdot 10^4$ cells/well and were cultured as outlined for AEC. In addition, the wells were coated with gelatine (Sigma, St. Louis, MO, USA) before plating and 1 ng/ml basic fibroblast growth factor (Promega, Madison, WI, USA), 100 μg/ml Heparin (H-3149, Sigma, St. Louis, MO, USA) and 0.5 μg/ml amphotericin (Biochrom) were added to the medium. The obtained monolayers proved to be more than 95% endothelial in origin and exhibited properties regarded to be typical for the blood–brain barrier (presence of tight junctions, γ-glutamyl transpeptidase-, alkaline phosphatase- and angiotensin-converting enzyme activities, Factor VIII antigen) [17].

2.5. Uptake experiments

After removing the culture medium, the cells were rinsed twice at 37°C with PBS–Dulbecco's (Biochrom), supplemented with 1 g/l glucose (DPBSG) and subsequently exposed to the substrates dissolved in DPBSG (150 μl/well) for the indicated periods. Subsequently, the substrate solution was aspirated off and the cells were washed four times with ice cold PBS (within 2 min) and lysed by allowing them to stand for about 20 h at room temperature in a 0.1% solution of Triton X-100 containing 10 mM trifluoroacetic acid (200 μl/well). For performing efflux experiments, the preloaded and washed cells were incubated with DPBSG (200 μl/well) at 37°C for indicated periods prior to lysis. For determination of the acid-sensitive bound proportion of cell-associated peptide, in some cases, an acid-wash step (incubation with 200 μl/well of 0.2 M acetic acid/0.05 M NaCl for 5 min at 0°C) was inserted between the normal wash process and lysis. The obtained extracts or lysates were utilized without further treatment for HPLC quantitation or liquid scintillation counting.

Parts of the Triton-lysates were used for determining the protein content, according to the method of Bradford [18]. In order to correlate the amount of protein with cell number, monolayers, grown under identical conditions in separate wells, were trypsinized and counted. For AEC, a ratio of 200 μg protein/10^6 cells was found by this procedure. The average volume of AEC was determined to be 1.4 pl by means of a coulter-ZM counter (Coulter Electronics, Luton, UK).

2.6. HPLC analysis

HPLC was performed using a Bischoff HPLC gradient system (Leonberg, Germany) equipped with a Kromasil 100 C_{18}, 5-μm column (250 × 4 mm I.D.), precolumns containing the same sorbent and a Rheodyne-RH 8125 injection valve with a 50-μl sample loop. The elution was performed with 0.01 M trifluoroacetic acid (TFA) (A) and acetonitrile–water (9:1, v/v) (B) (gradient from 30 to 60% B within 20 min). Detection was performed by fluorescence using a fluorescence detector RF-551 (Shimadzu, Japan) at 520 nm after excitation at 445 nm for prepurified FLUOS-labelled peptides (see below), at 350 nm after excitation at 285 nm for tryptophan-containing peptides and at 540 nm after excitation at 470 nm for NBD-labelled derivatives. The peaks corresponding to the intact peptides and their des-tyrosine metabolites were quantified using a Hyperdata Integration Workstation S I (Bischoff, Leonberg, Germany) on the basis of calibration lines established previously with pure references. If not specified otherwise, the so-determined quantities of incorporated peptide are generally indicated as the sum of intact peptide and detyrosinated metabolite.

The cells' own fluorescently active components did not interfere with detection of the peptides, even in the case of tryptophan-containing derivatives, so

that pretreatment of the cell lysates could be avoided at the cost of a somewhat more frequent replacement of the precolumn.

2.7. FLUOS derivatization

To 70 μl of the sample solution, 10 μl of 2 M K_2HP0_4, 10 μl of 23 μM cyclohexylalanine solution (internal standard) and 5 μl of a solution containing 4.9 mg of 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (Boehringer) in 100 μl of dimethylsulfoxide were added and allowed to react for about 20 h at room temperature. Subsequently, the solution was mixed with 4 μl of 8 M NaOH and, after standing for at least 30 min, with 11 μl of 1.4 M H_3PO_4. HPLC was first carried out by eluting with 0.04 M phosphate buffer, pH 7.4 (A) and acetonitrile–water (9:1, v/v) (B) with gradients from 9–28% B (0–20 min) and 28–85% B (20–30 min) at a flow-rate of 1.0 ml/min. UV Detection was at 242 nm. The eluates containing the derivatized peptides and their aminopeptidase degradation products (16 to 21 min) were collected and concentrated to dryness by means of a vacuum centrifugal evaporator (Jouan, Unterhaching, Germany). Residuals, corresponding to 1 ml of the eluate, were dissolved in 100 μl of 0.5 M TFA containing 25% acetonitrile and subjected to the HPLC protocol described above for quantitation.

2.8. Confocal laser scanning microscopy (CLSM)

Cells ($1 \cdot 10^4$) were plated on 22×22 mm coverslips, glued (with silicones RTV 615A/615B, Paul Hellermann, Pinneberg, Germany) above the hole (15 mm D) of punched plastic Costar culture dishes (35 mm D; Integra Biosciences, Germany) and cultured for four days, as described above. After removing the culture medium, the cells were exposed to solutions of the fluorescently labelled peptides in DPBSG (10–500 μM) for 60 min at 37°C. Subsequently, the cells were washed four times with ice-cold PBS and covered with about 200 μl of PBS. Microscopy was conducted within 10 min at room temperature using an LSM 410 inverted confocal laser scanning microscope (Carl Zeiss Jena, Jena, Germany). Excitation was performed at 488 nm by means of an argon–krypton laser and a dichroitic mirror (FT 510) for wavelength selection. Emission was measured at 515 nm using a cut-off filter (LP 515) in front of the detector. For optical sectioning in the *z*-direction, sixteen frames were made, with a thickness of 1 μm. Subsequent HPLC investigations of the incubation solution and of Triton X-100 lysates of the cells gave no indication of any metabolic breakdown of the peptides.

3. Results

3.1. Evidence for peptide incorporation after millimolar exposure of endothelial cells to enkephalin-derived peptides

With the initial intention of examining the possibility of whether or not natural amino acid transporters could be exploited for translocating small central active peptides through the blood–brain barrier, we exposed aortic- (AEC) and brain microvessel endothelial cells (BEC) to several enkephalin-derived peptides. Surprisingly, in all cases, we observed high degrees of peptide association onto the cells (Fig. 1). Incubation at 0–4°C (2 mM peptide, 60 min; precooling the cells for 30 min) resulted in virtually no association to the cells (< 0.1 nmol/mg protein) so that non-specific adsorption could be ruled out as contributing noticeably and actual uptake into the cell interior had to be taken into consideration.

Since both derivatives bearing an intact α-carboxy- and α-amino group (for recognition by amino acid

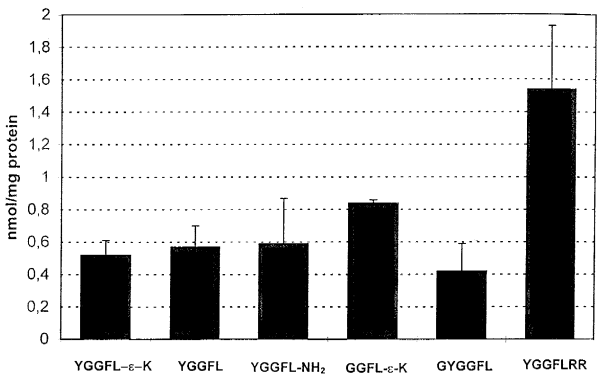

Fig. 1. Quantity of cell-associated peptide after exposure of aortic endothelial cells to 2 mM solutions of various enkephalin-derived peptides in DPBSG for 30 min at 37°C. Each bar represents the mean of three samples ± SEM.

transporters, according to Smith [19]) and unmodified peptides exhibited comparable degrees of association to the cells (Fig. 1), the observed peptide uptake appeared to resemble more general phenomena, unlike our initial intention. Therefore, we extended our investigations with the goal of gaining insights into the mode and structural requirements for the transport events that were found.

In order to facilitate detection and to render the degree of degradation by plasma membrane-associated peptidases negligible, millimolar peptide concentrations (0.2–10 mM) were employed in the first experiments. That the observed transport phenomena did not reflect artifacts, accounted for by these unphysiologically high peptide concentrations, was ascertained in subsequent investigations conducted in the low micromolar range using tritium-labelled peptides (see below).

3.2. Time dependency of peptide translocation

The uptake by the cells reached an equilibrium at about 20 min (Fig. 2). The efflux out of preloaded cells exhibited a complex course, indicating location of the cell-associated substrate within distinct compartments (Fig. 3). From BEC, the release of the incorporated substrate proceeded considerably more rapidly than that from AEC (Fig. 3), suggesting a relation to the lower degree of uptake into BEC (Fig. 3).

3.3. Influence of the peptide structure on cellular uptake

In order to obtain information about the structural requirements for the found peptide import, we modified the N- and C-terminal amino- and carboxy-groups of Leu-enkephalin, respectively, and incorporated basic and acidic residues and glycine into the N-terminal, C-terminal and middle regions of the molecule. Phenylalanine was generally replaced by tryptophan in this series to permit fluorescence detection during HPLC analysis. Since amidation of the C-terminus did not attenuate the uptake, in most cases, the more conveniently available amides were used (Table).

The performed structural modifications only slightly influenced peptide import (Table), indicating poor structural specificity for the transport mechanism. Generally, a positive effect of basic modification and, more surprisingly, of incorporation of glycine, is suggested by the data displayed in Table 1.

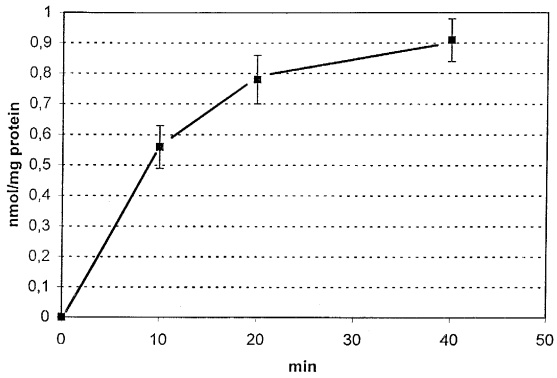

Fig. 2. Quantity of cell-associated peptide after exposing AEC to 2 mM YGGFL-ϵ-K at 37°C for different periods of time. Each point represents the mean of three samples ± SEM.

3.4. CLSM imaging after cell exposure to fluorescently labelled peptides

The poor structural specificity that was found should justify the use of N-terminally fluorescently labelled derivatives for directly demonstrating peptide uptake by CLSM, in spite of substantial alterations to the charge and lipophilicity, which were associated with the introduction of fluorescence labels into peptides. We, therefore, performed CLSM

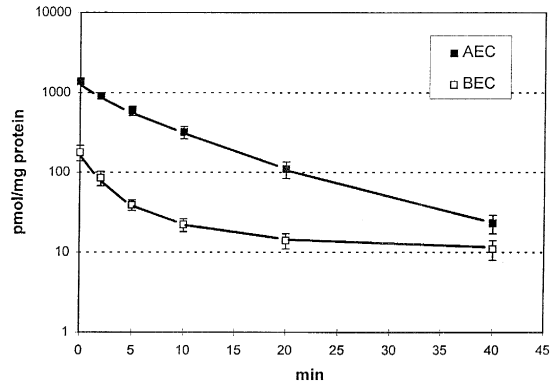

Fig. 3. Liberation of cell-associated peptide at 37°C from AEC and BEC, which had been previously exposed to 2 mM YGGFL-ϵ-K for 60 min at 37°C. Each point represents the mean of three samples ± SEM.

with AEC that had been previously exposed to the fluorescently labelled peptides NBD-GGFL, NBD-GGFL-ε-K, NBD-RR-all-D-LWGGY-NH_2 and all-D-RPRPQQFO-(FLUOS)GLM-NH_2 (all-D-FLUOS-SP). The CLSM images in all cases revealed actual peptide import into the cell interior and exhibited a similar pattern to that displayed by NBD-GGFL in Fig. 4. Analogous results to those found with AEC were also obtained with BEC (NBD-GGFL-ε-K), primary hypophysis cells (NBD-RR-all-D-LWGGY-NH_2; Furkert et al., in prep.) and mast cells (FLUOS-SP and all-D-FLUOS-SP; Lorenz et al., in prep) (not shown).

3.5. Cell viability and peptide degradation

No differences were detectable between controls and cells treated with 2 mM peptide according to the protocol outlined above with respect to trypan blue exclusion and the MTT-viability test [20].

Due to the high peptide concentrations used, the proportion of the peptide that was degraded by peptidases during exposure to the cells remained clearly below 10%, so that the influence of enzymatic breakdown upon the uptake was neglected. Correspondingly, the addition of 0.4 mM bestatin, a potent inhibitor of aminopeptidase N, the predominant peptidase activity residing on the surface of AEC [21,22], did not influence the amount of cell-associated peptide under the conditions used in this study.

Table 1
Quantity of cell-associated substrate after exposure of aortic endothelial cells to various enkephalin-derived peptides [a]

Peptide	nmol/mg protein
YGGFL	0.57(±0.13)
YGGWL	0.60(±0.17)
N-Methyl-GYGGWL-NH_2	0.71(±0.05)
N-Dimethyl-GYGGWL-NH_2	0.63(±0.19)
SuccinylYGGWL-NH_2	0.80(±0.04)
YGGWLE-NH_2	0.67(±0.13)
DAMGO	0.89(±0.09)
AcYGGWL-NH_2	0.97(±0.22)
YGGWL-NH_2	1.12(±0.11)
EYGGWL-NH_2	1.30(±0.13)
GYGGWL-NH_2	1.29(±0.26)
all-D-YGGWL-NH_2	1.48(±0.27)
YGGEWL-NH_2	1.61(±0.19)
RYGGWL-NH_2	1.65(±0.56)
KYGGWL-NH_2	1.90(±0.08)
AcKYGGWL-NH_2	1.85(±0.27)
YGGWLR-NH_2	1.98(±0.23)
YGGRWL-NH_2	2.12(±0.65)
all-D-LWGGY-NH_2	2.56(±0.36)
YGGWLG-NH_2	2.40(±0.26)
YGGGWL-NH_2	2.99(±0.24)

[a] Incubation with 2 mM peptide dissolved in DPBSG for 30 min at 37°C (mean of three samples ± SEM).

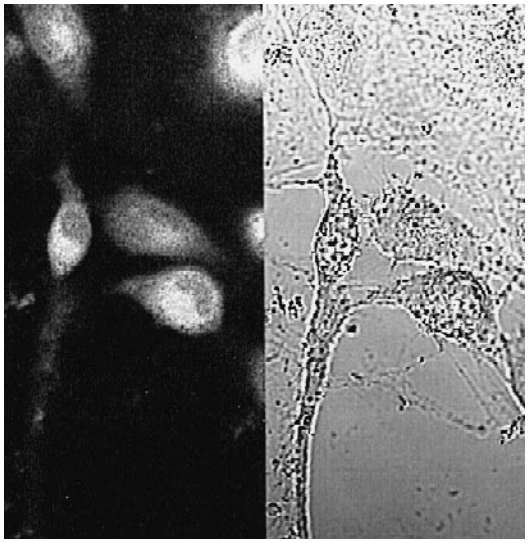

Fig. 4. Confocal laser scanning microscopy image of a 1-μm thick central horizontal optical section through AEC that had previously been exposed to 0.7 mM NBD-GGFL in DPBSG for 30 min at 37°C. Inset: The same section viewed in transmission mode.

In order to obtain information about the possible effects of endogenous peptidases on the internalized substrate, YGGFL-ε-K and YGGFL were exposed to Triton X-100 lysates of AEC or BEC at pH 7.2 [$1 \cdot 10^5$ cells, 1% Triton X-100 in 0.5 ml PBS–H_2O (1/9, v/v); 0.2 mM peptide; 60 min, 37°C]. The modified peptide proved to be substantially more stable than leucine enkephalin under these conditions. About 50% of YGGFL-ε-K remained intact, whereas 95% of YGGFL was degraded. After exposure of the cells to acidic lysates (10 mM TFA in 0.1% Triton X-100), no cleavage of the peptides could be detected within 24 h at ambient temperature.

3.6. Reproduction of the translocation events in the micromolar range

Beside the metabolically stable enkephalin analog, [^{3}H]DAMGO, we utilized several tritiated peptides

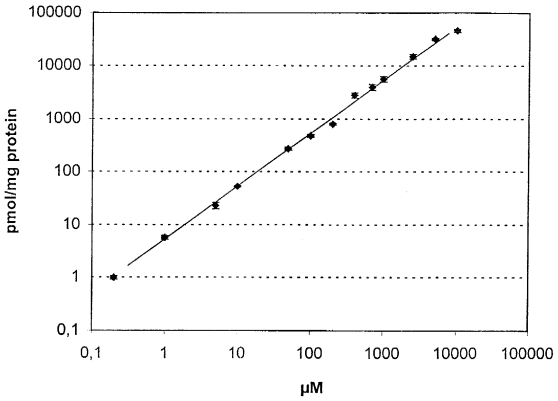

Fig. 5. Quantity of cell-associated peptide after exposing AEC to different concentrations of tritiated DAMGO for 30 min at 37°C. Each point represents the mean of three samples ± SEM.

that differed with respect to charge, size and modification of the N- or C-terminus (R-P-K-P-G-[^{3}H]Y-OH; R-K-E-V-[^{3}H]Y-OH; AcR-AcK-E-V-[^{3}H]Y-OH and [^{3}H]buserelin) for conducting transport experiments in the micromolar concentration range, in a manner analogous to that used in the millimolar range.

To minimize the influences of enzymatic degradation, we generally performed the uptake experiments in the presence of 0.2 mM bestatin. Under these conditions, more than 90% of the radioactivity in the incubation solution was attributable (by HPLC) to the intact peptide after exposure to the cells. From the cell-associated peptide portion, at least 40% coeluted with the intact peptide during subsequent HPLC analysis, indicating uptake of the intact peptide.

3.7. Concentration dependency of uptake

The uptake of the enkephalin analog, DAMGO, into AEC proved to be linear from 10^{-2} up to 10^{-7} M (Fig. 5), rendering peculiarities for the high concentration range unlikely. Uptake investigations performed with the other labelled and unlabelled peptides in the range $5 \cdot 10^{-4}$ to 10^{-7} M and from 10^{-2} to $2 \cdot 10^{-4}$ M led to analogous results (not shown).

Generally, the quantities of internalized peptide approached values corresponding to an equilibrium between intra- and extracellular peptide concentrations (taking into account the cell volume of 1.4 pl

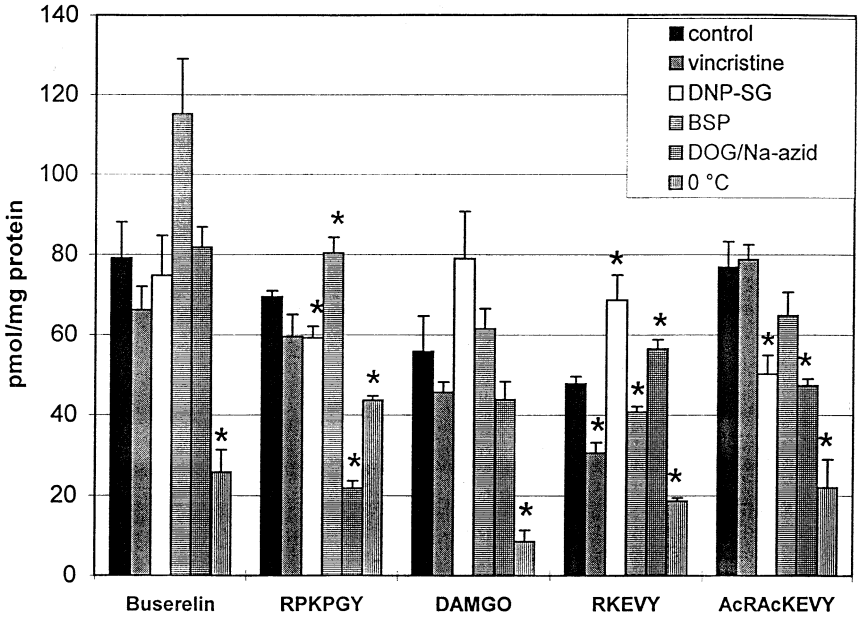

Fig. 6. Quantity of cell-associated peptide after exposing AEC, pretreated with DPBSG or 25 mM 2-deoxyglucose–10 mM sodium azide in DPBS, respectively, for 60 min at 37 and 0°C, to 10 µM tritiated peptide in DPBSG without additives and in the presence of 25 mM 2-deoxyglucose–10 mM sodium azide in DPBS, 20 µM vincristine in DPBSG, 100 µM 2,4-dinitrophenyl-*S*-glutathione (DNP-SG) in DPBSG and 100 µM sulfobromophthalein (BSP) in DPBSG for 60 min at 37 and 0°C. Each bar represents the mean of three samples ± SEM. The differences between the respective controls and the asterisk-labelled bars are statistically significant at $P \leq 0.05$ (Student's *t*-test).

and the protein content of 200 μg protein/10^6 cells given in the Section 2).

3.8. Influences of metabolic and transport inhibitors

To gain an insight into the mechanism of the observed peptide uptake, we exposed the cells to the peptides in the presence of various agents that are known to affect metabolism and transport events. Energy depletion and treatment with vincristine, an inhibitor of the multidrug resistance mediating efflux pump, P-glycoprotein [23], in most cases resulted in a slight reduction in uptake (Fig. 6). The presence of DNP-*S*-glutathione (DNP-SG) and bromosulfophthalein, known to serve as substrates for multidrug resistance-associated protein, glutathione conjugates- and large anion transporters [24–27], augmented or attenuated, respectively, the peptide incorporation, depending on the respective substrate (Fig. 6). This complex behaviour suggests the involvement of multiple energy-consuming transporters exhibiting distinct preferences for the inward or outward direction, respectively, and prevents clear kinetic characterization.

That these reagents also influence the efflux out of the cells is illustrated in Fig. 7 for DNP-SG-treated cells. Conclusions concerning the reduced efflux rate in the presence of DNP-SG, which is apparent in Fig. 7 for the period from 5 to 30 min, and its relation to the enhanced uptake, can be drawn, however, only with caution. Because of a delayed onset of the effects, the agents had to be added prior to the preloading of cells, which resulted in different peptide levels for control- and reagent-treated cells and, probably, in the occupation of distinct compartments by the peptides.

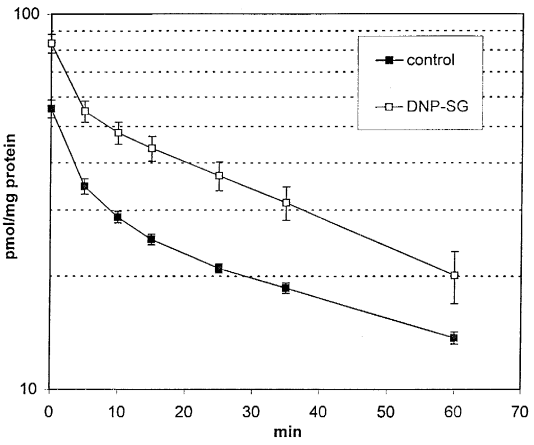

Fig. 7. Liberation of cell-associated peptide at 37°C from AEC into DPBSG without additives and in the presence of 100 μM 2,4-dinitrophenyl-*S*-glutathione (DNP-SG), exposed previously to 10 μM tritiated DAMGO for 60 min at 37°C alone or in the presence of 100 μM DNP-SG, respectively. Each point represents the mean of three samples ± SEM.

Brefeldin A, an inhibitor of vesicular transport [28], did not significantly affect the uptake (not shown), which argues against considerable contributions being made by endocytic processes.

The major part of the observed peptide uptake, however, remained uninfluenced by energy depletion and, therefore, must proceed in an energy independent way (Fig. 6). The substantially reduced uptake at 0°C renders simple diffusion as unlikely to be involved and points to facilitated diffusion as the mechanism for this energy-independent transport. Evidence for cotransport of protons or Na^+, however, often found associated with such a mechanism [29], could not be found. Alterations of the pH in the range of 7.2–5.5, treatment with the acidotropic agent, monensin (20 μM) [30], and replacement of Na^+ by choline, performed in this context, had no effect on peptide uptake (not shown).

3.9. Discrimination between acid-sensitive and acid-resistant portions of cell-associated peptide by acid washes

The levels of cell-associated peptide attained after incubation with 10 μM peptide at 0–4°C (Fig. 6) were relatively higher than those found in the millimolar concentration range, suggesting enhanced contributions of non-specific adsorption in the low concentration range. To obtain information about the degree of this non-specific adsorption, we performed an additional wash step with acetic acid–NaCl for 5 min at 0°C, commonly thought to strip off adsorbed peptides [31]. Fig. 8 displays the cell-associated peptide, which was split into acid-sensitive and an acid-resistant (internalized) parts. Generally (except for RPKPGY at 0°C), the acid-sensitive portion remained clearly below 50% of the total amount, which argues for an actual uptake of the greater part of the observed cell-associated peptide. Moreover, the signifi-

cant reduction in the acid-sensitive bound peptide portion observed in several cases after energy depletion (Fig. 8) raises questions regarding the assumption that the acid extract contains only surface-bound peptide. More likely, this finding suggests export from the cell interior, proceeding also throughout the extraction period at 0°C. That the cells exert residual transport activities at 0°C is also suggested by the considerable amount of acid-resistant bound (internalized) peptide found after incubation at this temperature (Fig. 8).

3.10. Reproducing the transport events with other cell types

In order to rule out the possibility that the found transport phenomena would reflect a peculiarity of AEC, we exposed several cell lines originating from different species and tissues to 10 μM tritiated DAMGO at 37°C and 0°C and after energy depletion. The results of these experiments are displayed in Fig. 9 and reveal, despite clear quantitative differences, considerable analogy to that found previously with AEC. This finding supports the notion that the observed transport phenomena would reflect a more general behaviour of mammalian cells, which has been suggested also by the above outlined analogies found between the results obtained with AEC and BEC, respectively, in the millimolar range, and of AEC and various cell types in CLSM studies.

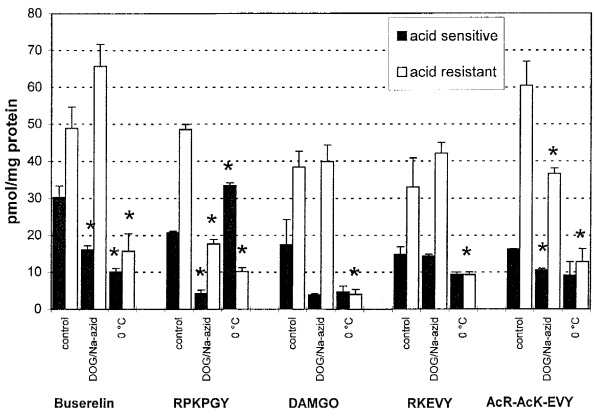

Fig. 8. Acid-sensitive and acid-resistant quantities of cell-associated peptide after exposing AEC, pretreated with DPBSG or 25 mM 2-deoxyglucose–10 mM sodium azide in DPBS, respectively, for 60 min at 37 and 0°C, to 10 μM tritiated peptide in DPBSG without additives and in the presence of 25 mM 2-deoxyglucose–10 mM sodium azide in DPBS for 60 min at 37 and 0°C. The acid-sensitive and acid-resistant proportions of the cell-associated peptide were assessed after incubating the washed cells in 0.2 M acetic acid containing 0.05 M NaCl for 5 min at 0°C before lysing the cells and measuring the radioactivities of both the acid extract and the lysate. Each bar represents the mean of three samples ± SEM. The differences between the respective controls and the asterisk-labelled bars are statistically significant at $P \leq 0.05$ (Student's *t*-test).

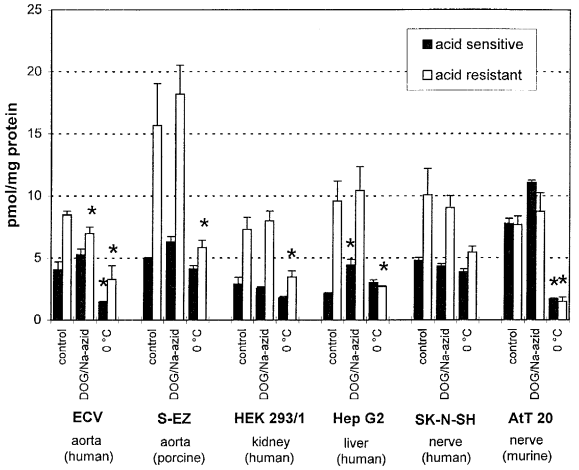

Fig. 9. Acid-sensitive and acid-resistant quantities of cell-associated peptide after exposing various cell types originating from different species and organs to 10 μM tritiated DAMGO, according to the conditions specified in the legend to Fig. 8. Each bar represents the mean of three samples ± SEM. The differences between the respective controls and the asterisk-labelled bars are statistically significant at $P \leq 0.05$ (Student's *t*-test).

4. Discussion

This study provides evidence that oligopeptides containing more than five amino acid residues are capable of extensively crossing plasma membranes of living cells. So far, such an ability was demonstrated only for di- and tripeptides [6,32,33] and for a limited number of oligopeptides possessing specific transporters at the blood–brain barrier [8,9].

Our findings contradict prevailing opinion that the repeatedly reported inefficiency of externally administered oligopeptides against recognition domains of signal proteins or intracellular enzymes would be accounted for primarily by poor permeability of peptides across the plasma membranes and point to

export processes being a more decisive factor in this context.

This notion appears reconcilable with recent speculation about the residence of, as yet undetected, peptide transporters of the ABC-transporter type in the plasma membrane of mammalian cells being operative preferably as efflux pumps [34–36]. The influence of energy depletion on the cellular uptake process, and of the P-glycoprotein- and MRP-substrates, vincristine and DNP-SG, respectively, [23–25], observed in the current work, support such speculations. Likewise, these observations provide the first clues to the mechanism for one part of the found translocation events.

The majority of the peptide transport described here, however, proceeded by energy-independent mechanisms. The energy independence, in conjunction with the levels of internalized peptide, approaching an equilibrium between external and internal concentrations, and the substantially reduced uptake at 0°C, point to facilitated diffusion as the mechanism for this part of peptide translocation.

Non-specific adsorptive endocytosis or pinocytosis can only partly explain the transport phenomena found in the present study, taking into consideration the high intracellular peptide levels attained and presuming a total volume for endocytic vesicles of only about 10% of that of the entire cell [37]. An explanation by these mechanisms is further compromised by the observed high exchange rates, of the order of minutes, considering that periods of 1–2 h are commonly required for the turnover of endocytic vesicles between a cell's interior and the plasma membrane [38]. Moreover, brefeldin A, an inhibitor of vesicular transport [28], had no influence upon peptide uptake.

The very specific transporters for enkephalin-like peptides [8,9] can be ruled out as accounting for the transport phenomena observed here, because of the lack of saturability and poor structural specificity. These features tempt one to speculate that the peptides might be capable of exploiting transport facilities that were originally destined for non-peptidic substrates. Preliminary experiments point to amino acid transporters and putative protein channels as probable candidates for mediating such transport. Other preliminary experiments suggest an analogy to the behaviour of several membrane-permeable amphipathic peptides, which have been reported to extensively cross the plasma membrane of mammalian cells by an unknown mechanism [39–41]. These issues will be addressed in future studies.

This study provides evidence for the existence of efficient and non-specific facilities for translocating small peptides in the plasma membranes of mammalian cells. The ability of peptides to extensively cross plasma membranes calls for altered strategies to attain intracellular activity that consider not only metabolic stability and propensity to traverse lipidic membranes, the currently most widespread practice, but also efforts to minimize peptide export out of the cells.

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