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CULTURE CONDITIONS INFLUENCE UPTAKE AND INTRACELLULAR LOCALIZATION OF THE MEMBRANE PERMEABLE cGMP-DEPENDENT PROTEIN KINASE INHIBITOR DT-2

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1. ABSTRACT

The membrane-permeable peptide DT-2 which utilizes the HIV-Tat membrane translocation sequence is known to inhibit cGMP-dependent protein kinase (PKG) effectively *in vitro* and in various cell lines and tissue preparations. However, the uptake characteristics of DT-2 have not been studied in detail. We investigated the intracellular uptake and localization of fluorescein-labeled DT-2 (fDT-2) in cultured C6-glial cells and vascular smooth muscle cells (VSMCs) as well as VSMCs in intact arteries. To avoid fixation-induced fluorescence, live unfixed cells and arteries were incubated with fDT-2 and examined using conventional and confocal fluorescence microscopy. In non-differentiated cultured VSMCs, uptake appeared vesicular with nuclear exclusion, consistent with an endocytotic internalization mechanism. Inhibition of endocytosis by phenylarsine oxide (PAO), low temperature or disruption of actin polymerization by cytochalasin-D or lantrunculin-A showed a residual non-endocytotic fDT-2 translocation with diffuse cytosolic and nuclear uptake. Similarly, differentiated contractile VSMCs within the medial layer of intact cerebral arteries also showed a distinctively different, more diffuse cytosolic uptake and time dependent nuclear localization. To verify the morphology dependency of fDT-2 uptake, VSMCs were reconstituted in fibrillar collagen matrices. The cells adopted a differentiated morphology and fDT-2 translocation was similar to cells in intact arteries. These results demonstrate that VSMCs cells utilize distinct cellular uptake mechanisms depending on their phenotype.

2. INTRODUCTION

Several membrane permeable peptides (MPPs) have been identified which are reported to cross cellular membranes and thus gain access to cytoplasmic spaces. Of these peptides, perhaps the most well-studied is the Tat protein found in HIV-1 (1). Early studies found that a fragment of this protein (YGRKKRRQRRRPP) was able to traverse biological membranes in a rapid and nonspecific fashion (2,3). After the discovery of Tat, other MPPs were soon identified and synthesized. In addition to the Tat peptide, the third helix of the antennapedia homeodomain peptide was also shown to be permeable to cell membranes (4). Initial studies using Tat and antennapedia peptides containing fluorescent tags suggested the uptake of these peptides was rapid and energy-independent. However, these early reports now appear to be confounded by the presence of fixation-induced artifacts (see ref. 5 for review). Current evidence suggests that membrane translocation occurs either via endocytosis, peptide oligomerization with resultant channel formation, or via the formation of inverted micelles in the membrane which transport these peptides across the membrane (6,7).

Although the mechanism(s) of action with which these peptides cross cellular membranes remains unclear, the utility of MPPs has been widely reported. The Tat sequence has been used to facilitate the delivery of DNA, RNA, proteins and small molecules (8-11). Interestingly, Schwarze *et al.* used the Tat transduction domain to facilitate the uptake of beta-galactosidase into all tissues of mice including brain (12). Thus, there is considerable evidence that membrane-permeable peptides, specifically Tat, can cross lipid membranes and thereby facilitatie the delivery of biomolecules into cells and tissues.

It has been reported that the rate of uptake as well as the effects of temperature on MPP uptake can vary widely, suggesting that the cargo and/or cell type can affect the uptake, localization and mechanism of translocation (13). Due to the variability between peptides and cells, this study focused on the uptake effects of a single peptide in rat vascular smooth muscle cells (VSMCs) specifically, the uptake of a PKG-inhibiting, Tat-based membrane permeable peptide known as DT-2 (14). We have previously and successfully used MPP sequences to create peptide inhibitors of cGMP-dependent protein kinase (PKG) (15). DT-2, the prototype PKG inhibitor resulting from these studies, was synthesized to contain a Tat sequence with an additional PKG substrate-site inhibitor (LRKKKKKH). The complete amino acid sequence for DT-2 is thus YGRKKRRQRRRPPLRKKKKKH. DT-2 has been used to inhibit PKG in a variety of cellular and tissue preparations (15-16).

Previous studies have established DT-2 as a useful tool in investigating cGMP and PKG-mediated events (15-16). To date however, the specific uptake characteristics of DT-2 have not been studied. To visualize uptake, fluorescein-labeled DT-2 (fDT-2) was synthesized (14). Early studies using cell fixation to investigate DT-2 uptake proved to be misleading as uptake and localization of fDT-2 was variable and changed considerably depending on the conditions used during imaging. Attempts to standardize the imaging of fDT-2 demonstrated that uptake of fDT-2 is more complex than initially anticipated. These data suggest that the uptake of fDT-2 is multi-faceted. Cell morphology appears to be a strong determinant of the uptake pattern of fDT-2. Furthermore, the use of endocytosis inhibitors affects the intracellular localization of fDT-2. Investigating the actions of fDT-2 uptake is essential to further elucidate the actions of this kinase inhibitor and may also shed light on the uptake of similar membrane permeable peptides.

3. MATERIALS AND METHODS

3.1. Peptide Synthesis

Peptides were synthesized as described previously (15). Briefly, solid-phase synthesis of the peptides was carried out on a Pioneer automatic peptide synthesizer (Applied Biosystems) employing Fmoc chemistry with TBTU-activation. Peptides were cleaved from the resin and deprotected by a 3 hour treatment with TFA. After precipitation with t-butylmethyl ether, the resulting crude peptides were purified by preparative HPLC. N-terminal fluorescein-labeled DT-2, DT-3, Tat(47-59) and Ant(43-59) were synthesized by treating ß-alanine and cysteine elongated peptides with a 1.2 fold excess of fluorescein maleimide (Molecular Probes, Eugene, Oregon) for 2 h in 1 M phosphate buffer pH 7.0 containing 20% DMSO. The labeled products were purified by preparative HPLC and characterized by MALDI-MS.

3.2. Animal and Tissue Preparation

Male adult Sprague-Dawley rats (200 g) were euthanized with an injection of sodium pentobarbital (120 mg/kg, i.p.) and subsequent decapitation, in accordance with the University of Vermont Institutional Animal Care and Use Committee and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Brains were removed and placed in cold (4°C) physiological saline solution (PSS) containing 119 mM NaCl, 4.7 mM KCl, 24 mM NaHCO3, 1.2 mM KH2PO4, 0.03 mM EDTA, 1.2 mM MgSO4, 1.6 mM CaCl2, and 10.6 mM glucose, pH 7.4. Cerebral arteries and thoracic and abdominal aortas were dissected, cleaned of fat and connective tissue, and treated as indicated below.

3.3. Cell culture of dissociated vascular smooth muscle cells

Aortic vascular smooth muscle cells were enzymatically dissociated using a modification of the protocol from Burnstock et al. (17). The aorta was placed in digestion medium consisting of HBSS containing 1.25 units/ml elastase (Worthington) and 175 units/ml collagenase (Sigma) for 30 min. The digestion was stopped with the addition of Dulbecco's modified Eagle's media (DMEM) containing 10% fetal bovine serum (FBS) and the tunica adventitia was gently pulled away from the tunica media and discarded. The tunica media was allowed to recover over night in 20% FBS in DMEM at 37°C, 5% CO2. Thereafter, segments of the tunica media were further digested for 2 hours (h) in digestion medium containing 2.5 units/ml collagenase until a single cell suspension was obtained. Cells were washed twice with DMEM-FBS and were suspended in DMEM containing 10% FBS and grown in culture dishes containing glass coverslips, or in collagen matrices.

3.4 Smooth muscle cell culture in fibrillar collagen

Freshly dissociated cells were mixed with a solution of 20 g/ml chilled type-I native collagen from rat tail (Biomedical Technologies Inc) at pH 7.4 and subsequently plated on tissue culture dishes. Dishes were immediately transferred to 37°C to initiate polymerization of the collagen. All cells were maintained in DMEM plus 10% FBS at 5% CO2 at 37°C and were allowed to grow for 4-8 days. Cells were trypsinized to ensure collagen embedment. Thereafter, cells embedded in collagen were incubated with 5 micromolar fDT-2 for 1.5 h, and washed three times with PBS. The matrices containing cells were transferred to glass slides, covered with cover slips, and imaged (40x) with the 488-nm line of a BioRad MRC 1024ES confocal microscope and LaserSharp 2000 software (BioRad).

3.5. Cell viability experiments

Cultured VSMCs were treated with 0.1 to 100 microM of DT-2 or fDT-2 for 4 h at 37 °C. The cells were

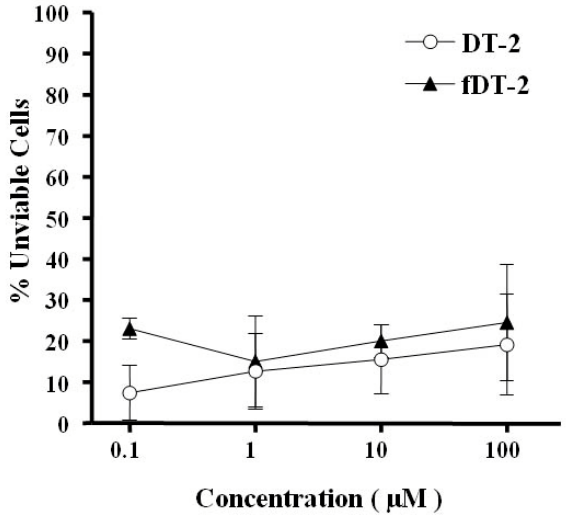

Figure 1. Toxicity of DT-2 and fDT-2. VSMCs were incubated for 4 h with 0.1 to 100 microM DT-2 (open circles) or fDT-2 (closed triangles), respectively. The percentage of trypan-blue positive (unviable) peptide-treated cells was established by determining the ratio of unviable cells vs. total cells minus the ratio of control cells not treated with peptide. Percentages were the mean ± SEM of 4 experiments each performed in duplicate.

then trypsinized and a sample of the cell suspension was diluted 1:5 with 0.4% trypan-blue. Using a hematocytometer, the total number of cells and stained cells was counted. The percentage of unviable cells was calculated by dividing the number of stained cells by the total number of cells treated. The percentage of unviable cells was normalized by subtracting the average value found in the controls (VSMCs not treated with peptide).

3.6. Uptake and imaging of fDT-2

To evaluate fixative effects on uptake and internalization of fDT-2, C6 glial cells were grown in glass-bottomed culture dishes, incubated with 10microM fDT-2 for 30 minutes (min) and imaged using an inverted Nikon Diaphot 200 microscope with a 40X oil immersion fluorescence objective. Thereafter, the cells were fixed with methanol, isopropenol or 4% paraformaldehyde and re-imaged. The microscope was outfitted with an ORCA ER cooled CCD camera (Hamamatsu). Excitation (480 nm) was provided by a Lambda LS Xenon Arc Lamp. Image acquisition (emission 530 nm) was performed using Metafluor 4.64 software from Universal Imaging (Media, PA). Camera settings were held constant throughout a given experiment in order for each plate to be compared to a control plate containing no peptide.

To examine specific internalization of fDT-2 in live cells, VSMCs were incubated in 2 microM fDT-2 in serum-free media for 1, 4 or 18 h at 37 oC. After fDT-2 treatment, a nuclear stain was performed using DRAQ5 (5 microM, 20 min; Biostatus ltd., Leicestershire, UK) diluted in PBS. Coverslips containing the treated cells were mounted on slides using Aqua Poly/Mount (polysciences, Warrington, PA). Imaging was performed using a BioRad MRC 1024ES confocal microscope. Fluorescein and DRAQ5 nuclear staining excitation was imaged using 488 nm and 647 nm settings, respectively.

Posterior cerebral arteries dissected from rat brains were incubated with 5 microM fDT-2 in serum-free DMEM and placed in an incubator at 37 °C and 5% CO2 for 3 to 90 min. Nuclear staining was performed using TOTO-3 iodide (2 microM, 20 minutes; Molecular Probes) and imaged using 647 nm settings. Arteries were washed 5 times in PBS before imaging. All experiments were performed at least three times. Average slice thickness was approximately 1.0 micrometer. Single optical sections were acquired with 3 Kalman averages.

3.7. Actin Staining and Endocytosis Inhibition

VSMCs grown on glass coverslips were treated with different concentrations of endocytosis inhibitors for 4 h. After treatment, cells were washed twice with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature (RT). Thereafter, cells were permeabilized using PBS containing 0.1% Triton X-100 for 20 min. Nonspecific binding was blocked using PBS containing 3% dehydrated skim milk and 0.1% Triton X-100 for 1 h at RT. The cells were incubated with monoclonal smooth muscle alpha-actin antibody (1:500, 2 h; Sigma) followed by a fluorescein-conjugated anti-mouse secondary antibody (1:1000, 1 h; Molecular Probes). Nuclear staining was performed with DRAQ 5 before imaging. Uptake of fDT-2 was investigated after pretreatment with the endocytosis inhibitors: 5 and 50 microM phenylarsine oxide (PAO), 0.5 and 5 microM cytochalasin-D and 0.5 and 5 microM latrunculin-A (Sigma). After 10 min incubations with various inhibitor concentrations, 2 microM fDT-2 was added and cells were imaged as described above. Promidium iodide (PI) was added to cells 20 min before imaging in order to identify any toxic effects of endocytosis inhibitors which could affect fDT-2 uptake. PI staining was demonstrated in cells killed via 5 second microwave heating (data not shown). Each experiment was performed at least three times.

4. RESULTS

4.1. DT-2 cytotoxicity

To assess the cellular uptake and intracellular distribution of DT-2 we employed the fluorescein-labeled derivative fDT-2 (see Materials and Methods). We have previously shown that DT-2 and fDT-2 have identical PKG inhibitory and membrane translocating properties (14-15). However, the cellular toxicity of DT-2 and fDT-2 has previously not been determined. Cell viability could affect uptake or distribution of fDT-2, thus trypan-blue was used to assess the vitality of DT-2 and fDT-2-treated cells. Figure 1 shows the cytotoxicity of DT-2 and fDT-2 on vascular smooth muscle cells (VSMCs). When incubated for 4 h with concentrations ranging from 0.1 to 100 microM, cell death was negligible for both DT-2 and fDT-2. Although 20% toxicity was seen in cells treated with fDT-2, there was no concentration dependent toxicity observed, suggesting that VSMCs have a moderately high

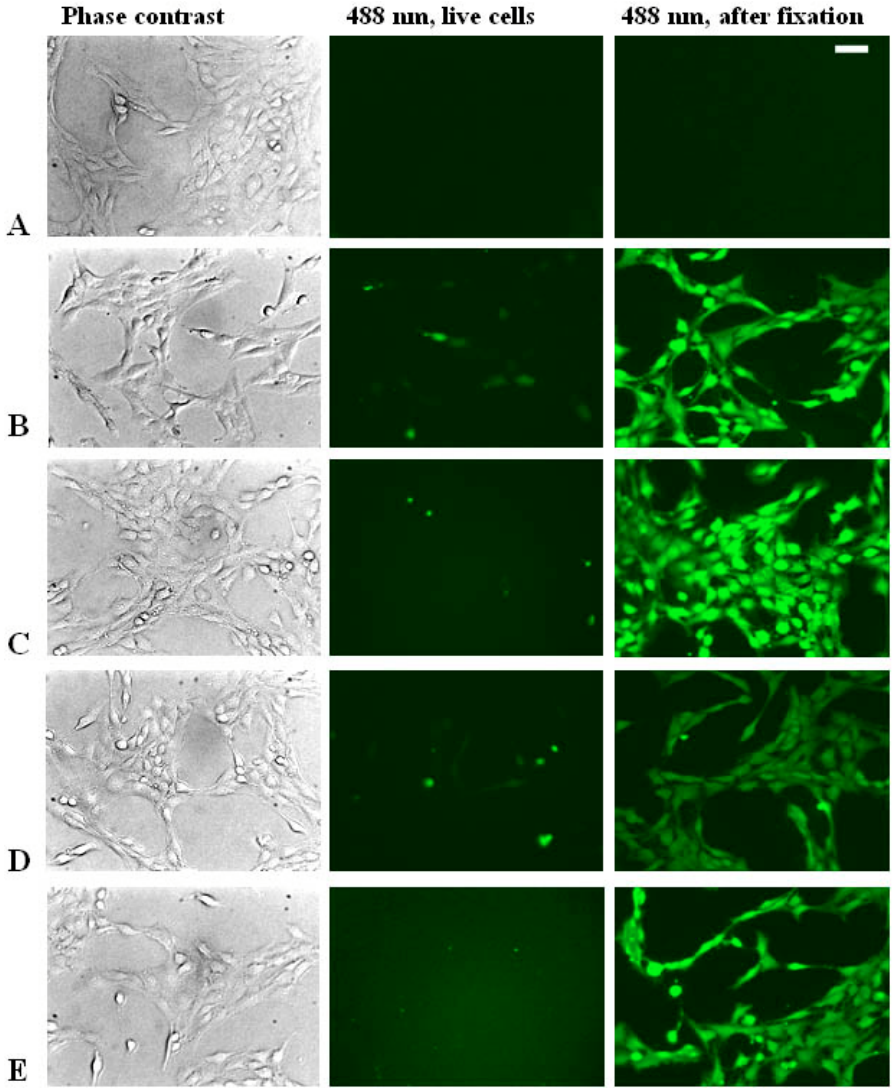

Figure 2. Effects of fixation on fluorescent-peptide internalization. C6-glial cells (phase contrast, left column) were incubated with vehicle (A), 2 microM of fluorescein-labeled peptides fDT-2 (B), fDT-3 (D) and the control fluorescein-labeled peptides fTat (C) and fAnt (E) for 2 h. Fluorescence intensity of the cells was followed at 488 nm in the absence (middle column) and presence of methanol fixation (right column).

percentage of unviable cells present but that this lethality is not significantly increased with higher concentrations of fDT-2 or DT-2.

4.2. Effects of fixation on DT-2 uptake

Next, we employed fDT-2 to study cellular uptake patterns in a variety of cell types. Initial experiments suggested that peptide internalization was cell type dependent. However, fixation of mammalian cells using alcohol-based fixatives caused dramatic increases in fluorescence over and above that of live cells. This fixation artifact has also been reported by others (18). Figure 2 demonstrates the effect of methanol fixation on C6 glial cells treated with a variety of fluorescein-labeled peptides. Near confluent cells (left column) were incubated with the fluorescein labeled peptides fDT-2, fDT-3 and their corresponding MPP carriers fTat and fAnt (Figure 2 B-E) and illuminated at 488 nm (middle column). Moderate peptide uptake was observed for all peptides after 30 min incubations; however fluorescence was dramatically increased after fixation. Fixation of the cells under identical capture settings with 95% methanol increased fluorescence instantly and peptides were identified in both cytoplasmic and nuclear spaces (right column). This fixation effect was also seen with ethanol, isopropyl alcohol and paraformaldehyde on a variety of other mammalian cell types including vascular smooth muscle cells (VSMCs) and HEK293 cells (data not shown). As a consequence, all future fDT-2 imaging experiments were performed only on live, unfixed cells.

4.3. Uptake and cellular localization of fDT-2 in two-dimensional cultured VSMCs

To further investigate the cellular uptake patterns of fDT-2 we used primary, dissociated, early passage cultured vascular smooth muscle cells (VSMCs) from rat

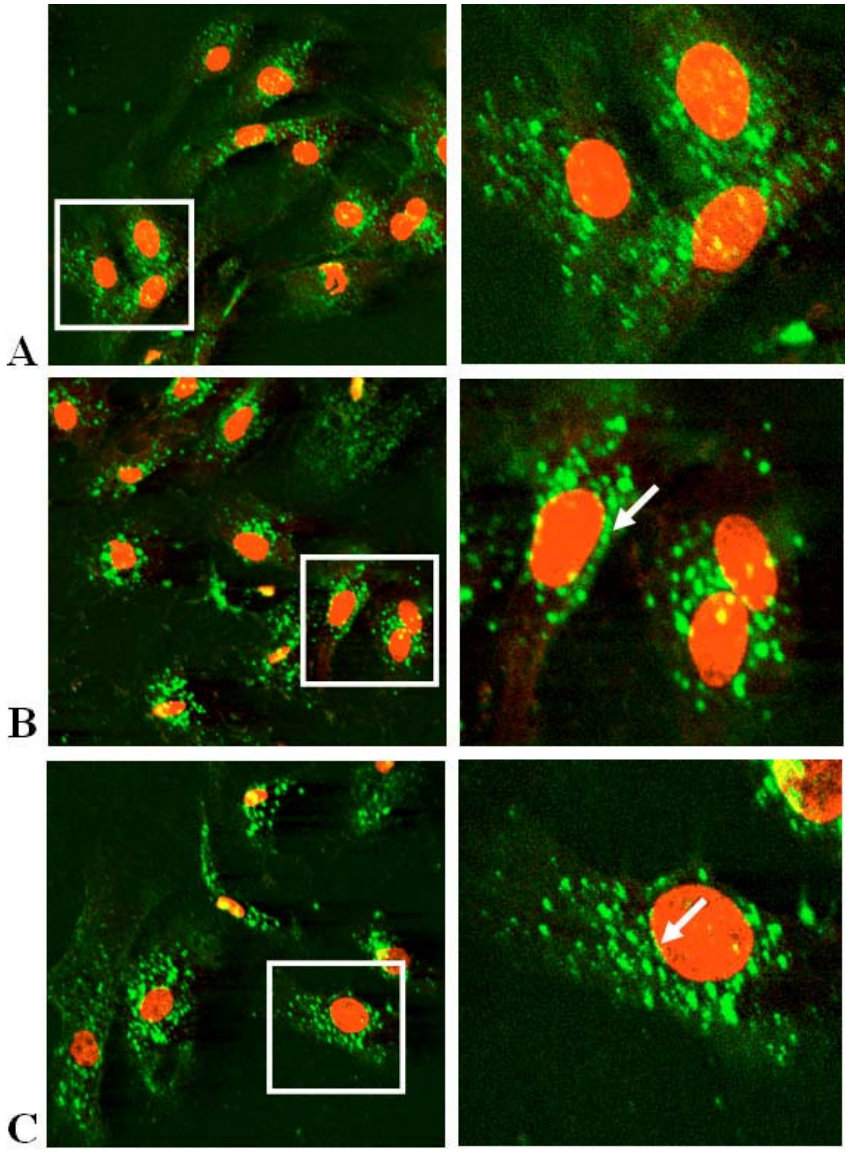

Figure 3. Uptake of fDT-2 in two-dimensional cultured VSMCs.. Cells were isolated from adult rat aorta and cultured on glass cover slides as described in Materials and Methods. Cells were incubated with 2 □M fDT-2 (green) for 1 hour (A), 4 hours (B) and 18 hours (C). Nuclear staining was performed using DRAQ5 (red). Insets are magnified at the right column. Arrows show peri-nuclear staining. Scale bar = 50 micrometers.

aorta. VSMCs represent an ideal cellular model system to study the kinetic effects of PKG inhibitors such as DT-2 and the effects of cell culture conditions on phenotype modulation (14, 19). VSMCs kept in conventional culture, immediately adopt an undifferentiated, secretory or synthetic phenotype in the presence of serum-derived growth factors (20-22). Figure 3 shows the peptide uptake patterns of cultured VSMCs incubated with 2 microM fDT-2 for one, four and 18 h. Peptide translocation appeared punctate and showed nuclear exclusion. In these undifferentiated cells the uptake distribution suggested fDT-2 was enclosed within vesicles and distributed throughout the cytoplasm. This distribution pattern is characteristic of an endocytotic process. In addition, the uptake did not appear to be time-dependent. For each time point, identical punctate distribution patterns were observed when regions were viewed more closely (insets in Figure 3). Although some peri-nucelar staining was apparent (arrows in Figure 3) no nuclear staining could be observed.

4.4. Effects of endocytosis inhibitors on fDT-2 uptake

To examine the affect of actin-dependent endocytosis on fDT-2 uptake, cultured VSMCs were treated with different endocytosis inhibitors (Figure 4). First, we investigated the effects of endocytosis inhibitors on the actin structure. Figure 4, left columns shows the actin-disrupting effects of Phenylarsine Oxide (PAO), Cytochalasin-D and Latrunculin-A on VSMCs, immunostained for alpha smooth muscle actin. No differences in alpha smooth muscle actin structure were

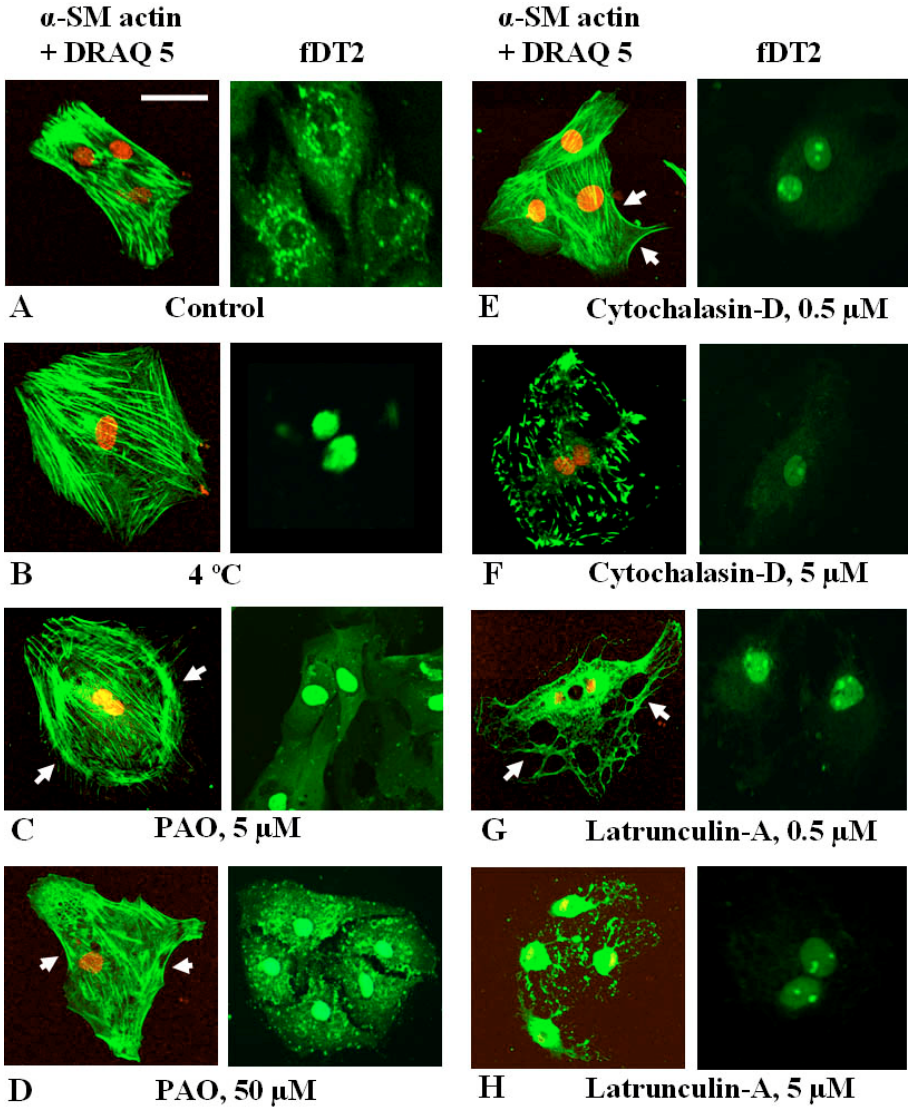

Figure 4. Effects of endocytosis inhibitors on actin and fDT-2 uptake. VSMCs were treated with PBS (A), 5 microM and 50 microM phenyl arsine oxide (PAO) (C, D), 0.5 microM and 5 microM cytochalasin-D (E, F), and 0.5 microM and 5 microM latrunculin-A (G, H) for 4 h at 37oC, or with PBS for 1 h at 4ºC (B). Cells shown in the left columns were fixed and immuno-stained for smooth muscle alpha-actin (green) and nuclei were stained using DRAQ 5 (red). In parallel experiments cells were incubated in the presence of 2 microM fDT-2 (right columns). Arrows indicate moderate actin filament disorganization at different endocytosis inhibitor concentrations. Scale bar = 50 micrometers.

observed in cells pretreated with fDT2 (10microM; 60 min) compared with untreated cells (data not shown). Next, the effects of these inhibitors on fDT-2 uptake on unfixed cells were investigated (right columns, Figure 4). Cells were pretreated with different concentrations of PAO, Cytochalasin-D or Latrunculin-A for 10 min. After pretreatment, cells were incubated with 2 microM fDT-2. Since the viability of the cell could presumably affect the uptake distribution, promidium iodide (PI) was also added to asses the viability of cells after treatment. Only cells which did not contain PI were imaged (data not shown).

Figure 4 A shows actin arrangement and fDT-2 localization in untreated VSMCs. Although endocytosis is temperature dependent, no change in actin structure was observed when cells were incubated at 4°C (Figure 4 B). However the distribution of fDT-2 after incubation at 4°C was markedly different than control. When VSMCs were incubated with 5 microM PAO, actin reorganization was visible in the form of reduced stress fibers (arrows). When 50 microM PAO was used, a general decrease in actin polymerization was noted (Figure 4 C, D). Both concentrations of PAO affected fDT-2 localization. Unlike control cells (Figure 4 A and Figure 3), fDT-2 was found within the nucleus after PAO treatment (Figure 4 C, D). A concentration of 0.5 microM cytochalasin-D resulted in only minor actin filament disorganization, whereas 5 microM completely blocked actin polymerization (Figure 4

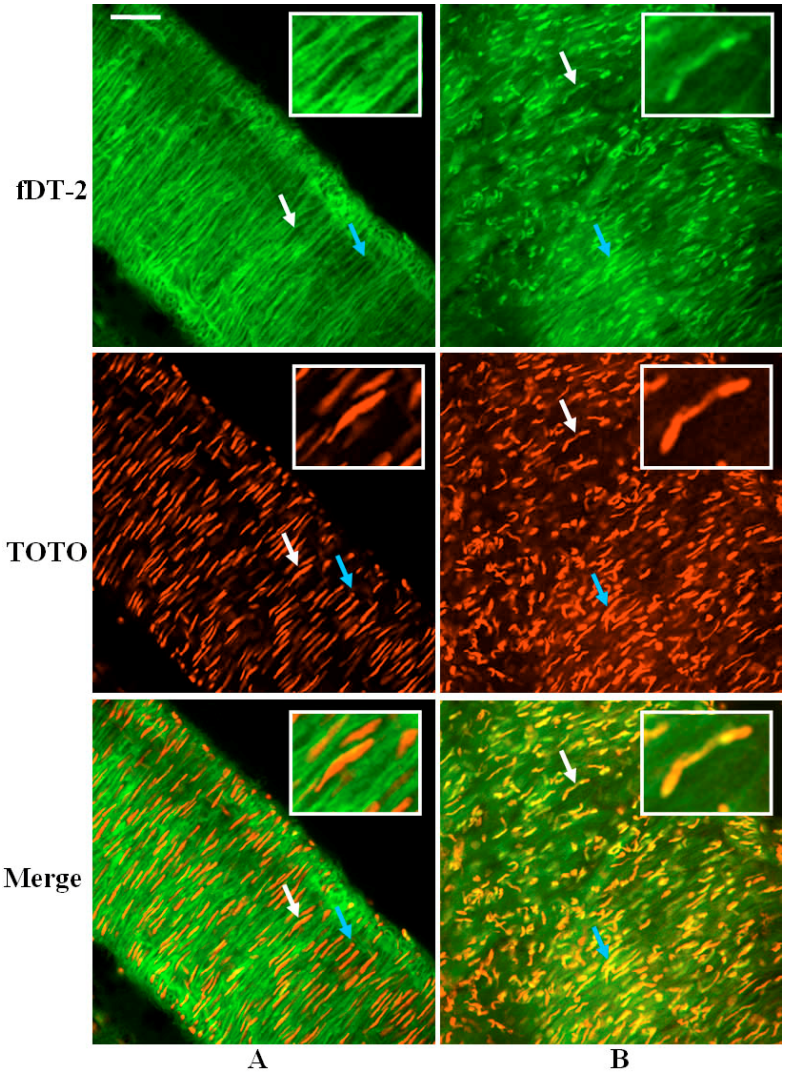

Figure 5. Uptake of fDT-2 in intact cerebral arteries. Posterior cerebral arteries from rat brains were incubated with 5microM fDT-2 (green) for 10 min (A) or 90 min (B). Nuclear staining performed using TOTO-3 (red). Arrows in panel A depict primary cytosolic DT-2 localization (merged image). White and blue arrows represent examples of cytosolic staining and nuclear translocation (merged image) in panel B, respectively. Insets: magnification of areas specified by white arrows. Scale bar = 50 micrometers.

E, F). Latrunculin-A (0.5 microM and 5 microM) also disrupted the actin cytoskeleton at concentrations of 0.5 and 5 microM (Figure 4 G, H). Similarly, fDT-2 localization shifted from the cytoplasm into the nuclei of cells after treatment with these agents. It appears that nuclear localization of fDT-2 in VSMCs only occurred when endocytosis was disrupted.

4.5. Peptide distribution in VSMCs from intact arteries

To examine uptake characteristics of fDT-2 in a physiologically significant environment, whole cerebral arteries were incubated with fDT-2 (Figure 5). In contrast to VSMCs grow in culture dishes, arteries incubated with fDT-2 showed nuclear localization of the peptide. In addition, the uptake and subsequent cellular localization of fDT-2 also appeared to be time dependent, with the peptide being targeted to the nucleus after longer incubations with the peptide. Arteries incubated with fDT-2 at early time points (10 min., Figure 5A) showed an accumulation of fluorescence occurring mainly at the cytoplasm. Nuclear staining using TOTO-3 revealed little or no co-localization of the dyes. In contrast, longer incubation periods of 1.5 h and longer (not shown) demonstrated an overlap of cytosolic (blue arrows) and nuclear (white arrows) fDT-2 localization (Figure 5B). Compared with the distribution of peptide in VSMCs grown in culture dishes (Figure 3), fDT-2 fluorescence was not punctuate but rather, appeared highly diffuse throughout the cytosol. This result suggests a non-endocytosis dependent mechanism of internalization. Consequently, differences in the cellular environment, including effects on cell morphology appear to be strong determinants on the uptake distribution pattern of membrane permeable peptides.

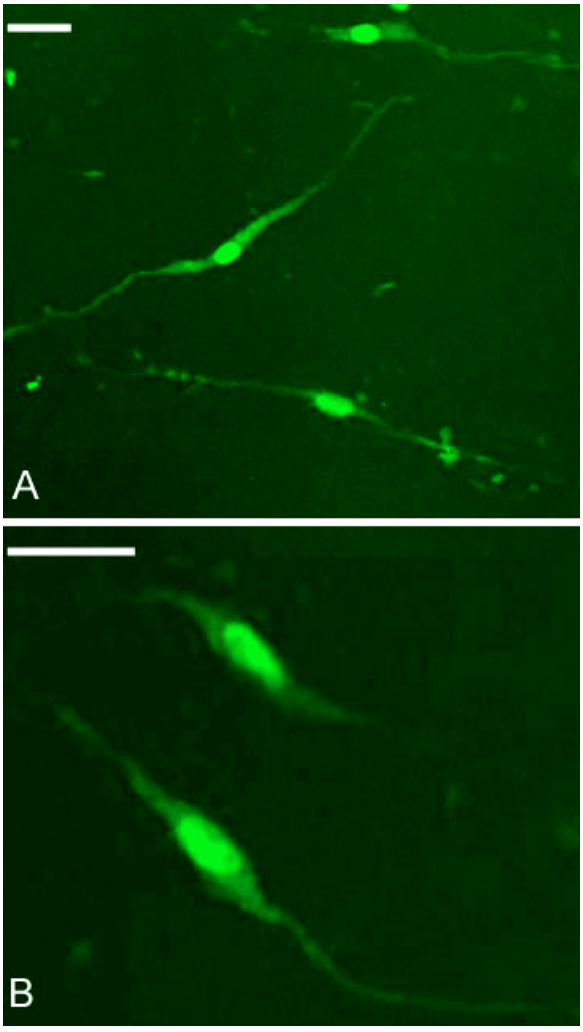

Figure 6. Uptake of fDT-2 in three-dimensional cultured VSMCs. Cells were reconstituted in fibrillar collagen matrixes, incubated with 5 microM fDT-2 for 1.5 h, and were subsequently transferred to glass cover slides as described in Materials and Methods. Whole cell peptide uptake and cell morphology is depicted in (A). Subcellular and nuclear staining is demonstrated in (B). Scale bar = 50 micrometers.

4.6. VSMCs reconstituted in three-dimensional matrices and fDT-2 uptake

In order to elucidate the possible effect of cellular morphology and phenotype on peptide uptake, freshly dissociated VSMSc were seeded in fibrillar collagen matrices and allowed to grow. In contrast to VSMCs cultured on dishes, VSMCs kept in fibrillar collagen immediately adopted a contractile, spindle shape and differentiated phenotype (Figure 6). In addition, Figure 6 demonstrates that cells kept in the context of a three-dimensional culture space had fDT-2 uptake characteristics similar to that of VSMCs in intact cerebral arteries. Moreover, unlike cells grown on culture dishes, VSMCs grown in collagen displayed non-punctate fDT-2 fluorescence and a more diffuse cytoplasmic distribution. Similar to VSMCs in the media of intact cerebral arteries, fDT-2 was present in nuclear and cytosolic spaces of cells grown in collagen. However as Figure 6 B indicates, nuclear fDT-2 translocation differed from intact tissue preparations in the staining of sub-nuclear particles (nucleoli). In order to ensure the cells growing in collagen matrices were completely embedded in a 3-D matrix, any cells growing on the surface of the collagen were removed by brief trypsinization prior to treatment with fDT-2.

5. DISCUSSION

The finding that vascular smooth muscle cells (VSMCs) internalize MPPs (DT-2) by different mechanisms depending on their phenotype and culture conditions is a novel observation. The initial observations on the dramatic uptake and distribution of fDT-2 in fixed cells, led to investigations on the effects of fixation as well as the inherent toxicity of the peptides fDT-2 and DT-2 (Figures 1 and 2). Our findings are consistent with those reported by others concerning artificial cellular import of fusion proteins during fixation (18, 23, 24). After VSMCs grown in conventional culture were fixed they displayed a large increase in fluorescence, likely due to the ability of the positively charged peptide to rapidly diffuse through the disrupted cell membrane and bind to the nuclear envelope or chromosomes. Alternatively, the intense nuclear uptake of fDT-2 in fixed cells could be due to the ability of non-vesicularized fDT-2 to translocate across nuclear pores. Furthermore, the nuclear accumulation appears consistent with studies of fluorescently-labeled Tat, which contains a nuclear localization sequence and subsequent RNA binding activity (3). The dramatic observations of fDT-2 uptake in fixed cells (Figure 2) led to the inquiry of the inherent toxicity of both DT-2 and fDT-2 (Figure 1). The use of trypan blue staining showed that both DT-2 and fDT-2 were non-toxic, even when used at high concentrations. These results confirm that toxic effects of DT-2 played no role in previously published reports concerning the actions of DT-2 on cells and tissues (15-16) and further supports the use of DT-2 *in vivo*.

The punctuate uptake of fDT-2 in cells grown in conventional culture (Figure 3) led to the conclusion that fDT-2 may be internalized via clathrin-dependent endocytosis (25). In addition, fDT-2 uptake by cells grown in 2-D culture showed nuclear exclusion, indicating that the peptide was unable to enter the nucleus. Interestingly, confocal images of rat aortic VSMCs grown in 2-D culture, showed that PKG itself appears to be restricted to vesicle-like structures (26). Thus it is possible that specific intracellular binding of non-vesicular fDT-2 to PKG could also result in punctuate fluorescence.

To further elucidate if the mechanism of fDT-2 translocation was endocytosis dependent, various inhibitors of endocytosis were employed. Since actin polymerization is essential for endocytic transport (27, 28) fDT-2 uptake was examined in the presence of several actin specific endocytosis inhibitors. The concentrations and incubation times used for these inhibitors were in accordance with previous studies (29). Cytochalasin-D prevents actin

polymerization (30) and Latrunculin-A sequesters G-actin (31). A third endocytosis inhibitor, phenylarsine oxide (PAO), was also used. PAO is a protein-tyrosine phosphatase inhibitor known to inhibit actin polymerization (32). In addition to these agents, incubation at 4^{o}C was also used since endocytosis is temperature dependent (33). Figure 4 shows the effect of Cytochalasin-D, Latrunculin-A and PAO on fDT-2 internalization. All agents caused a loss of punctate cytosolic fDT-2. In addition, one hour incubation of fDT-2 at 4^{o}C also showed the same effect. In the case of cells treated with cytochalasin-D and latrunculin-A, the fDT-2 uptake-inhibition seems to be dose dependent, with an almost complete lack of fDT-2 in the cytosol. The PAO treatment only partially disrupted fDT-2 uptake. As shown in Figure 4, different concentrations of PAO do appear to alter actin organization. The weaker effect of PAO on fDT-2 uptake may be due to the more complex mechanism of actin inhibition caused by this phosphatase inhibitor.

When membrane dependent uptake of fDT-2 is disrupted, the peptides may enter the cell via a different mechanism. In cells where endocytosis has been disrupted, fDT-2 is present within the nucleus. This idea is supported by observations showing that peptides containing a nuclear localization sequence must be able to bind a nuclear import receptor in the cytoplasm (34). If the peptide is enclosed in a vesicle, it may be unable to bind to nuclear import receptors. Why both patterns of uptake (punctate uptake and nuclear staining) don't occur in the same cell is perplexing and requires further study. Although DT-2 has been shown to be active in intact cerebral arteries (16) and VSMCs grown in conventional culture (14) the specific molecular mechanism and subcellular trafficking of DT-2 has so far remained elusive. Surprisingly, fDT-2 uptake varied greatly in VSMCs enclosed in the media of intact arteries compared with peptide uptake by cells grown in 2-D culture. Although MPPs have been used in whole-animal studies, to our knowledge, relatively few studies exist in which MPPs were evaluated in intact tissue samples. In smooth muscle cells enclosed in the media of whole cerebral arteries, peptide fluorescence appeared non-endocytotic, however the time dependency of internalization (Figure 5) clearly suggests an endocytotic component of peptide internalization. We are currently investigating the functional ability of DT-2 to inhibit downstream targets of PKG in VSMCs in conventional cell culture and intact arteries. Conversely, the targeting of fDT-2 to the nucleus of VSMCs over time is consistent with a non-endocytosis mechanism of fDT-2 internalization.

Similar to differentiated VSMCs located within the media of intact arteries, VSMCs grown in 3-D fibrillar collagen matrices showed diffuse cytosolic uptake and nuclear localization of fDT-2 (Figure 6). Since actin polymerization is essential for endocytotic transport (27, 28) it is likely that changes in the cytoskeleton could have effects on endocytosis. The regulation of the cytoskeleton is critical to normal cell function. When enzymatically isolated from tissues, VSMCs lose their normal extracellular surrounding and subsequently, their original filament architecture. Conversely, enzymatically dissociated VSMCs can maintain similar filament architecture to cells *in vivo*, when they are maintained in 3-D extracellular matrix culture (35). Accordingly, we have found that uptake by VSMCs enclosed in the media of intact arteries and those grown in 3-D extracellular matrices ECM show similar characteristics in the uptake and localization of fDT-2.

Our results support an endocytosis-independent mechanism of DT-2 internalization in differentiated smooth muscle cells. To inhibit PKG, DT-2 must be able to bind the substrate binding site of PKG. Previously published data showing the effects of DT-2 on PKG-dependant cellular processes within living cells and tissues (16) suggest that this peptide is available to bind to PKG after translocation into the cell. Furthermore, the rapid actions by which DT-2 elicits vasoconstriction in cerebral arteries (16) further support the hypothesis of an endocytosis-independent mechanism of DT-2 internalization in differentiated smooth muscle cells.

Taken together, these data demonstrate that both endocytotic and nonendocytotic uptake pathways are involved in the internalization of the cell penetrating peptide fDT-2 in live cells. Moreover, cellular orientation and the presence of a 3-D matrix are strong determinants in the uptake and cellular localization of the cell penetrating peptide fDT-2. These results provide important implications for the study of other cationic membrane permeable peptides.

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