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Membrane Transduction of Oligoarginine in HeLa Cells Is Not Mediated by Macropinocytosis

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Abstract: The mechanism in which small cationic oligopeptides are able to reach the cytosol of cells is controversial. Macropinocytosis has been recently suggested as a major mechanism for internalization of these peptides. In this report, the involvement of macropinocytosis on cytosolic localization of oligoarginine was quantitatively investigated in HeLa cells. Using a method which allows for the separate measurement of cytosolic versus vesicular oligopeptide, the results show that neither macropinosome nor filopodia formation correlates with cytosolic delivery of oligoarginine. Additionally, unlike macropinocytosis, the cytosolic delivery of oligoarginine was not inhibited by incubation at 16 °C, or by treatment with amiloride. Oligoarginine treatment does not contribute to leakage from endocytic vesicles, indicating the lack of endosomolytic properties. Finally, the amount of oligoarginine found in the cytosol was not substantially increased after coincubation with EGF, a known stimulator of macropinocytosis. Taken together, these data indicate that membrane transduction of oligoarginine occurs separately from macropinocytosis in HeLa cells.

Keywords: Membrane transduction; cationic peptides; oligoarginine; macropinocytosis; quantitative measurement

Introduction

Short sequences of cationic amino acids known as cell penetrating peptides, CPPs[†], or membrane transduction peptides, MTPs, have been shown to have the unique ability to facilitate the delivery of macromolecules into the cytosol of cells. This direct transport across cellular membranes, termed "membrane transduction", is a highly controversial subject. The mechanism in which MTPs gain access to the cytosolic compartment of cells is still unknown. There have been several publications which suggest that "membrane transduction" does not exist, and that these MTPs are merely entering the cells through an endocytic mechanism with subsequent release into the cytosol. These recent studies have implicated the involvement of several different types of endocytosis, including macropinocytosis, in the internalization of MTPs with varying results.[1−7]

Macropinocytosis is a type of endocytic mechanism which results in the formation of large endocytic vesicles (0.2−3

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μM), or macropinosomes, by the closure of lamellipodia and ruffling membranes. Macropinocytosis occurs as a constitutive process in macrophages and in some tumor cell lines, or as an inducible process in other cell lines such as epithelial cells, fibroblasts, and neutrophils by treatment with growth factors or mitogenic agents (reviewed in ref 8). In human cervical carcinoma cells (HeLa), for example, macropinosome formation is stimulated by treatment with epidermal growth factor (EGF), resulting in an increased uptake of fluid phase markers.[9,10] In this paper, macropinocytosis and membrane transduction were measured separately in HeLa cells to determine the involvement of these two cellular processes by using a previously described quantitative method.[11,12]

Experimental Section

Peptide Synthesis and Labeling. $YG(R)_9$ and $YG(K)_9$ were synthesized by Genemed (South San Francisco, CA). The tyrosine moiety was labeled with $Na^{125}I$ (ICN, Irvine, CA) using the Chloramine-T method.[13] The labeled oligopeptides were purified by size exclusion chromatography using Sephadex G-15 (Sigma, St. Louis, MO) gel matrix. The specific radioactivity for the radioiodinated $YG(R)_9$ and $YG(K)_9$ was 1.0×10^5 and 1.7×10^5 cpm/μg, respectively.

Cell Culture. Human cervix carcinoma cells (HeLa) (ATCC, Manassas, VA) were grown to confluence in Dulbecco's modified minimum essential medium (DMEM) (GIBCO-BRL, Carlsbad, CA) containing 10% fetal bovine serum. The cells were incubated at 37 °C, 5% CO_2, and replenished with fresh medium the day before confluence, at which time the assays were performed.

Cytosolic Localization Assays. The measurement of cytosolic localization of MTPs was performed with a previously reported procedure.[11] Briefly, confluent HeLa monolayers grown in T75 flasks (Corning, Acton, MA) were incubated in serum-free medium containing 5 μg/mL of ^{125}I-labeled oligopeptide, 0.1 mg/mL FITC-dextran (70 kDa) (FD) (Sigma), and protease inhibitor cocktail (PI) (Sigma). After treatment for 15 min at either 16 or 37 °C, the monolayers were washed three times with cold PBS and detached by treatment with trypsin-EDTA at 37 °C for 5 min, and the isolated cell pellets were washed with 0.5 mg/mL heparin-PBS followed by PBS. The cell pellets were then homogenized in buffer (HB) containing 0.25 M sucrose, 2 mM EDTA, and 10 mM HEPES, pH 7.4 using a Balch cell press (H & Y Enterprises, Redwood City, CA).[14] The cell homogenate was centrifuged at 600 g at 4 °C for 10 min, and the postnuclear supernatant was fractionated using Sephacryl S-500 (Amersham, Piscataway, NJ) size exclusion chromatography (1 × 13 cm column dimensions) with HB as the eluting buffer. One milliliter fractions were collected and assayed for ^{125}I-oligopeptides using a Gamma counter (Packard, Downers Grove, IL), for FD using fluorescence spectroscopy (Hitachi, Tokyo, Japan) (Ex 494 nm Em 519 nm), and for protein content using the Pierce protein assay kit. The amount of oligopeptide internalized by endocytosis versus transduction was calculated using the equations previously described.[11] For the macropinocytosis inhibitor assays, the localization assay method was performed after pretreatment of HeLa cell monolayers at 37 °C with serum-free medium containing 100 μM amiloride for 30 min, followed by a 15 min coincubation with ^{125}I-oligopeptide and FD and the respective inhibitors. The localization assay method was also repeated by treating HeLa cell monolayers at 37 °C with serum-free medium in the presence and absence of 100 ng/mL EGF coincubated with ^{125}I-oligopeptide and FD. For measurement of total FD internalization, HeLa cell monolayers grown in 6-well culture plates (Corning, Acton, MA) were incubated in serum-free medium containing 5 μg/mL oligopeptide and 1 mg/mL FITC-dextran (70 kDa) for 15 min at 37 °C. The cell pellets were washed with PBS, isolated following treatment with trypsin-EDTA, washed with PBS, and dissolved in 0.4% Triton X-100. The amount of FD internalized was measured by fluorescence spectroscopy (Ex 494 nm Em 519 nm), and the total cell protein content was determined by the Pierce protein assay.

Induction of Macropinocytosis. Hela cells were incubated with 1 mg/mL FD in the presence or absence of 100 ng/mL epidermal growth factor (EGF), 5 μg/mL $YG(R)_9$, or 5 μg/mL $YG(K)_9$ at either at 16 or 37 °C, and macropinosome formation and F-actin staining was quantified by fluorescence microscopy, in which approximately 150−200 cells were counted in randomly selected fields. Cells were considered

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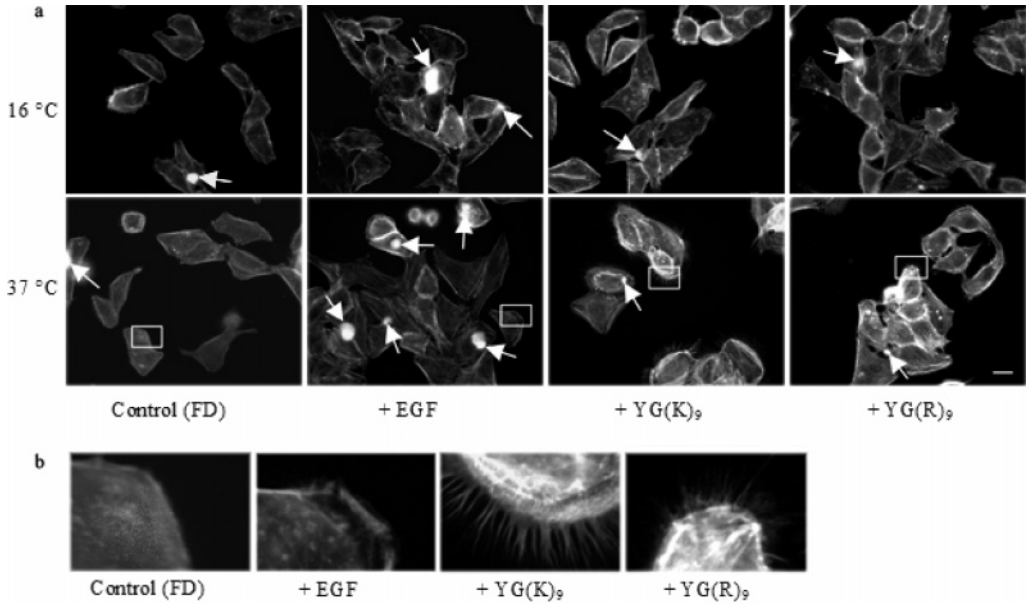

Figure 1. Investigation of macropinocytosis in HeLa cells. (a) HeLa cultured cell monolayers were incubated with 1 mg/mL FITC-dextran in the presence or absence of 100 ng/mL EGF, 5 μg/mL $YG(R)_9$, or 5 μg/mL $YG(K)_9$ for 15 min at 16 or 37 °C. Cells were fixed, stained for F-actin, and visualized by fluorescence microscopy. Arrows indicate macropinosomes; boxed regions indicate areas of filopodia formation; scale bar indicates 20 μm. (b) Filopodia formation in the presence or absence of EGF, $YG(K)_9$, and $YG(R)_9$. Enlarged images of boxed regions in panel a.

to be macropinosome-positive if they contained at least one FD filled, relatively large vesicle (0.2−5 μm in diameter).[15]

Assay of Endosomolytic Properties. The endosomolytic properties of oligoarginine were investigated by comparison of the amount of FD released from the vesicles into the cytosolic fraction (% RV), calculated in the presence and absence of 5 μg/mL $YG(R)_9$ or 100 ng/mL EGF.

Results

Induction of Macropinosome and Filopodia Formation in HeLa Cells. Macropinosome and filopodia formation was determined in HeLa cells following treatment with EGF as a positive control, $YG(R)_9$, and $YG(K)_9$ at 16 and 37 °C (Figure 1). The results presented in Figures 1 and 2 show that macropinosome formation is not increased in the presence of either oligoarginine or oligolysine. Additionally, treatment with both oligoarginine and oligolysine resulted in a significant increase in filopodia formation, at both 16 and 37 °C, while treatment with EGF resulted in only a slight increase in filopodia formation (Figures 1 and 3).

Effect of Inhibitors of Macropinocytosis on Transduction of Oligoarginine. The temperature sensitivity of macropinocytosis was investigated at 16 and 37 °C. The results shown in Figures 1 and 4 demonstrate that the induction of macropinosome formation by EGF was completely abolished at 16 °C in comparison to incubation at 37 °C. The internalization of oligoarginine via membrane transduction

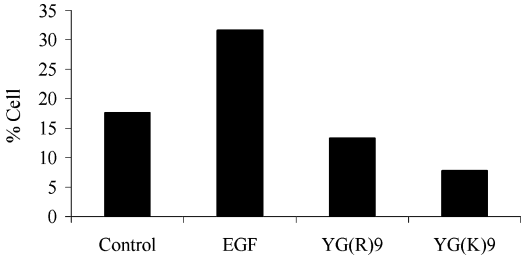

Figure 2. Stimulation of macropinosome formation. HeLa cultured cell monolayers were incubated with 1 mg/mL FITC-dextran in the presence or absence of 100 ng/mL EGF, 5 μg/mL $YG(R)_9$, or 5 μg/mL $YG(K)_9$ for 15 min at 37 °C. Cells were fixed, and macropinosome formation was quantified by fluorescence microscopy in which approximately 150−200 cells were counted in randomly selected fields. Cells were considered to be macropinosome-positive if they contained at least one FD filled, relatively large vesicle (0.2−5 μm in diameter).

was measured at 16 and 37 °C. In contrast to the results obtained for macropinocytosis of EGF, membrane transduction of oligoarginine is not inhibited by incubation at 16 °C. As expected, the endocytosis of oligoarginine at 16 °C is inhibited as compared to the 37 °C control by 75% (Figure 5). Additionally, the effect of the specific macropinocytosis inhibitor, amiloride, on oligoarginine transduction was determined. As seen in Table 1, treatment with amiloride did not inhibit the transduction of oligoarginine, while endocytosis was inhibited by 40%. As a positive control, the stimulation of internalization of FD by EGF was shown to be inhibited in the presence of amiloride (Table 2).

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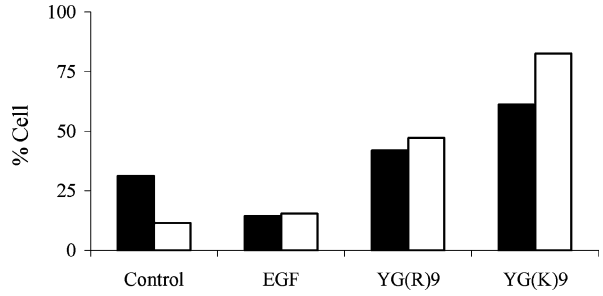

Figure 3. Stimulation of filopodia formation. HeLa cultured cell monolayers were incubated with 1 mg/mL FITC-dextran in the presence or absence of 100 ng/mL EGF, 5 μg/mL YG-$(R)_9$, or 5 μg/mL YG$(K)_9$ for 15 min at 16 °C (closed bars) or 37 °C (open bars). Cells were fixed and stained for F-actin, and filopodia formation was quantified by fluorescence microscopy.

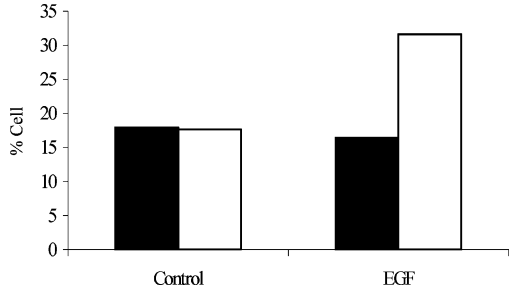

Figure 4. Temperature sensitivity of macropinocytosis. HeLa cultured cell monolayers were incubated with 1 mg/mL FITC-dextran in the presence or absence of 100 ng/mL EGF for 15 min at 16 °C (closed bars) or 37 °C (open bars). Cells were fixed, and macropinosome formation was quantified by fluorescence microscopy.

Effect of EGF on Oligoarginine Transduction. Since EGF is a stimulator of macropinocytosis, it is conceivable that coincubation with MTPs would increase transduction if it involved macropinosome formation. Therefore, HeLa cells were treated with ^{125}I-YG$(R)_9$ in the presence and absence of EGF, and the amount internalized by membrane transduction and endocytosis was determined. As shown in Figure 6, treatment with EGF does not greatly increase transduction of YG$(R)_9$.

Internalization of FITC-Dextran in the Presence and Absence of Oligoarginine. Since induction of macropinocytosis results in the increase of internalization of fluid-phase markers, the internalization of FITC-dextran in the presence and absence of oligoarginine was investigated in HeLa cells. As shown in Table 2, treatment with oligoarginine does not increase the internalization of FITC-dextran. Additionally, the involvement of macropinocytosis in the cytosolic localization of MTPs requires that the MTP be released from the macropinosome, which should also result in the corelease of the fluid-phase marker when coincubated. Therefore, the endosomolytic property of oligoarginine was investigated by the comparison of the elution profiles of FITC-dextran (FD) in the presence and absence of oligoarginine. The amount of FD retained in the vesicles (% RV) was calculated as described in the Experimental Section and compared after incubation in the presence and absence of 5 μg/mL YG$(R)_9$. The results presented in Table 2 demonstrate that the amount of FD located in the cytosolic fraction is similar in the

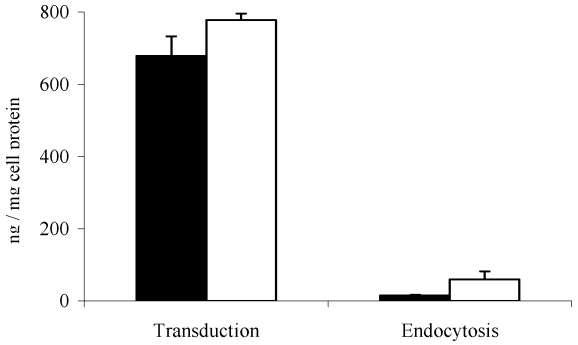

Figure 5. Temperature sensitivity of membrane transduction and endocytosis of YG$(R)_9$. HeLa cultured cell monolayers were incubated with serum-free medium containing FITC-dextran, 5 μg/mL ^{125}I-YG$(R)_9$, and protease-inhibitor cocktail at 16 °C (closed bars) or 37 °C (open bars) for 15 min. The cell monolayers were detached by treatment with trypsin, and the cell pellets were washed with heparin-containing PBS, followed by PBS, and homogenized using a Balch cell press, and the PNS was separated on an S-500 gel filtration column. The resultant fractions were assayed for radioactivity using a Gamma counter, and for fluorescence using a fluorescence spectrophotometer to determine amount internalized by endocytosis or transduction. Data represents mean ± standard deviation with $n = 3$.

Table 1. Effect of Amiloride on Internalization of YG$(R)_9$[a]

treatment	transduction (ng/mg cell protein)	endocytosis (ng/mg cell protein)
YG$(R)_9$, control	778 ± 18	60 ± 22
+ amiloride	939 ± 20	36 ± 3

[a] HeLa cells were incubated in serum-free medium containing either FITC-dextran (FD) and YG$(R)_9$ (control); or FD, YG$(R)_9$, and amiloride following pretreatment with amiloride. The amount of oligoarginine internalized by transduction and endocytosis was determined as described in the Experimental Section. Data is presented as average ± standard deviation with $n = 3$.

Table 2. Stimulation of Fluid-Phase Endocytosis and Investigation of Endosomolytic Properties

treatment	total FD (RFU/mg cell protein)[a]	% RV[b]
FITC-dextran, control	26.7 ± 2.0	40 ± 1
+ YG$(R)_9$	23.0 ± 6.5	42 ± 0
+ EGF	41.2 ± 6.0	44 ± 1
EGF + amiloride	29.6 ± 3.7	not measured

[a] HeLa cells were incubated in serum-free medium containing FITC-dextran (FD) (control); FD and YG$(R)_9$; FD and EGF; or EGF and amiloride following pretreatment with amiloride. The total amount of FD internalization was determined as described in the Experimental Section. Data is presented as average ± standard deviation with $n = 3$. [b] HeLa cells were incubated in serum-free medium containing 1 mg/mL FITC-dextran (FD) (control); FD and YG$(R)_9$; or FD and EGF for 15 min at 37 ° C. The amount of FD retained in vesicles (% RV) was calculated as described. Data is presented as average ± standard deviation with $n = 3$.

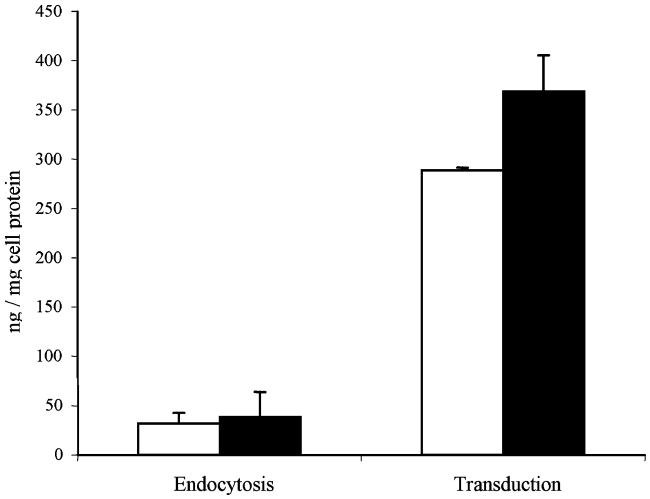

Figure 6. Effect of stimulation of macropinosome formation on membrane transduction of $YG(R)_9$. HeLa cultured cell monolayers were incubated with serum-free medium containing FITC-dextran, 5 μg/mL ^{125}I-$YG(R)_9$, and protease-inhibitor cocktail in the absence (closed bars) and presence (open bars) of 100 ng/mL EGF at 37 °C for 15 min. The cell monolayers were detached by treatment with trypsin, and the cell pellets were washed with heparin-containing PBS, followed by PBS, and homogenized using a Balch cell press, and the PNS was separated on an S-500 gel filtration column. The resultant fractions were assayed for radioactivity using a Gamma counter, and for fluorescence using a fluorescence spectrophotometer to determine the amount internalized by endocytosis or transduction. Data represents mean ± standard deviation with $n = 3$.

presence and absence of oligoarginine, indicating that oligoarginine is not released into the cytosol due to endosomolytic properties. Additionally, % RV was also similar in the presence and absence of EGF, verifying that macropinosomes are not ruptured during the homogenization process.

Discussion

The stimulation of macropinocytosis by treatment with EGF was compared to oligoarginine and oligolysine at 16 and 37 °C following a 15 min incubation period. This incubation period was chosen since the stimulation of macropinocytosis is a rapid process.[8−10] Longer incubation times will result in the accumulation of FITC-dextran in the late endosomes and lysosomes. These organelles are of a size comparable to that of macropinosomes, thereby potentially confounding the identification of macropinocytic uptake structures from other endocytic vesicles. The results show that oligoarginine and oligolysine do not stimulate macropinosome formation in HeLa cells, and that the macropinosome formation induced by EGF in HeLa cells is completely abolished at 16 °C in comparison to the 37 °C control. This result is expected since membrane fusion is required for closing the membrane ruffle formed at the cell surface, and membrane fusion events are inhibited at this temperature.[16] Alternatively, it has been demonstrated in CHO cells that while endocytosis of $YG(R)_9$ is inhibited at 16 °C, the amount of oligoarginine delivered to the cytosol is not affected.[12] Therefore, the effect of incubation at 16 °C in HeLa cells was also determined, showing similar results. While membrane transduction is only slightly inhibited (12%), endocytosis is significantly decreased at 16 °C in comparison to the 37 °C control by 75%. The lack of inhibition of membrane transduction at 16 °C is a strong indication that this process does not occur through macropinosome formation.

In addition to macropinosome formation, the process of macropinocytosis can also be investigated by the formation of filopodia on the cell surface. Filopodia are F-actin enriched, dynamic protrusions formed at the surface of the cell, and are necessary for active ruffling and macropinosome organization in epithelial cells.[17] After treatment of HeLa cells with $YG(R)_9$ or $YG(K)_9$, the cells showed a significant increase in filopodia formation which was not inhibited at 16 °C as seen for macropinocytosis (Figures 1 and 3). Although the change in membrane protrusions following treatment with these oligopeptides was striking, the results indicate that it is not involved with membrane transduction via macropinocytosis. First, for $YG(R)_9$ and $YG(K)_9$, the filopodia formation did not result in formation of macropinosomes, and did not show similar temperature dependence similar to that shown by macropinosome formation. While stimulation of macropinosome formation was abolished at 16 °C, filopodia formation was only slightly inhibited (Figures 3 and 4). Additionally, it has previously been shown that while oligoarginine is preferentially internalized by transduction, oligolysine is primarily endocytosed in CHO cells.[11] Similar results were obtained when the transduction and endocytosis in HeLa cells were measured (data not shown). Therefore, membrane transduction does not correlate with filopodia formation since oligolysine, which is not efficiently transduced, shows an even greater stimulation of filopodia formation than oligoarginine (Figure 3). On the basis of these results, the filopodia formation induced by oligoarginine and oligolysine is more likely to be attributed to the typical cell-spreading characteristic seen when growing cells on polylysine-coated plates. The procedure to plate cells onto polylysine coated glass coverslips is common to allow for attachment. It has been shown that when cells are plated onto coated coverslips, the cells display irregular morphology with numerous filopodia-like extensions,[18] similar to those seen after treatment with the oligopeptides here. Additionally, although membrane protrusions and macropinocytosis are closely linked,[19,20] filopodia formation may be necessary, but

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not be sufficient, for macropinosome formation. For example, it has been suggested regarding the stimulation of macropinocytosis using growth factors, that the membrane ruffling events are a prerequisite for macropinosome formation, however, additional activities may be required for transformation of the membrane ruffles to closed intracellular vesicles. It is known that macrophage colony-stimulating factor (M-CSF) and phorbol myristate acetate (PMA) stimulate both ruffling and macropinocytosis in macrophages.[19,20] However, M-CSF or PMA-treated cells in the presence of wortmannin, an inhibitor of the catalytic subunit of mammalian phosphoinositide 3-kinase (PI 3-kinase), fail to close membrane ruffles into macropinosomes, indicating that PI 3-kinase is necessary for the completion of actin-dependent endocytosis, but not for membrane ruffling.[21] Similarly, it is possible that oligolysine and oligoarginine may initiate the initial step of filopodia formation, but not the subsequent signaling cascade required for the closure of the filopodia into the formation of intracellular vesicles.

The effects of known modulators of macropinocytosis on the membrane transduction of oligoarginine were further investigated. In agreement with many published findings,[9,10] macropinosome formation can be induced in HeLa cells by treatment with EGF (Figures 1 and 2). To determine if this increase in macropinocytosis can increase the amount of oligoarginine delivered to the cytosol of cells, cultured HeLa cell monolayers were treated with $YG(R)_9$ in the presence and absence of EGF, and the amount internalized by endocytosis and transduction was determined. As shown in Figure 6, the membrane transduction of oligoarginine is only slightly increased in the presence of EGF, indicating that stimulation of macropinosome formation cannot account for the majority of the cytosolically delivered oligoarginine. The presence of multiple pathways for MTPs to reach the cytosol may explain the slight increase in membrane transduction following treatment with EGF. For oligoarginine, the major pathway to reach the cytosol is via membrane transduction. However, different MTPs or the conjugation of cargo molecules may alter internalization, which may be an important consideration when investigating other macromolecules which may be preferentially internalized by alternate pathways.

Additionally, for oligoarginine to reach the cytosol via macropinocytosis, the oligopeptide would need to be released from the macropinosome following internalization. The possibility that the MTPs are entering the cytosol through endosomal rupture was investigated by the comparison of the elution profiles of the fluid-phase endocytosis marker, FD. The elution profile of the FD contains two peaks, the first being the amount of FD localized in the intracellular vesicles, and the second peak being the amount of FD that leaked into the soluble fraction due to vesicular rupture by shear forces during the homogenization process. The elution profile of FD in cell preparations containing endosomolytic agents would show a higher amount of FD localized in the cytosolic fraction. Therefore, the elution profiles of FD in the presence and absence of oligoarginine were compared. As shown in Table 2, the amount of FD retained in the vesicles is similar in the presence and absence of oligoarginine. The lack of increase in the amount of FD released to the cytosol in the presence of oligoarginine indicates that the MTP is not endosomolytic and does not reach this cytosol via this process. Finally, macropinocytosis can also be investigated by the response to drugs such as amiloride, an inhibitor of Na^+/H^+ exchange, that act on the intracellular pH.[22] However, the presence of this inhibitor did not decrease the membrane transduction of oligoarginine, while endocytosis was inhibited by 40%.

Taken together, the results presented in this report strongly indicate that macropinocytosis is not the major mechanism involved in the membrane transduction of oligoarginine in HeLa cells. These results are in agreement with previous findings that show that transduction and endocytosis are indeed two distinct processes of cellular entry.[12,23] However, the possibility that there are other minor pathways involved, such as macropinocytosis, in membrane transduction cannot be ruled out. These alternative pathways may become significant only if the direct transduction across the plasma membrane is hindered by factors such as the size, charge, and hydrophobicity of the cargo molecule that is conjugated to the MTP. In this case, a drastic reduction of membrane transduction of MTP should be detected.

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