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Zinc uptake into MCF-10A cells is inhibited by cholesterol depletion

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Abstract

The mechanism for cellular Zn uptake was investigated by depleting cell cholesterol levels, a treatment that disrupts lipid rafts/caveolae-dependent processes and inhibits coated-pit budding. Incubation of MCF-10A human breast epithelial cells with hydroxypropyl-β-cyclodextrin significantly lowered cell cholesterol levels and significantly inhibited cellular zinc uptake measured at 10 min, but had no effect on 2-deoxyglucose uptake. Replacing potassium for sodium in the uptake buffer significantly stimulated Zn uptake by 20%. The effects of potassium depletion and chlorpromazine on Zn uptake were investigated to determine the contribution of coated-pit endocytosis. Potassium depletion following hypotonic shock significantly inhibited Zn uptake into MCF-10A cells approximately 15%. Chlorpromazine at 20 μg/ml inhibited uptake approximately 30%. The data support the hypothesis that Zn uptake into MCF-10A cells involves lipid rafts/caveolae. The relatively mild effects of potassium depletion and chlorpromazine suggest that a small portion of Zn uptake may require coated pit endocytosis.

Keywords: Breast epithelium; Cholesterol; Endocytosis; Lipid rafts; Zinc transport

1. Introduction

Zn is a component of over 300 enzymes and proteins, serving in a structural, catalytic, or regulatory capacity [1]. In spite of its important role in cellular metabolism, the mechanism of zinc transport has not been fully resolved. The identification of several mammalian Zn uptake transporters has been important in advancing understanding of the cellular transport mechanism. Two of these transporters, human ZIP1 and 2, have been characterized further [2,3]. Both transporters increase Zn uptake in cell transfectants and compartmentalize to the plasma membrane of K562 erythroleukemia cells. However, human ZIP2 is not endogenously expressed in K562 cells [3] and human ZIP1, though endogenously expressed in K562 and other cell types [2,4], does not locate to the plasma membrane of the cells other than K562 [4]. Recently, the gene SLC39A4 has been implicated in the human Zn deficiency disease, acrodermatitis enteropathica [5]. This gene encodes a histidine-rich protein hZip4 and is located on the chromosomal region 8q24.3 [6]. The protein exhibits similarity to the other Zip Zn transporters and therefore may be involved in transmembrane Zn transport [5].

Cells use both coated pit-dependent and non-coated pit-dependent endocytosis to acquire extracellular ligands. Studies with endothelial cells indicate that albumin uptake, and Zn uptake from medium containing albumin, are inhibited by treatments that inhibit coated pit endocytosis [7]. The non-coated pit uptake mechanism utilizes lipid rafts and/or caveolae. Albumin receptors localize in caveolae [8–10]. Certain caveolae, upon internalization, have been observed to fuse with endosomes arising from clathrin-coated pits [10]. The role these mechanisms play in Zn uptake is not known, though treatments such as N-ethylmalemide or nystatin, which disrupt the non-coated pit pathway, also inhibit Zn uptake [11,12].

The purpose of this report is to test whether Zn transport is reduced in cells depleted of their cholesterol. The depletion of cell cholesterol disrupts lipid raft/caveolae formation

Abbreviations: CD, hydroxypropyl-β-cyclodextrin; 2-DG, 2-deoxyglucose; HGS, 10 mmol HEPES/L, 5.6 mmol glucose/L, 138 mmol NaCl/L, 7 mmol KCl/L, 1.3 mmol $CaCl_2$/L, pH 7.5; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid; ZIP, ZRT/IRT-related protein; hZIP2, human ZIP2; IRT, iron regulated transporter; ZRT, zinc regulated transporter.

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[13,14], and inhibits the formation of vesicles from clathrin-coated pits [15,16]. Hydroxypropyl-β-cyclodextrin was used to deplete plasma membrane cholesterol content. Our results indicate that reducing cholesterol levels inhibits Zn uptake, but not the uptake of 2-deoxyglucose, which does not require vesicle formation. The assembly of the coat protein clathrin at the site for coated pit formation is inhibited by potassium depletion [17,18]. This treatment inhibited Zn uptake slightly. The addition of chlorpromazine to the cells, which inhibits the Golgi transport and the recycling of receptors in the coated-pit endocytic pathway [19], slightly inhibited Zn uptake. The data presented here provide evidence for a predominant role for the non-coated pit pathway (lipid rafts/caveolae) in cellular Zn uptake, and indicate that a small portion of Zn uptake requires receptor recycling to a receptor-mediated endocytic compartment.

2. Materials and methods

MCF-10A cells were obtained from the American Type Culture Collection (Rockville, MD). Dulbecco's minimum essential medium, F12 (Ham) medium, insulin, hydrocortisone, and mevastatin were bought from Sigma (St. Louis, MO). Horse serum, cholera toxin, epidermal growth factor, penicillin, streptomycin, and amphotericin B were obtained from Gibco/Life Technologies (Rockville, MD). Hydroxypropyl-β-cyclodextrin (CD) was acquired from Aldrich Chemical Co. (Milwaukee, WI). Tissue culture flasks and dishes were purchased from Corning (Corning, NY). Carrier-free ^{65}Zn was bought from NEN Life Sciences (Boston, MA). ^{14}C-labeled 2-deoxyglucose (2DG) was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid (MTT) was acquired from Roche (Indianapolis, IN).

2.1. Cell culture procedures

The spontaneously immortalized MCF-10A breast epithelial cells originated from a patient with fibrocystic disease [20]. Cells were used for these experiments between passages 150 and 200. The cells were grown as a monolayer in Dulbecco's minimum essential medium/F12 (Ham) medium (1:1) supplemented with 5% horse serum and 10 mg insulin/L, 20 μg epidermal growth factor/L, 100 μg cholera toxin/L, 500 μg hydrocortisone/L, 100,000 U penicillin/L, 100 mg streptomycin/L, 250 μg amphotericin B/L. The cells were subcultured at confluency at a density of 2 x 10^5 cells/cm^2 every three to four days into 75 cm^2 flasks, or into 9.6 cm^2 or 3.8 cm^2 culture dishes for measuring uptake.

2.2. Time-dependent uptake of ^{65}Zn

MCF-10A cells were subcultured into 9.6 cm^2 culture dishes and allowed to grow in normal growth medium to confluence. The cells were then incubated for 4 h in serum-free medium. At 4 h, the cells were rinsed twice with 10 mmol HEPES/L, 5.6 mmol glucose/L, 138 mmol NaCl/L, 7 mmol KCl/L, 1.3 mmol $CaCl_2$/L, pH 7.5 (HGS) at 37°C. The uptake of 10.8 μmol ^{65}Zn/L (37 MBq/L) in HGS was measured following incubation at 37°C for 30 sec to 90 min.

2.3. Depletion of cell cholesterol

Cells were subcultured into 3.8 cm^2 culture dishes and allowed to grow in normal growth medium for 24 h, then treated with mevastatin for 42 h. On the day of the experiment (66 h after subculturing) the medium was changed to serum-free growth medium containing 10 μmol mevastatin/L with or without CD. The confluent monolayer of cells was incubated for an additional 4 h after exchanging the medium, and Zn uptake was measured or the cells processed for cholesterol determination.

Following their incubation with CD, cells were lifted from the growing surface with 1 ml 0.4% trypsin. Trypsinization was stopped with the addition of 1 ml complete growth medium. The cells were pelleted by centrifugation (2500 $\times$ g at 4°C for 5 min) and washed twice, then resuspended, in phosphate-buffered saline. Following sonication an aliquot of the cell suspension was removed for protein analysis [21]. The cell lipids were extracted into chloroform and the cholesterol content of the cells was measured using HPLC [22]. The columns were composed of 7 μm cyanopropyl Zorbax packing material (Phenomenex, Torrence, CA). Cholesterol was eluted using 0.1% isopropanol in heptane as the eluant, at a flow rate of 1 ml/min. The cholesterol was quantitated using a Sedex 45 light scattering detector (Sedere, Vitry Sur Seine, Cedex, France) attached to a HP 3394A Integrator (Hewlett-Packard, Avondale, PA).

2.4. Concentration-dependent uptake of ^{65}Zn

The confluent monolayer of cells was treated as described above with mevastatin alone or mevastatin plus CD (5.5, 8, or 11 mmol/L). At the end of the 4 h incubation, the medium was removed and the monolayers were rinsed twice with HGS. After the second rinse, 1 ml of ^{65}Zn-labeled HGS medium (37 MBq/L; 0.8 μmol Zn/L to 20.8 μmol Zn/L) was added to the culture dishes and the cells incubated for 10 min at 37°C. Following this incubation, the culture dishes were placed on ice and the labeled medium was removed. The cells were rinsed three times with ice-cold HGS. The cells were then incubated with 1 ml ice-cold 10 mM EDTA in 150 mM NaCl for approximately 30 s. The reaction was stopped with the addition of 1 ml HGS. The solution was then removed from the culture dishes and the cells rinsed twice with ice-cold HGS. After the second rinse, the cells were incubated with 1 ml ice-cold acid saline medium (150 mmol NaCl/L, 40 mmol acetic acid/L, pH 4.0) for 30 s. The acid saline was diluted with 1 ml ice-cold HGS and the solution removed. The cells were rinsed a second

time with 1 ml ice-cold HGS and then lysed with 1 ml 0.2 mol NaOH/L, 0.2% (w/v) sodium dodecyl sulfate for at least 15 min. The cell lysate was placed in gamma counting tubes and the activity of ^{65}Zn was measured.

2.5. Uptake of 2-deoxyglucose (2-DG)

Cells were grown and treated as above for the Zn uptake experiments with CD. Following the 4 h CD treatment, the medium was removed and the cells rinsed twice with 1 ml phosphate-buffered saline (PBS) at 37°C. After the second rinse, ^{14}C-labeled 2-DG (Amersham Pharmacia Biotech, Piscataway, NJ) was added to the culture dishes (37 MBq/L; 1 and 2 mmol 2-DG/L) and incubated with the cells at 37°C for 10 min. Placing the culture dishes on ice stopped the reaction. The labeled medium was removed and the cells were rinsed twice with ice-cold PBS. The cells were then lysed by the addition of 0.25 mmol NaOH/L and incubated for 15 min at room temperature, then placed at -20°C overnight. The next morning, the cell protein solution was thawed and a 50 μl aliquot was removed for protein determination. The remainder of the sample was measured for radioactivity by scintillation counting.

2.6. ^{65}Zn uptake following treatment with inhibitors of clathrin-dependent endocytosis

The formation of clathrin-coated pits is inhibited by potassium depletion [17,18]. Confluent cells were incubated in HGS buffer diluted 1:1 with water (37°C) for 5 min, followed by incubation for 30 min at 37°C in isotonic HGS buffer without potassium. Zn uptake was then measured in the same buffer containing 10.8 μmol ^{65}Zn/L (37 MBq/L). Zn uptake was also measured following the exchange of potassium for sodium in the HGS buffer, so that the buffer contained 145 mmol Na/L instead of 138 mmol Na/L with a potassium concentration of 7 mmol/L. The cells were incubated for 30 min in this buffer, then Zn uptake was measured as described.

Chlorpromazine inhibits the Golgi transport and the recycling of receptors in the coated-pit endocytic pathway [19]. Confluent cells were incubated in HGS buffer containing 0, 5, 10, 20 μg chlorpromazine/L for 30 min at 37°C, followed by measurement of Zn uptake in the same buffer containing 10.8 μmol ^{65}Zn/L (37 MBq/L).

2.7. Protein determination

The protein concentration was measured using the bicinchoninic acid method [23] or the Lowry method [21]. Bovine serum albumin was used as the standard.

2.8. Statistical analysis

Values within the figures and tables are expressed as the mean ± the pooled SEM. The data were analyzed by ANOVA using the Student-Newman-Keuls method or the Student's t-test (Statmost 1.01, DataMost, Salt Lake City, UT). Differences were considered significant when $p < 0.05$.

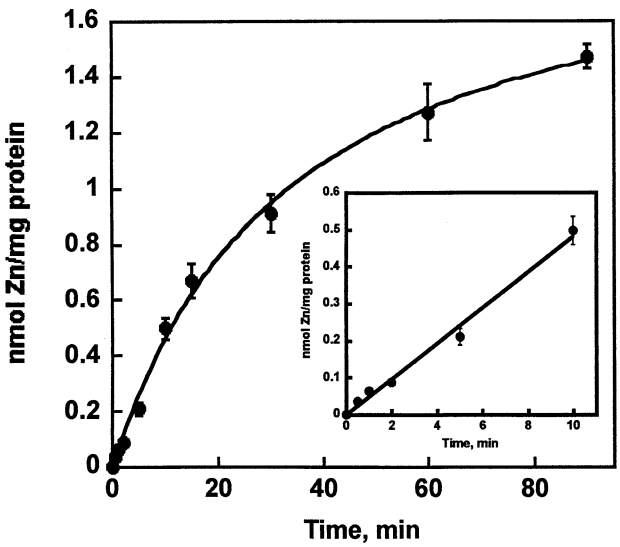

Fig. 1. Time course for Zn influx. MCF-10A cells were subcultured into 9.6 cm^2 culture dishes at a density of 2×10^5 cells/cm^2. The influx of 10.8 μmol ^{65}Zn/L was measured following incubation for 0.5 to 90 min at 37°C. Each data point represents the mean ± the pooled SEM of 3 to 6 replicates.

2.9. Data analysis

The data for ^{65}Zn uptake as a function of time were fit to the nonlinear regression equation, $y = (at)/(b + t)$ (from 0 to 90 min), or fit to a least squares linear regression, $y = a + bt$ (0 to 5 min), where a is the y-intercept, b is the slope, and t is the time in min. The kinetic parameters, apparent K_m and V_{max}, were calculated using the nonlinear regression equation, $y = (V_{max}\ x\ [Zn])/(K_m + [Zn])$.

3. Results

3.1. Time-dependent uptake of ^{65}Zn

The uptake of 10.8 μmol ^{65}Zn/L into MCF-10A cells was measured up to 90 min (Fig. 1). ^{65}Zn uptake was linear up to 15 min, and began to plateau between 30 min and 90 min. The inset shows ^{65}Zn uptake up to 5 min. The 10 min time point was chosen for all further ^{65}Zn uptake measurements.

3.2. Cell cholesterol levels following treatment with cyclodextrin

Cellular cholesterol levels were measured following treatment with CD concentrations ranging from 0 mmol/L to 11 mmol/L (Fig. 2). Cholesterol concentrations decreased to 60% of control levels at 8 mmol CD/L, and did not

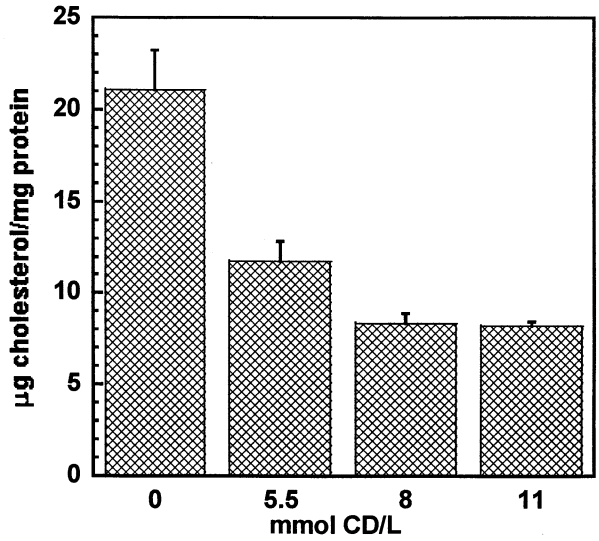

Fig. 2. Cell cholesterol content as a function of cyclodextrin (CD) concentration. MCF-10A cells were subcultured into 3.8 cm^2 culture dishes at a density of 2×10^5 cells/cm^2. After 24 h the cells were treated with 10 μmol mevastatin/L for 42 h in normal growth medium containing 5% horse serum. On the day of the experiment the medium was changed to serum-free medium containing mevastatin with or without hydroxypropyl-β-cyclodextrin. The total cell cholesterol content was assayed by HPLC following treatment of the cells with different concentrations of cyclodextrin. The values are expressed the mean ± the pooled SEM of 3 replicates.

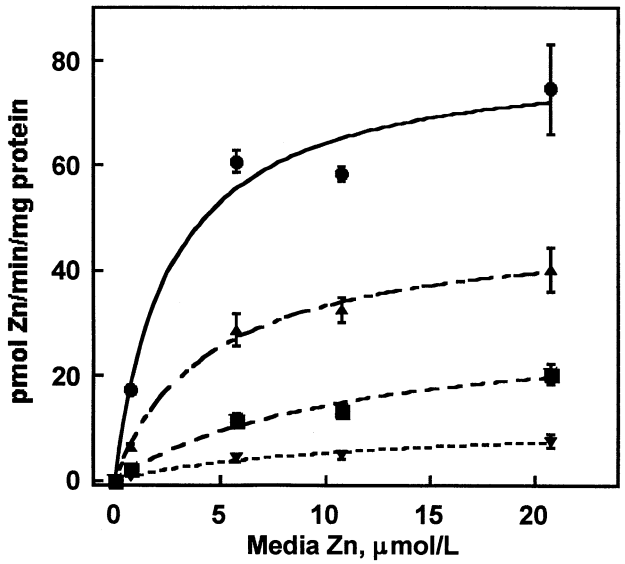

Fig. 3. Inhibition of Zn uptake into MCF-10A cells treated with cyclodextrin (CD). The cells were treated as described in Fig. 2. The concentrations of CD used were 0 mmol/L ●, 5.5 mmol/L ▲, 8 mmol/L ■, 11 mmol/L ▼. ^{65}Zn uptake was measured following rinsing the cells with EDTA and acidic saline. Cellular Zn uptake was defined as the amount of ^{65}Zn remaining with the cells. Each data point represents the mean ± the pooled SEM of 6 replicates.

decrease further when the CD concentration was increased to 11 mmol/L. There was no signficant effect of CD treatment on cell viability as measured by trypan blue exclusion. CD treatment also did not significantly affect the accumulation of formazan using the MTT assay, which also measures cell viability by mitochondrial dehydrogenase [24]. The mean assay absorbances (N = 8) ranged from 0.718 to 0.75 with an average variance of 4.5%.

3.3. Concentration-dependent ^{65}Zn uptake following cholesterol depletion

Treating the cells with CD significantly inhibited Zn uptake (Fig. 3). Zn uptake was measured using concentrations from 0.8 to 20.8 μmol/L for 10 min. Three CD concentrations, 5.5, 8, and 11 mmol/L were chosen for the Zn transport experiments below. Significant inhibition of Zn uptake was observed at all cyclodextrin concentrations.

Both the apparent K_m and the apparent V_{max} were significantly affected by increasing the cyclodextrin concentrations (Table 1). The apparent K_m increased by 3.9-fold with 11 mmol CD/L. The uptake velocity (apparent V_{max}) was reduced to 12% of the control value with 11 mmol CD/L.

3.4. Cholesterol depletion and the uptake of 2-deoxyglucose

It is possible that the depletion of cell cholesterol also significantly affects other nutrient uptake mechanisms not associated with caveolae or coated pits. To investigate this possibility, the uptake of 2-DG was measured in our cholesterol depletion model (Fig. 4). CD treatment did not inhibit the uptake of 1 or 2 mmol 2-DG/L.

3.5. ^{65}Zn uptake following treatment with inhibitors of clathrin-dependent uptake

Depleting plasma membrane cholesterol also affects coated-pit formation [15,16]. If clathrin-dependent endocytosis were a major component of the Zn uptake mechanism, then disrupting coated-pit formation by potassium depletion would significantly inhibit Zn uptake. Removal of potassium from the extracellular uptake medium (-KM) stimulated Zn uptake by approximately 20%. If the cells were

Table 1
Influence of cyclodextrin on zinc uptake into MCF-10A breast epithelial cells

mM Cyclodextrin	Apparent K_m	Apparent V_{max}
0.0	2.46 ± 0.44^{a}	77.43 ± 5.04^{a}
5.5	4.11 ± 1.02^{b}	$43.97 \pm 2.97^{a,b}$
8.0	$12.60 \pm 3.16^{a,b,c}$	$33.01 \pm 4.30^{a,c,d}$
11.0	$9.49 \pm 2.10^{a,b,d}$	$9.62 \pm 1.36^{a,b,d}$

The kinetic parameters apparent K_m and V_{max} were estimated by fitting the data to the nonlinear regression equation $v = (V_{max}\ app \times [Zn])/(K_m\ app + [Zn])$ using KaleidaGraph software (Synergy Software, Reading, PA). Values are mean ± pooled SEM. For each kinetic parameter, values with the same superscript are significantly different from each other, ($P > 0.05$).

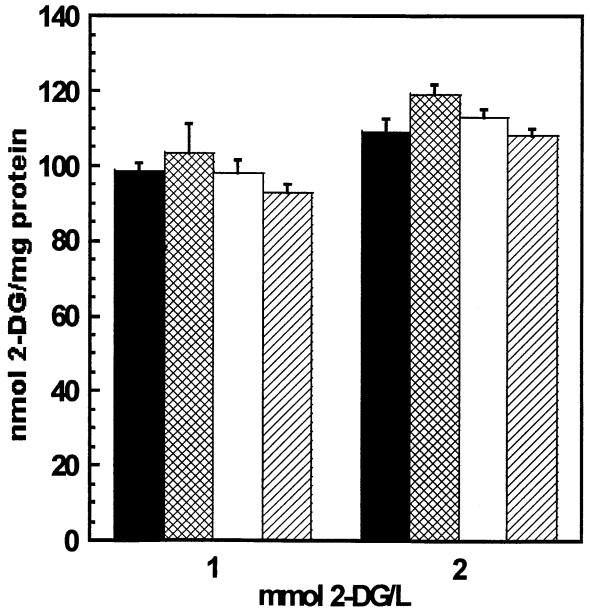

Fig. 4. 2-Deoxyglucose (2-DG) uptake following cyclodextrin (CD) treatment. The cells were treated as in Fig. 2, except that the uptake of ^{14}C-labeled 2-DG was measured. The concentrations of CD used were 0 mmol/L ■, 5.5 mmol/L ▨, 8 mmol/L □ and 11 mmol/L ▨. Values are expressed as the mean ± the pooled SEM of 3 replicates.

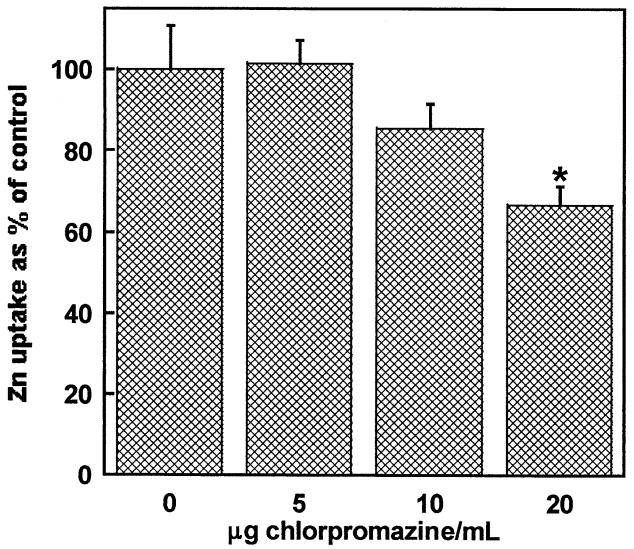

Fig. 6. Zn uptake following chlorpromazine treatment. MCF-10A cells were subcultured into 3.8 cm^2 culture dishes at a density of 2×10^5 cells/cm^2. Following rinsing, the cells were incubated with HGS medium containing from 0 to 20 μg/ml chlorpromazine for 30 min. Uptake of Zn was measured as in Fig. 5. Values are expressed as the mean % of control ± the pooled SEM of 3 replicates. Asterisks (*) over the bars indicate significance ($P < 0.05$).

subjected to hypotonic shock prior to incubation in potassium-depleted medium (-KS) Zn uptake was reduced by 15% (Fig. 5). Similarly, chlorpromazine treatment (20 μg/ml) significantly inhibited Zn uptake by approximately 33% (Fig. 6).

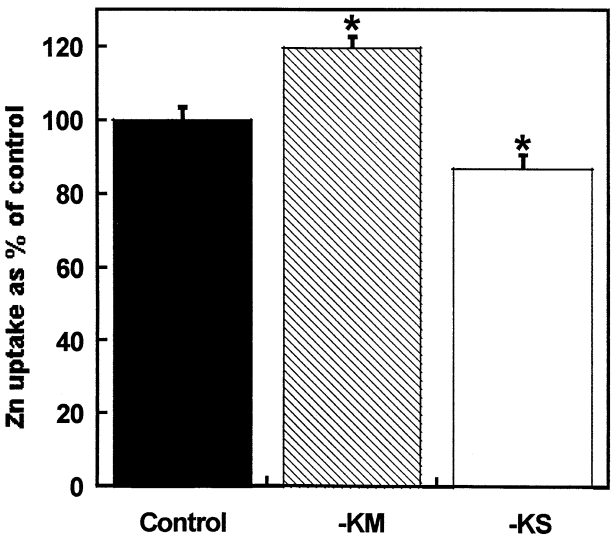

Fig. 5. Zn uptake following potassium depletion. MCF-10A cells were subcultured into 3.8 cm^2 culture dishes at a density of 2×10^5 cells/cm^2. Following rinsing, the cells were incubated with HGS medium in which potassium was replaced with sodium (-KM), or the cells were subjected to hypotonic shock then incubated with HGS in which sodium replaced potassium (-KS). Incubation was for 30 min, after which the uptake of 10.8 μmol/L ^{65}Zn was measured. Values are expressed as the mean % of control ± the pooled SEM of 3 replicates. Asterisks (*) over the bars indicates significance ($P < 0.05$).

4. Discussion

Numerous investigators have used cyclodextrins to alter membrane cholesterol levels [13,25–27]. β-Cyclodextrins exhibit a high degree of specificity for plasma membrane cholesterol over other lipids [28,29] and efficiently remove it from the plasma membrane of cells [26]. We observed that increasing cyclodextrin concentrations to 8 mmol/L resulted in an approximately 60% reduction in cell cholesterol levels from approximately 21.1 μg/mg protein to 8.3 μg/mg protein. Our data are consistent with the observations of others concerning the use of this type of reagent in scavenging cholesterol from cells [26]. Treating the cells with the cyclodextrin concentrations used in these experiments did not affect membrane integrity as measured by trypan blue exclusion, nor did the treatments affect cell viability as measured by an MTT assay. The treatments also did not inhibit the uptake of 2-DG, a molecule taken up by cells using a mechanism other then coated pit or non-coated pit endocytosis.

Over 90% of the cell cholesterol is reported to be associated with the plasma membrane [30]. The relation between plasma membrane cholesterol and the formation of caveolae is well established. Caveolin, the protein coat of caveolae, binds cholesterol and translocates with cholesterol from the Golgi apparatus to the plasma membrane [13]. One

can visualize the cholesterol-dependent formation of caveolae using caveolin antibody [14].

Our data show that cellular Zn uptake occurs via a process involving cholesterol, an observation consistent with the disruption of the non-coated pit endocytosis and with the disappearance of caveolae [13]. Treating cells with increasing concentrations of CD resulted in a corresponding decrease in cell cholesterol and significantly inhibited the rate of Zn uptake. The apparent V_{max} decreased, and the apparent K_m increased, as the cholesterol content decreased. The increase in the dissociation constant with decreasing cholesterol content may indicate that the environment surrounding the transporter is important in its function, possibly through maintaining its proper three-dimensional structure or, upon forming endocytic vesicles, the appropriate pH for Zn binding. Although cell cholesterol content did not decrease beyond the 8 mmol CD/L level, Zn uptake, but not the apparent K_m, was reduced further following treatment with 11 mmol CD/L (Fig. 3 and Table 1). At these concentrations of cyclodextrin, there may be other Zn-transport-related cholesterol pools that have become redistributed within the plasma membrane thereby inhibiting the transport function of the protein, but not affecting further its affinity for Zn. Consequently, a decrease in the apparent V_{max}, but not the apparent K_m, was observed.

The observed effect of cholesterol depletion on Zn uptake does not preclude a role for clathrin coated-pit endocytosis, since endosomal formation from the coated pits requires cholesterol [15,16]. If coated-pit endocytosis were required for Zn uptake in our cell culture model, then it would be expected that potassium depletion or chlorpromazine treatment would radically inhibit its uptake process similar to that observed following cholesterol depletion. However, we observed only a slight, albeit significant, decrease in Zn uptake with hypotonic shock/potassium depletion (Fig. 5; -KS) or with chlorpromazine (Fig. 6). Other cell types, i.e. endothelial cells, exhibit as much as a 70% decrease in Zn uptake with the same treatments [7]. Consequently, the particular contribution that coated pit endocytosis plays in Zn uptake may depend on the type of cell.

The data in Fig. 5 can be interpreted apart from the effects of potassium depletion on clathrin coated-pits. Several groups have reported that cellular Zn uptake requires Zn^{2+}/K^+ counter-transport [31,32]. Reducing the intracellular potassium levels by >75% with potassium ionophores significantly reduced Zn uptake. These treatments would affect the transmembrane potassium gradient similar to the hypotonic shock/potassium depletion treatment used in our experiments. Our data indicate that simply replacing the extracellular potassium in the medium with sodium significantly stimulated Zn uptake, consistent with the observations of others using human fibroblasts [31,32]. Further, cells subjected to hypotonic shock followed by incubation in potassium-depleted medium exhibited reduced uptake. However, our data indicate that, for this breast epithelial cell culture model, the Zn^{2+}/K^+ counter-transport mechanism plays only a minor role in Zn uptake.

In summary, the data presented here are consistent with a role for caveolae or caveolae-like plasma membrane microdomains (lipid rafts) in cellular Zn uptake. These compartments were initially identified as important for the uptake of folate [33–37]. Other important molecules have been found associated with caveolae including a calcium pump ATPase [38], the GLUT4 transporter [39], and various signal transduction molecules [40,41]. Additionally, caveolae function in the transcytosis of albumin [8–10]. The identification of melanotransferrin as a glycosylphosphatidylinositol-linked protein implicates these plasma membrane compartments in an aspect of cellular iron uptake as well [42]. The identification of specific Zn transporter molecules within these membrane compartments is currently unknown. Several Zn transporters, involved in Zn uptake or efflux, have been identified to date [2,3,5,43]. It will be interesting to determine whether their activities are cholesterol-dependent, and whether those that are located in the plasma membrane are also within caveolae/lipid rafts.

Acknowledgments

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