Calcitonin Stimulates Lysosomal Enzyme Release and Uptake in LLC-PK₁ Cells

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Abstract. Renal tubular targeted hormones increase urinary excretion of a lysosomal enzyme, N-acetyl-β-D-glucosaminidase (NAG). To elucidate the mechanism of this event, the calcitonin effect on NAG handling by LLC-PK₁ cells was examined. Calcitonin (1 nM to 10 µM), phorbol myristate (10 nM to 1 µM), and ionomycin (1 to 10 µM) promoted NAG release without any increase in lactate dehydrogenase release or any reduction of mitochondrial dehydrogenase activity. Treatment with 100 nM calphostin C or 50 µM KN-93 partially reversed the calcitonin effect on NAG release. Calcitonin promoted secretion of fluorescence ceramide, a reporter of protein transport from Golgi apparatus to cell surface. Calcitonin-stimulated NAG release was partially inhibited by 10 µg/ml brefeldin A, a blocker of protein transport through the Golgi apparatus. Calcitonin accelerated cellular uptake of exogenous NAG, which was inhibited by low temperature, 0.1 mM monodansyl cadaverine (receptor-mediated endocytosis inhibitor), and 10 mM mannose-6-phosphate. Furthermore, calcitonin promoted progression of intracellular membranes stained by a fluorescence membrane marker, styryl pyridinium dye, from cell periphery to perinuclear regions (commonly referred to as recycling vesicles) and increased dye release from preloaded cells. Fluorescence release from the cells pre-loaded with FITC-labeled NAG or albumin was also stimulated by calcitonin. These calcitonin effects on endocytotic and re-exocytotic pathways were inhibited by 100 nM cytochalasin D, 100 nM nocodazole, 0.1 to 1 µM bafilomycin A₁, or 0.1 mM monodansyl cadaverine. Increased urinary NAG excretion has been considered to reflect renal tubular damage. However, it was demonstrated here that stimulation of secretory and recycling pathways may be an alternative mechanism for calcitonin-induced enzymuria, which will become a new indicator of renal tubular response to this hormone.

Newly synthesized molecules from biosynthetic pathways and exogenous materials carried through endocytic pathways are transferred to lysosomes. Therefore, lysosomes have been considered to be the final destination of unidirectionally transported soluble macromolecules. However, retrograde trafficking from lysosomes can occur (1) and lysosomes may be maintained by a repeated series of transient fusion and fission processes with late endosomes/prelysosome compartments (2). Moreover, a model of secretory lysosomes fusing directly with the plasma membrane was proposed for mediating lysosomal enzyme release (3).

Renal proximal tubular cells contain high amounts of N-acetyl-β-D-glucosaminidase (NAG), one of the lysosomal glycolytic enzymes (4). Urinary excretion of this enzyme has been used as a marker of renal tubular damage. However, it shows diurnal variation in healthy subjects similar to the timing of circadian rhythm in urinary free cortisol (5). Parathyroid hormone (PTH) promotes urinary NAG excretion in healthy subjects but to a lesser extent in the patients with renal tubular resistance to PTH (pseudohypoparathyroidism) (6). Calcitonin also increases urinary NAG excretion (6). Therefore, lysosomal enzyme excretion may be modified by renal tubular targeted hormones and may be useful as a marker of renal tubular responsiveness to these hormones.

LLC-PK₁ cells, which are derived from porcine kidney, express calcitonin receptors, which couple to both adenyl cyclase and phospholipase C (7), and have retained several features of proximal tubular cells, including high NAG activity (8,9). In this study, we investigated the regulatory effects of calcitonin on NAG release, cellular uptake of externally supplied NAG, protein transport from Golgi apparatus to cell surface, intracellular transport of vesicular membranes, and re-exocytotic pathway in LLC-PK₁ cells. The results will help us further understand hormonal regulation of lysosomal enzyme handling by renal tubular cells.

Materials and Methods

Materials

NAG extracted from bovine kidney was purchased from Boehringer Mannheim Biochemicals (Mannheim, Germany); N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-(3-triethylammonium-propyl)-4-(4-(dibutylamino) styryl) pyridinium dibromide (FM 1-43) were from Molecular Probes (Eugene, OR); KN-93 was from Seikagaku Co. (Tokyo, Japan); Dulbecco’s modified Eagle’s medium (DMEM) was from Life Technologies BRL (Gaithersburg, MD); fetal
calf serum was from Hyclone Laboratories (Logan, UT); all other materials were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

LLC-PK₁ (ATCC CRL 1392) cells were obtained from American Type Culture Collection (Rockville, MD) and maintained in DMEM supplemented with 10% fetal calf serum. Confluent monolayers were split once a week and the medium was changed twice a week.

NAG Release Experiments

We examined the effect of salmon calcitonin (sCT) on NAG release from LLC-PK₁ cells and studied the contribution of protein kinase C (PKC), protein kinase A (PKA), and Ca²⁺/calmodulin-dependent protein kinase II (CaMK II) to this action. Cells were grown in 10% serum-supplemented medium on 24-well plates for 4 d followed by an additional 18 h of preincubation in serum-free medium containing 0.1% bovine serum albumin (BSA). The stock solution of 10% BSA in phosphate-buffered saline (PBS) was heated at 50°C for 2 h to inactivate bovine NAG. On the experimental day, cells were incubated for 30 min with indicated agents and NAG release into the medium was measured at 30-min intervals.

N-Acetyl-β-d-Glucosaminidase Activity Assay

NAG activities were determined using 4-methylumbelliferyl-N-acetyl-β-d-glucosaminide as substrate, from which NAG liberates 4-methylumbellif erone at pH 4.5 (10). One unit of NAG activity was defined as the amount of NAG catalyzing the liberation of 1 μmol of 4-methylumbellif erone per minute at 37°C. NAG release was expressed as nU/μg cell protein per min. Protein was determined by a modified method of Bradford using Bio-Rad Protein Assay (Bio-Rad, Richmond, CA).

LDH Activity Assay and Mitochondrial Dehydrogenase Activity Assay

To determine whether cell integrity was affected by the exposure to sCT, phorbol myristate (PMA), and ionomycin, LDH activity was measured in the incubation medium and cell lysates. Cells were treated with 1 μM sCT, 100 nM PMA, or 10 μM ionomycin for 30 min. LDH released into medium during the next 3 h was measured using LDH-cytotoxic test Wako (Wako Junyaku, Osaka Japan). The reduction of mitochondrial dehydrogenase activity is an early sign of functional impairment (sublethal injury) of cells. The enzyme activity, the ability to reduce tetrazolium salt WST-1 (succinate-tetrazolium reductase activity), during 3 h incubation after 30 min preincubation with sCT, PMA, or ionomycin was measured using a Cell Counting kit (Dojindo, Kumamoto, Japan).

Endocytosis Experiments

The endocytotic pathway may also be involved in the modulatory effects of sCT, PMA, and ionomycin on lysosomal enzyme targeting. After 30 min preincubation with 1 μM sCT, 100 nM PMA, or 10 μM ionomycin, cells were incubated for 30 min with FITC-labeled NAG (200 μg/ml) or FITC-labeled albumin (200 μg/ml) after the indicated time intervals. Incubation was stopped by rinsing the cells 6 times with Hanks’ balanced salt solution containing 10 mM Hepes, pH 7.4, and cells were solubilized by 0.1% Triton X-100 in PBS. The intracellular fluorescence was determined using a spectrofluorometer at excitation 495 nm/emission 520 nm. Cellular uptake of FITC-albumin and FITC-NAG were expressed as μg fluorescence/mg cell protein per 30 min. Binding of FITC-NAG or FITC-albumin to plasma membrane was determined at 4°C using the same procedure as for endocytotic experiments.

FITC-Albumin and FITC-NAG Release Assay

The effects of calcitonin on re-exocytotic pathway were also studied. Cells were pulsed for 2 h with FITC-albumin (500 μg/ml) or FITC-NAG (200 μg/ml). This medium was removed and cells were washed 6 times with fresh medium. Thereafter, cells were incubated with the indicated agents for 30 min. At 30-min intervals, conditioned medium was collected and replaced with fresh media. The fluorescence of conditioned medium was measured by a spectrofluorometer at excitation 495 nm/emission 520 nm. Fluorescence release was expressed as μg equivalent FITC-albumin or FITC-NAG/mg cell protein per 30 min.

FITC-Labeling of NAG

NAG (3 mg) extracted from bovine kidney (Boehringer Mannheim, Mannheim) was incubated with FITC isomer 1 (1 mg) in 0.1 M sodium carbonate-bicarbonate buffer, pH 9.0, for 2 h at 20°C. FITC-conjugated NAG was purified by eluting Sephadex G-25 column with PBS. Fluorescein/NAG molar ratio was 6.

Monitoring Transport from Golgi Apparatus to Cell Surface

As a reporter for secretory activity, we used BODIPY FL C₅₆-ceramide, which enters cells via spontaneous transfer and specifically labels the Golgi complex before its metabolites are transported to the cell surface (11). For staining of the Golgi apparatus, cells were incubated for 2 h with 5 μM BODIPY FL C₅₆-ceramide. After labeling, cells were washed 3 times with medium and treated with 1 μM sCT for 30 min. Cellular fluorescence was observed with a confocal laser microscope (Leica, Heidelberg, Germany) excited with a 488-nm laser and detected with 530-nm band-pass filter. Cellular fluorescence intensity was measured with the cells grown on 96-well plates using fluorometer (Fluoroskan Ascent; Libsystems, Helsinki, Finland) at excitation 485 nm/emission 538 nm.

Monitoring Endocytosis and Exocytosis using Membrane Markers

Using the fluorescent dye FM 1-43, which is virtually nonfluorescent in aqueous medium and is inserted into the outer leaflet of surface membrane where it becomes intensely fluorescent (12), we investigated the effects of calcitonin on endocytosis and exocytosis. In endocytosis experiments, cells were exposed to 5 μM FM 1-43 with or without 1 μM sCT for 30 min and then incubated with FM 1-43 for the indicated additional time. For monitoring exocytosis, cells were stained with 5 μM FM 1-43 for 4 h followed by rapid twice washing with fresh medium and then treated with 1 μM sCT for 30 min. Membrane-incorporated FM 1-43 was observed with confocal microscope excited with 488 nm laser and detected with 605-nm-long pass filter. The changes in cellular fluorescence intensity were monitored with the cells grown on 96-well plates using fluorometer at excitation 485 nm/emission 590 nm.

Statistical Analyses

All values are presented as means ± SD. The significance of differences was tested by one-way ANOVA. Differences were considered significant if P < 0.05.
Results

Effect of sCT, PMA, and ionomycin on NAG Release and Ceramide Transport from Golgi Apparatus to the Cell Surface

Treatment with sCT stimulated NAG release from LLC-PK₁ cells in a dose-dependent manner (Figure 1). PMA and ionomycin mimicked the calcitonin action. Ionomycin reproduced the initial increase in NAG activity, whereas PMA reproduced the calcitonin action in a more sustained manner. PKA activators (100 μM forskolin, 100 μM dibutyryl cAMP, 100 μM SpcAMP, 100 μM 8-bromo cAMP) had no significant effect on NAG release (data not shown). Calcitonin-induced NAG release was suppressed by the pretreatment with 100 μM H-7 (nonspecific serine/threonine kinase inhibitor) (data not shown) and 100 nM calphostin C (selective PKC inhibitor) (Table 1), whereas 100 μM RpcAMPS (PKA inhibitor) has no effect on the calcitonin action on NAG release (data not presented). The effects of sCT and ionomycin on NAG release were significantly reduced in the presence of 50 μM KN-93 (CaMK II inhibitor) (Table 1). Calcitonin promoted release of BODIPY FL C5-ceramide fluorescence from the preloaded cells (Figure 2). Brefeldin A is a potent drug that blocks coated vesicle formation from Golgi apparatus and induces a reversible block of protein secretion. The effects of sCT and PMA on NAG release were partially reversed by this agent (Table 1).

Effect of sCT, PMA, and Ionomycin on Cell Integrity and Viability

Treatment with sCT, PMA, or ionomycin had no effect on LDH release. Ionomycin had no effect on mitochondrial dehydrogenase activity. PMA and sCT did not reduce but rather increased the enzyme activity (135% of control cells).

Table 1. Inhibitory effects of calphostin C, KN-93, and brefeldin A on the action of salmon calcitonin (sCT), phorbol myristate (PMA), and ionomycin on N-acetyl-β-D-glucosaminidase (NAG) release from LLC-PK₁ cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>NAG Release (nU/μg cell protein per min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Control</td>
<td>4.7 ± 0.5</td>
</tr>
<tr>
<td>Calphostin C (100 nM)</td>
<td>3.6 ± 0.5b</td>
</tr>
<tr>
<td>KN-93 (50 μM)</td>
<td>3.9 ± 0.2b</td>
</tr>
<tr>
<td>Brefeldin A (10 μg/ml)</td>
<td>3.2 ± 0.4b</td>
</tr>
</tbody>
</table>

* LLC-PK₁ cells were treated with sCT, PMA, or ionomycin for 30 min after a 30-min preincubation with calphostin C or KN-93. In the brefeldin A case, experiments were conducted in the presence of brefeldin A after a 30-min preincubation with brefeldin A. n = 8.

b P < 0.05 versus control.

c P < 0.05 versus sCT treatment.

d P < 0.05 versus PMA treatment.

e P < 0.05 versus ionomycin treatment.
tively, these observations indicated that cell viability was not significantly affected in these experimental conditions.

Effects of sCT on Albumin and NAG Uptake

Treatment with sCT, PMA, or ionomycin promoted cellular uptake of externally supplied NAG and albumin with the maximum effect at 60 to 90 min (Figure 3). Binding of FITC-NAG or FITC-albumin to plasma membrane determined at 4°C was not affected by these agents (Figure 3). Monodansyl cadaverine (MDC) inhibits clustering and internalization of the ligand-receptor complexes into clathrin-coated vesicles. We used this agent to investigate the possible role of receptor-mediated endocytosis in NAG uptake. MDC strongly inhibited baseline and calcitonin-induced uptake of NAG and albumin (Table 2). NAG contains mannose-6-phosphate (M6P), which is a recognition marker for mannose-6-phosphate receptor (MPR). LLC-PK1 cells may reuptake NAG through receptor-mediated endocytosis via MPR on the plasma membrane. To test this possibility, endocytosis experiments were conducted in the presence of M6P. Treatment with M6P partially blocked both baseline and calcitonin-stimulated NAG uptake but had no effect on albumin internalization (Table 2). We also investigated the role of proton pump, actin, and microtubule in the stimulatory effect of calcitonin using Bafilomycin A1, cytochalasin D, or nocodazole. Treatment with these antagonists inhibited calcitonin-stimulated uptake of albumin (Table 2).

Figure 2. Effects of calcitonin on the release of BODIPY FL C5-ceramide fluorescence from the preloaded cells. (A) Calcitonin promoted the release of BODIPY FL C5-ceramide fluorescence from the preloaded cells. Top panel, control; Bottom panel, sCT-treated cells (1 μM). (B) Cellular fluorescence intensity measured using fluorometer are expressed by the ratio with the 0 min value as 1. ● calcitonin; ○, control. n = 8. *P < 0.05 versus control.
Baseline and calcitonin-stimulated albumin uptake was also partially inhibited by brefeldin A.

**Effects of Calcitonin on the Intracellular Progress of Endocytosed Membranes**

Intracellular membranes stained by FM 1-43 were found in a cluster of vesicles in both peripheral (probably early endosomes) and perinuclear regions (commonly referred to as recycling vesicles) of the cell. During endocytosis, FM 1-43 progressed from cell periphery to perinuclear region with little loss in fluorescence intensity. Treatment with sCT promoted this progression of the fluorescence membranes to perinuclear regions. The fluorescence intensity of calcitonin-treated cells was higher at 60 to 90 min than control cells (Figure 4).

**Effect of sCT on the Release of the Fluorescence from the Cells Preloaded with FM 1-43, FITC-Albumin, or FITC-NAG**

In the fluorescence release experiments with the cells preloaded with FM 1-43, sCT promoted decrease of fluorescence intensity. Treatment with sCT, PMA, and ionomycin increased uptake of FITC-labeled albumin and FITC-labeled NAG. Binding of FITC-albumin or FITC-NAG on the plasma membrane determined at 60 to 90 min (arrows) was not affected with these agents. Treatment with 0.1 mM monodansyl cadaverine (MDC; ▼) suppressed cellular uptake of FITC-labeled albumin and FITC-labeled NAG. n = 8. *P < 0.05 versus control (○).

**Table 2. Effects of monodansyl cadaverine (MDC) and mannose-6-phosphate (M6P) on endocytosis and role of proton pump, actin, and microtubule in cellular uptake of albumin**

<table>
<thead>
<tr>
<th>Condition</th>
<th>FITC-Albumin Uptake (μg/mg cell protein per 30 min)</th>
<th>FITC-NAG Uptake (μg/mg cell protein per 30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control sCT (1 μM)</td>
<td>Control sCT (1 μM)</td>
</tr>
<tr>
<td>Control</td>
<td>1.93 ± 0.17</td>
<td>1.24 ± 0.06</td>
</tr>
<tr>
<td>MDC (0.10 mM)</td>
<td>1.34 ± 0.18b</td>
<td>0.89 ± 0.05b</td>
</tr>
<tr>
<td>M6P (10 mM)</td>
<td>2.14 ± 0.24</td>
<td>1.13 ± 0.11b</td>
</tr>
<tr>
<td>Bafilomycin A1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05 μM</td>
<td>1.36 ± 0.14b</td>
<td>1.87 ± 0.27c</td>
</tr>
<tr>
<td>0.1 μM</td>
<td>1.22 ± 0.16b</td>
<td>1.27 ± 0.20c</td>
</tr>
<tr>
<td>1 μM</td>
<td>1.13 ± 0.13b</td>
<td>1.31 ± 0.33c</td>
</tr>
<tr>
<td>Cytochalasin D</td>
<td>1.79 ± 0.18</td>
<td>2.56 ± 0.12c</td>
</tr>
<tr>
<td>Nocodazole</td>
<td>2.05 ± 0.18</td>
<td>2.54 ± 0.24c</td>
</tr>
<tr>
<td>Brefeldin A</td>
<td>1.69 ± 0.22b</td>
<td>2.43 ± 0.29c</td>
</tr>
</tbody>
</table>

*a Cells were treated with sCT for 30 min in combination with MDC, after a 30-min preincubation with Bafilomycin A1, cytochalasin D, and nocodazole, or in the presence of brefeldin A after a 30-min preincubation with brefeldin A. Cellular uptake of FITC-labeled albumin or FITC-labeled NAG was measured at 60 to 90 min. Endocytosis experiments were also conducted in the presence of M6P. n = 8.

b P < 0.05 versus control.

c P < 0.05 versus sCT treatment.
intensity of the cells (Figure 5). Treatment with sCT stimulated release of the fluorescence from the cells preloaded with FITC-labeled albumin or NAG (Figure 6). Ionomycin or PMA reproduced calcitonin-stimulated release of the fluorescence, but with a certain delay in PMA experiments. Pretreatment with KN-93 reduced both baseline and calcitonin-stimulated release of fluorescence (Figure 6). The stimulatory effects of calcitonin on the release of fluorescence were also inhibited by the pretreatment with Bafilomycin A1, cytochalasin D, or nocodazole (Table 3).

The Relationship between Exocytosis and Endocytosis

Endocytosis results in removal of membrane components from cell surface. Exocytosis may be required to complement the membrane deficit produced by endocytosis. Therefore, triggered exocytosis may be related to the triggered endocytosis. To determine the relationship between endocytosis and re-exocytosis, MDC were used. Pretreatment with MDC reduced both baseline and calcitonin-stimulated release of fluorescence from the cells preloaded with FITC-albumin or FITC-NAG (Figure 6). MDC (0.02 to 0.1 mM) also reduced sCT effects on endogenous NAG release by 12 to 21%.

Discussion

The major findings of the present study are that calcitonin increases NAG release and uptake, probably by promoting protein transport from the Golgi apparatus to the cell surface, receptor-mediated endocytosis, and re-exocytotic pathway. Calcitonin-induced NAG release was not associated with any increase of LDH release or reduction of mitochondrial dehydrogenase activity. Fluorescence release from the cells preloaded with FITC-labeled NAG or albumin was dependent on
microtubular function and blocked by receptor-mediated endocytosis inhibitor.

Lysosomal enzyme release is known to be modified by secretagogues, Ca\(^{2+}\) ions, PKC, or hormones in several cells. Recently, Rodriguez et al. demonstrated Ca\(^{2+}\)-dependent fusion of lysosomes with plasma membrane and, as a result, lysosomal content release from fibroblasts and epithelial cells (3). The results in the present study indicate that PKC and CaMK II constitute important mediators also in calcitonin-induced NAG release from LLC-PK\(_1\) cells.

Newly synthesized lysosomal enzymes reach lysosomes directly from the trans-Golgi network or indirectly via endocytosis from the cell surface. The experiment with BODIPY FL C\(_2\)-ceramide indicated that sCT promotes protein transport from Golgi complexes to plasma membrane in LLC-PK\(_1\) cells. Protein transport from endoplasmic reticulum and distal Golgi compartments to plasma membrane is known to be PKC-dependent (13) and is reversibly blocked by brefeldin A, which disassembles Golgi and prevents coated vesicle formation from the Golgi complex and trans-Golgi network (14,15). Inhibitory effects of brefeldin A on calcitonin- or PMA-regulated NAG release also indicate that protein traffic through Golgi complex is involved in the stimulatory effects of calcitonin and PMA on intracellular NAG traffic in LLC-PK\(_1\) cells.

PKC and intracellular Ca are known to be related to endocytic pathways in other cells (16,17). After lysosomes fuse with plasma membrane and lysosomal enzymes are secreted into extracellular space, cation-independent (Cl\(-\)) MPR appear on the plasma membrane and the released lysosomal enzymes can be internalized (18). Calcitonin, PMA, and Ca ionophore

Figure 5. Effects of calcitonin on the release of fluorescence from the endocytosed membranes. (A) Calcitonin promoted decrease of fluorescence intensity of the cells preloaded with FM 1-43. Top panel, control; Bottom panel, sCT-treated cells (1 \(\mu\)M). (B) Data are expressed by the ratio with the 0 min value as 1. ●, calcitonin; ○, control. n = 8. *P < 0.05 versus control.
stimulated endocytotic pathway as well as exocytotic traffic in LLC-PK₁ cells. Inhibitory effects of MDC and M6P on baseline and calcitonin-induced NAG uptake indicated that NAG uptake takes place by receptor-mediated endocytosis via MPR in LLC-PK₁ cells.

Several putative physiologic functions have been attributed to M6P-dependent endocytosis. One of the potential functions may consist in transfer of lysosomal enzymes from one cell type to another (19,20). A proportion of lysosomal enzymes in human urine are efficiently internalized by cultured hepatocytes via the CI-MPR (21,22), and NAG consists of about 25% of the lysosomal enzymes in urine containing the highest proportion of mannose-6-phosphorylated form (23). Calcitonin receptors distribute along the nephron from proximal straight tubule to cortical collecting duct (24). Lysosomal enzymes released from some portion of renal tubules can be recycled by the tubular cells at the more distal portion of nephron. Others have suggested that lysosomal enzymes bound to MPR at cell surface of fibroblasts mediate degradation of matrix proteoglycans (25). In the distal tubule, urine pH is less than 5 under appropriate conditions (26). Lysosomal acid hydrolase can actively degrade intraluminal materials at this site. Therefore, lysosomal enzyme release from proximal renal tubular cells may have two implications: enzyme reuse by more distal tubular cells and degradation of intraluminal substrates at the distal portion of the nephron.

Most receptor-ligand complexes accumulate at clathrin-coated pits of plasma membrane, which bud off to yield clathrin-coated vesicles. The vesicles distribute throughout the peripheral and perinuclear cytoplasm and transport membrane components, directly or indirectly, back to plasma membrane. The experiments using membrane marker FM 1-43 and release of the fluorescence from the cells preloaded with FITC-albumin or FITC-NAG indicated that calcitonin promotes the recycling pathway. Delivery of endocytosed proteins to the cell surface is known to be regulated by intracellular Ca (17,27). Inhibitory effects of KN-93 on baseline and calcitonin-stimulated release of fluorescence from the cells preloaded with FITC-albumin indicated that the return pathway from endosomes to cell surface is regulated by CaMK II also in LLC-PK₁ cells. Cytochalasin D and nocodazole experiments indicated that the re-exocytotic pathway is also dependent on the function of microfilaments and microtubules in these cells.

Table 3. Role of proton pump, actin, and microtubule in the stimulatory action of calcitonin on release of the fluorescence from the cells preloaded with FITC-labeled albumin

<table>
<thead>
<tr>
<th>Condition</th>
<th>FITC-Albumin Release (µg/ml cell protein per 30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Bafilomycin A1</td>
<td>1.12 ± 0.10</td>
</tr>
<tr>
<td>0.1 µM</td>
<td>0.85 ± 0.21b</td>
</tr>
<tr>
<td>1 µM</td>
<td>0.75 ± 0.17b</td>
</tr>
<tr>
<td>Cytochalasin D (100 nM)</td>
<td>1.16 ± 0.07</td>
</tr>
<tr>
<td>Nocodazole (100 nM)</td>
<td>1.02 ± 0.10</td>
</tr>
</tbody>
</table>

*After 2-h loading of 500 µg/ml FITC-albumin, LLC-PK₁ cells were treated with sCT after a 30-min preincubation with Bafilomycin A1, cytochalasin D, or nocodazole and then fluorescence release from the preloaded cells was measured. n = 8. b P < 0.05 versus control. c P < 0.05 versus sCT treatment.
din A is known to suppress not only protein transport through the trans-Golgi network but also early endocytic pathway (28,29). Inhibition of FITC-albumin uptake by brefeldin A indicated that endocytotic pathway in LLC-PK1 cells also requires normal function of a coatomer protein (β-COP).

Dual activation of endocytosis and exocytosis by calcitonin may be related to maintenance of a steady-state distribution of membrane components between cell surface and intracellular vesicles. Inhibition of re-exocytotic pathway by MDC, receptor-mediated endocytosis inhibitor, suggests that the possible role of calcitonin-stimulated exocytosis is to insert intracellular vesicle membrane into the plasma membrane deficit, which is produced by endocytosis.

Increased urinary NAG excretion has been considered to reflect the renal tubular cell damage. However, our study indicated that stimulation of secretory and recycling pathways may be an alternative mechanism for calcitonin-induced enzymuria, which will become a new indicator of renal tubular response to this hormone.

Acknowledgments

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References