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Original

Effect of ionomycin on interaction of calnexin with vesicular stomatitis virus glycoprotein is cell type-specific

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Abstract: Ionomycin is a calcium ionophore that induces release of calcium ions (Ca^{2+}) from cellular storage to cytoplasm and Ca^{2+} influx from the outside of the cell. We investigated the effect of ionomycin on endoplasmic reticulum (ER)-Golgi transport in the vesicular stomatitis virus glycoprotein (VSV-G) system. Ionomycin inhibited transport of VSV-G in a concentration-dependent manner in baby hamster kidney (BHK) cells and HeLa cells. Half-maximum inhibition was observed at 5 μM. The inhibitory effect of ionomycin was not dependent on the cytoplasmic portion. Chelation of Ca^{2+} in culture medium did not affect transport efficiency, but co-incubation with ionomycin completely shut off transport. These findings highlight the importance of Ca^{2+} release from cellular storage. Because the inhibitory effect of ionomycin was expected to be dependent on mutual interaction of VSV-G and the ER chaperone calnexin, we further investigated interaction kinetics. In HeLa cells but not BHK cells the interaction of VSV-G and calnexin was prolonged in the presence of ionomycin. Taken together, the present results indicate that, by releasing Ca^{2+} from cellular storage, ionomycin inhibits ER-Golgi transport by interfering with the release of VSV-G from calnexin in HeLa cells. A mechanism of cell type-dependent ER-Golgi transport regulation was revealed.
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Keywords: ionomycin; VSV-G; calnexin, Ca^{2+}.

Introduction

The endoplasmic reticulum (ER) is the major intracellular reservoir of calcium ions. The total concentration of calcium in the ER is on the order of millimoles. In comparison, the calcium ion concentration in cytoplasm is three orders of magnitude lower (1). Approximately 50% of calcium ions in the ER can be rapidly released by the action of inositol 4,5-bisphosphate. The fraction of stored calcium that is not sensitive to inositol 4,5-bisphosphate is released into the cytoplasm by calcium ionophores. Drainage of calcium ions from the ER by calcium ionophores can cause rapid secretion of ER-resident proteins (2,3). A previous report found that the ER-resident protein immunoglobulin joining chain (J-chain) was released from ER-retention mechanisms and secreted outside cells by incubating J-chain-transfected fibroblasts with the calcium ionophore ionomycin (4).

Vesicular stomatitis virus glycoprotein (VSV-G) is a type I membrane protein and has been used as a cargo molecule for research on ER-Golgi transport (5). VSV-G molecules reside in the ER until they are folded properly to form a homotrimer and exported outside the ER (6,7). Unfolded VSV-G molecules are retained in the ER and eventually degraded by the ubiquitin-proteasome system, a mechanism referred to as the ER quality control system

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(8,9). Chaperone molecules have a very important role in this system: they form a molecular complex with unfolded proteins in the ER lumen. When the cargo molecules are folded properly, this molecular complex is resolved and the cargo proteins are exported. The chaperone molecules responsible for VSV-G retention in the ER are immunoglobulin heavy chain-binding protein (BiP) and calnexin (10). Although both the interaction of chaperone with cargo molecule and the anterograde transport of VSV-G are regulated by calcium ions (11), the direct effects of calcium deprivation and ER-Golgi transport of VSV-G have not been carefully studied. In the present study, we investigated the effects of the calcium ionophore ionomycin on ER-Golgi transport of VSV-G. In both baby hamster kidney (BHK) and HeLa cells, ionomycin inhibited VSV-G transport. However, in contrast to findings for HeLa cells, interaction of VSV-G with calnexin was not responsible for delayed transport of VSV-G in BHK cells. These results indicate that calcium-dependent transport is regulated differently in BHK and HeLa cells.

Materials and Methods

Cells

BHK cells (a hamster-derived fibroblastic cell line) and HeLa cells (a human-derived fibroblastic cell line) were cultured with 10% fetal calf serum-Dulbecco's modified Eagle's medium (10% FCS-DMEM) supplemented with 50 U/mL penicillin and 50 µg/mL streptomycin.

Reagents

Ionomycin was purchased from Sigma-Aldrich (Tokyo, Japan).

Antibodies

Rabbit anti-calnexin polyclonal antibody (Ab) was purchased from Stressgen Bioreagents (Ann Arbor, MI, USA). Mouse anti-VSV-G monoclonal antibody (clone BW8G5) and rabbit anti-VSV-G antibody (T25I) were kindly provided by Dr. William E. Balch (The Scripps Research Institute, San Diego, CA, USA).

Plasmids

The plasmid encoding VSV-G, pAR-VSV-G, the cytoplasmic deletion mutant pAR-VSV-G (Del 1-29), and the green fluorescence protein (GFP)-tagged temperature-sensitive mutant GFP-pET11d-VSV-tsO45 were generously provided by Dr. William E. Balch (The Scripps Research Institute). The pT7-blue vector was purchased from Novagen (Darmstadt, Germany) and used as the mock transfection vector.

Transfection

Infection with recombinant vaccinia virus and transfection were performed as described previously (12). Briefly, before infection, cells were washed once with OPTI-MEM (Life Technologies, Tokyo, Japan) and then infected with vaccinia T7 RNA polymerase recombinant virus (vTF7-3) (12) at a multiplicity of 10 plaque-forming units/cell in 0.5 mL of OPTI-MEM for 30 min with intermittent rocking in a 37°C CO_2 incubator. The infection medium was removed, and the cells were washed twice with OPTI-MEM. Cells were then transfected with the Lipofectamine Plus transfection kit (Life Technologies) according to the manufacturer's instruction. Briefly, 1 µg of each plasmid DNA was mixed in 100 µL of OPTI-MEM with 6 µL of Plus reagent. At the same time, 6 µL of Lipofectamine was mixed in 100 µL of OPTI-MEM. Fifteen minutes later, the medium containing DNA was mixed with Lipofectamine medium and incubated at room temperature for 15 min. The transfection medium was then applied to the BHK or HeLa cells and incubated for 6 h at 37°C in a CO_2 incubator.

Transport experiment

After transfection, the cells were washed with labeling medium (Sigma-Aldrich) and incubated with 1 mL of the same medium for 15 min for starvation. The cells were then metabolically labeled with 30 µCi/mL of Tran-[^{35}S]-label (MP Biomedicals, Aurora, OH, USA) for 5 min at 37°C. The cells were washed once with 10% FCS-DMEM and further incubated with the same medium for the durations indicated. For the ionomycin treatment experiment, the cells were cultured in the presence of varying concentrations of ionomycin. To examine the effect of ethylene glycol tetraacetic acid (EGTA), 5 mM of EGTA was added to culture media. The cell lysates were harvested and subjected to immunoprecipitation (IP). Briefly, the cells were lysed with 500 µL of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.5% Triton X-100). The cell lysates were cleared by centrifugation (14,000 × g for 1 min) and transferred to new tubes. One microliter of anti-VSV-G antibody (BW8G5) was incubated with the samples for 18 h, followed by 10 µL of protein G-sepharose (GE Healthcare, Tokyo, Japan) for 1 h at 4°C. After the pellets were thrice washed with 500 µL of cell lysis buffer, the pelleted samples were resuspended in 10 mM Tris, pH 6.5/1% SDS solution and heated at 95°C for 10 min. The supernatants were collected in new tubes, and 7 µL of 10 × G5 buffer (0.5 M sodium citrate, pH 5.5) and 5 µL of 1,000 U/mL of endoglycosidase H (Endo H) (New England BioLabs, Beverly, MA, USA) were added. After

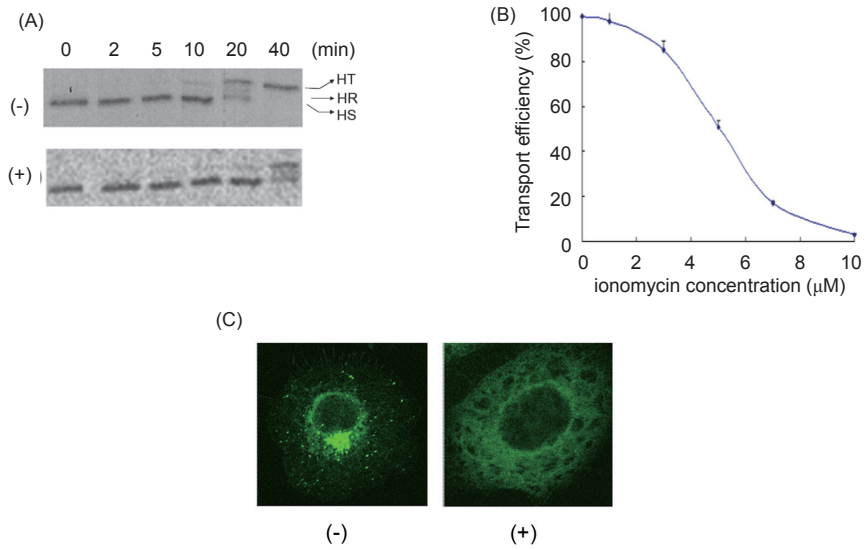

Fig. 1 Ionomycin inhibited ER-Golgi transport of VSV-G. **(A)** 3×10^5 of BHK cells were transfected with pAR-VSV-G for 6 h. After transfection, the cells were pulse-labeled with ^{35}S-methionine for 5 min. The cells were cultured in the presence or absence of 5 μM ionomycin for 0, 2, 5, 10, 20, and 40 min. The cell lysates were harvested and subjected to Endo H treatment. The samples were separated by 8% SDS-PAGE and exposed to X-ray film. HT: endo H-terminally glycosylated form, HR: endo H-resistant form, HS: endo H-sensitive form. **(B)** VSV-G-transfected BHK cells were cultured in the presence of various concentrations (0, 1, 3, 5, 7, 10 μM) of ionomycin. The cell lysates were harvested and subjected to Endo H treatment. The proportions of VSV-G converted to HT and HR at 0 μM ionomycin were set as 100%, and the relative transport rate was calculated with NIH Image. The data represent the means of three different experiments. **(C)** BHK cells were transfected with GFP-pET11d-VSV-tsO45 for 6 h at 39.5°C. After transfection, the cells were moved to permissive temperature (32°C) and cultured for 30 min. The cells were fixed, and the localization of GFP-tagged VSV-G was visualized.

24 h of incubation at 37°C, the samples were applied to 8% SDS-PAGE and autoradiographed. For the immunoprecipitation experiment with anti-calnexin antibody, cells were labeled for 3 h before transfection.

Immunoprecipitation and Western blotting

For the IP experiment, cell lysates were prepared from pAR-VSV-G-transfected BHK cells, as described above, and incubated with 1 μL of anti-calnexin Ab or BW8G5 Ab for 1 h at 4°C. Samples were further incubated with 10 μL of protein G-sepharose for 1 h at 4°C. The samples were thrice washed with lysis buffer and subjected to 8% SDS-PAGE. Samples were transferred to an Immobilon-P transfer membrane (Millipore, Tokyo, Japan) and blocked in 1% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS)/0.1% Tween 20 (blocking buffer) for 1 h at room temperature. The membranes were incubated with anti-calnexin Ab or T25I Ab for 1 h, followed by horseradish peroxidase (HRP)-goat-anti-rabbit IgG (H+L) Ab or HRP-goat-anti-mouse IgG (H+L) Ab, (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) (1/10,000 dilution in blocking buffer), respectively. The membranes were developed using ECL reagents (GE Healthcare).

Band intensity was measured using NIH Image.

Morphological analysis of transport

Morphological analysis of VSV-G intracellular transport was performed as previously described (4). BHK cells were plated on coverslips and transfected with a pET11d-GFP-tsO45. After 6 h of transfection at 39.5°C, the culture medium was replaced by CO_2-independent minimum essential medium (Sigma-Aldrich) and transferred to 32°C. The cells were further incubated for 30 min in the presence or absence of 5 μM of ionomycin. The coverslips were then mounted on glass slides and visualized. The images were viewed and photographed with an LSM510 confocal laser microscope (Carl Zeiss, Heidelberg, Germany).

Results

ER-Golgi transport was inhibited by ionomycin

Vectorial transport of VSV-G from the ER through sequential cis-, medial-, and trans-Golgi compartments is measured by processing the VSV-G-linked oligosaccharides acquired in the ER to various Golgi forms (13). These

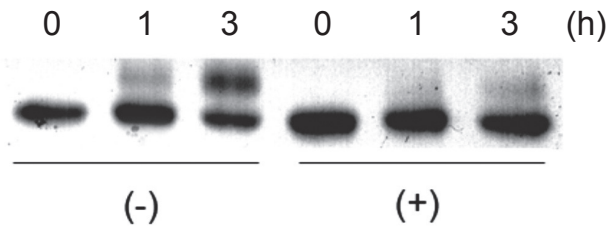

Fig. 2 Inhibitory effect of ionomycin is not dependent on the cytoplasmic portion of VSV-G. BHK cells were transfected with pAR-VSV-G (Del 1-29) for 6 h. The cells were labeled, and the transport experiment was performed as in Fig. 1A. The cells were cultured in the presence or absence of 5 μM ionomycin for 0, 1, and 3 h.

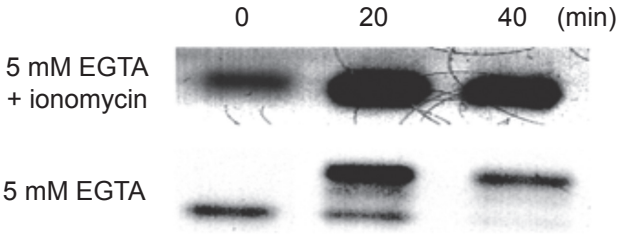

Fig. 3 EGTA shut off the VSV-G transport in combination with ionomycin. BHK cells were transfected with pAR-VSV-G for 6 h. After metabolic labeling, the cells were cultured with 5 mM EGTA alone or in combination with 5 μM ionomycin for the indicated times.

processing intermediates can be readily distinguished by SDS-PAGE followed by IP and Endo H treatment. First, we investigated the transport kinetics of VSV-G in BHK cells. VSV-G transfectants were labeled with ^{35}S-methionin and chased for the indicated times. As shown in Fig. 1A (upper panel), in the absence of ionomycin, VSV-G was gradually transported to the Golgi. After 10 min of chase, approximately 5% of total VSV-G was converted to an Endo H-resistant form (Endo HT), and 1 to 2% of total VSV-G was an Endo H-resistant intermediate form (Endo HR). After 20 min of chase, approximately 50% of total VSV-G was converted to the Endo HT and Endo HR form; at 40 min of chase, all VSV-G was transported to the Golgi, and no Endo-H-sensitive form (Endo HS) was detected. When the cells were chased in the presence of ionomycin, VSV-G transport was significantly affected. After 20 min of chase, most VSV-G remained in Endo HS, and only a few percent of VSV-G was converted to the Endo HT form. Even after 40 min of chase, not all VSV-G was transported to the Golgi, and the Endo HS and Endo HR forms were detected. Next, the transport assay was performed with the titrated concentration of ionomycin. VSV-G transport was inhibited by ionomycin in a dose-dependent manner (Fig. 1B). Fifty percent of inhibition was observed at an ionomycin concentration of 5 μM (Fig. 1B), and this concentration was used in the following experiments.

The inhibitory effect of ionomycin was also examined by a morphological method. For this purpose, a GFP-tagged, temperature-sensitive, VSV-G mutant (tsO45 VSV-G) system was used. The tsO45 has a point mutation in its luminal domain, and its thermosensitivity is due to the misfolding of tsO45 VSV-G at restrictive temperature (39.5°C) (14). Incubation at the permissive temperature (32°C) results in rapid ATP-dependent dissociation of the aggregate, trimerization, and export from the ER. The GFP-tagged tsO45 was transfected to BHK cells at restrictive temperature. After transfection, cells were moved to permissive temperature and chased for 30 min in the presence or absence of ionomycin. Although GFP-tsO45 successfully accumulated in the Golgi compartment in the absence of ionomycin (Fig. 1C, left panel), a reticular localization pattern was observed with ionomycin, thus confirming the biochemical data shown in Fig. 1B. In sum, these results indicate that ionomycin inhibited ER-Golgi transport of VSV-G.

Inhibitory effect of ionomycin is not dependent on the cytoplasmic portion of VSV-G

The VSV-G protein is a transmembrane protein and has a cytoplasmic portion. To examine whether the cytoplasmic portion of VSV-G is the target for the inhibitory effect of ionomycin, a transport assay was performed with the cytoplasmic deletion mutant of VSV-G (Del 1-29). Overall transport kinetics were reduced, and even after 3 h of chase only 40% of total VSV-G was converted to the Endo HT form (Fig. 2, left) and 60% remained in the Endo HS form. In the presence of ionomycin, transport efficiency was further reduced. After 3 h of chase, less than 5% of total VSV-G was converted to the Endo HT form; most VSV-G remained in the Endo HS form (Fig. 2, right). These results indicated that the inhibitory effect of ionomycin was not dependent on the cytoplasmic portion of VSV-G.

Chelation of calcium ion in the culture media completely abolished ER-Golgi transport

Ionomycin induces release of calcium ions from cellular storage such as the ER and mitochondria. At the same time, ionomycin activates calcium ion influx from outside cells. To examine the effect of the calcium influx activated by ionomycin on the transport kinetics of

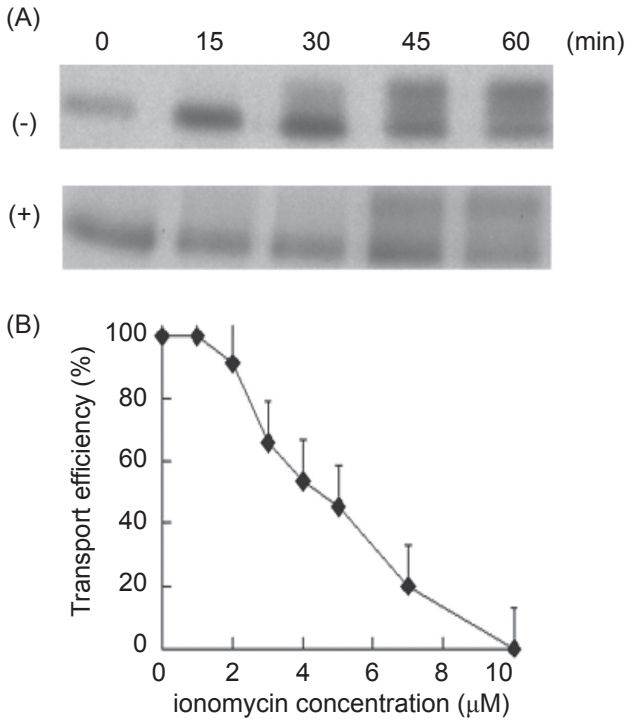

Fig. 4 Ionomycin inhibited ER-Golgi transport of VSV-G in HeLa cells. **(A)** 3×10^5 of HeLa cells were transfected with pAR-VSV-G for 6 h. The transport experiment was performed as in Fig. 1A. **(B)** Transport efficiency was measured as in Fig. 1B. The data represent the means of five different experiments.

VSV-G, a transport assay was performed in the presence or absence of EGTA in culture media. The addition of 5 mM of EGTA alone did not affect VSV-G transport (Fig. 3, lower panel). Surprisingly, however, VSV-G transport was completely abolished when cells were cultured in the presence of both ionomycin and EGTA (Fig. 3, upper panel). These results indicate that the ER-Golgi transport was reduced by ionomycin and that calcium influx is indispensable to compensate for basic cellular functions.

Cell type-independent inhibitory effect of ionomycin

To determine whether the inhibitory effect of ionomycin on VSV-G transport was cell type-specific, HeLa cells were used for a transport experiment, as in Fig. 1A. The transport kinetics were much lower in HeLa cells than in BHK cells. After 60 min of chase, less than 75% of VSV-G was transported to the Golgi (Fig. 4A). In the presence of ionomycin, transport kinetics were reduced dramatically, and the amount of VSV-G transported to the Golgi reached only 15% after 60 min of chase. These results demonstrate that ionomycin also elicits inhibitory effects in HeLa cells. To determine the optimal concentration of ionomycin to inhibit VSV-G transport, a transport experiment with varying concentrations of ionomycin was performed. As shown in Fig. 4B, the amount of VSV-G protein converted to the Endo HT form gradually decreased in a dose-dependent manner. When the amount of Endo HT form in the Golgi compartment after 60 min of chase (no ionomycin) was set as 100%, 50% inhibition was observed at 5 μM ionomycin. Transport was completely abolished when the cells were cultured with 10 μM ionomycin. Taken together, these results indicate that the inhibitory effect of ionomycin on VSV-G transport was also observed in HeLa cells and that half-maximum inhibition occurred at the same concentration as in BHK cells.

Prolonged interaction of VSV-G with calnexin

The VSV-G protein interacts sequentially with ER-resident chaperone molecules such as BiP and calnexin in proper folding and export (10). Thus, ionomycin treatment might disrupt interaction of VSV-G with these chaperones. To examine this possibility, the interaction of VSV-G with calnexin was investigated. Cell lysates from VSV-G-transfected and -untransfected BHK cells were collected and subjected to immunoprecipitation with anti-VSV-G (T25I) or anti-calnexin Ab, respectively. The samples were separated by 8% SDS-PAGE and subjected to Western blot. When the IP was performed with anti-VSV-G Ab, both VSV-G and calnexin were detected by Western blot only in VSV-G transfectant (Fig. 5A, upper panel). The reciprocal IP was performed with anti-calnexin Ab. Western blot demonstrated the existence of calnexin in both samples; however, VSV-G was only detected in VSV-G-transfected cells (Fig. 5A, lower panel). These results indicate reciprocal interaction between VSV-G and calnexin in the ER.

Next, we investigated the effect of ionomycin on the interaction of newly synthesized VSV-G and calnexin in the ER. For this purpose, the transfected BHK cells were metabolically labeled with ^{35}S-methionine and chased for the indicated times. The cell lysates were prepared and subjected to IP with anti-calnexin antibody. As shown in Fig. 5B (lower panel), in BHK cells VSV-G interacted with calnexin at the early time point. After 20 min of chase, no interaction was detected, indicating that VSV-G was released from calnexin and transported at this time point. Although the ER-Golgi transport was apparently inhibited in BHK cells, the interaction of VSV-G and calnexin was not affected by ionomycin.

The interaction was also examined in HeLa cells. VSV-G and calnexin interaction was successfully detected at the end of labeling (0 min) and was gradually reduced in the absence of ionomycin (Fig. 5B, upper). Only a small amount of VSV-G was co-precipitated with

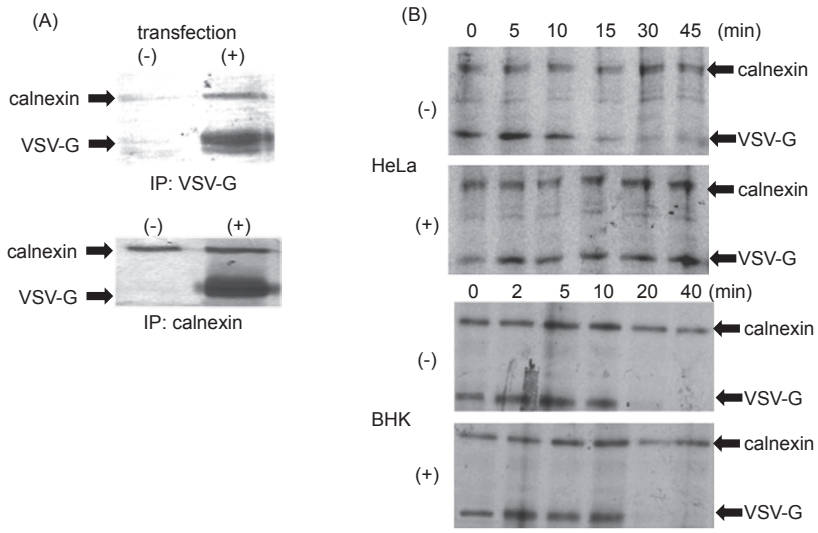

Fig. 5 Prolonged interaction of VSV-G and calnexin in HeLa cells. **(A)** BHK and HeLa cells were transfected with pAR-VSV-G for 6 h. After transfection, the cell lysates were prepared and subjected to IP. For IP experiments, the samples were incubated with anti-VSV-G Ab (upper panel) or anti-calnexin Ab for 1 h at 4°C, followed by protein G-sepharose. The samples were subjected to Western blotting with anti-calnexin Ab and anti-VSV-G Ab as primary Abs. **(B)** BHK and HeLa cells were labeled for 3 h before transfection. The cells were transfected with pAR-VSV-G for 6 h and labeled as in Fig. 1A. The cells were cultured in the presence or absence of 5 μM ionomycin. The cell lysates were harvested at the indicated times and subjected to IP with anti-calnexin Ab. The samples were applied to 8% SDS-PAGE and dried before exposure to X-ray film.

calnexin after 15 min of chase. At 45 min, no detectable amount of VSV-G was co-precipitated with calnexin. In contrast, in the presence of ionomycin VSV-G was co-precipitated throughout the chase period, and the interaction was detectable even after 45 min of chase (Fig. 5B, upper). These results indicate that ionomycin treatment resulted in the prolonged interaction of VSV-G and calnexin in HeLa cells but not in BHK cells.

EGTA in combination with ionomycin shut down ER-Golgi transport of VSV-G

To further confirm prolonged interaction, a transport assay was performed by titrating the concentration of ionomycin. VSV-G-transfected HeLa cells were incubated with various concentrations of ionomycin for the indicated times. After incubation, VSV-G and calnexin were co-immunoprecipitated. In the absence of ionomycin, the amount of VSV-G co-precipitated with calnexin was reduced time-dependently (Fig. 6A). After 40 min of chase, only a very small amount of VSV-G was co-precipitated with calnexin. In the presence of 5 μM ionomycin, however, VSV-G was detected throughout the chase period, and clear interaction was visible after 40 min of chase. The amount of VSV-G detected in the presence of 3 μM ionomycin was intermediate between that with 0 μM and 5 μM ionomycin. These results demonstrate that ionomycin prolongs the interaction of VSV-G and calnexin in a dose-dependent fashion.

In a previous experiment, EGTA in combination with ionomycin treatment completely shut down ER-Golgi transport. Next, the effect of EGTA on interaction of VSV-G and calnexin was examined. In the absence of ionomycin, clear interaction was observed at the end of labeling (Fig. 6B, lane 1). After 40 min of chase, only a small amount of VSV-G was observed, which indicates successful transport of VSV-G (lane 2). In the presence of EGTA, the interaction between VSV-G and calnexin and ER-Golgi transport was not affected (lane 4). In the presence of ionomycin, however, VSV-G was co-precipitated with calnexin (lane 3). The addition of EGTA plus ionomycin did not disrupt the interaction of VSV-G and calnexin (lane 5). These results indicate that EGTA shuts down the ER-Golgi transport of VSV-G in combination with ionomycin but does not affect the interaction between VSV-G and calnexin.

Discussion

Proper folding and homotrimerization is indispensable for VSV-G to be transported from the ER to Golgi (6,7). During folding, VSV-G must associate sequentially with two chaperones, BiP and calnexin (10). Calnexin is a lectin-like molecular chaperone that binds to newly synthesized glycoproteins in the ER (1). With calreticulin, calnexin constitutes the so-called “calnexin

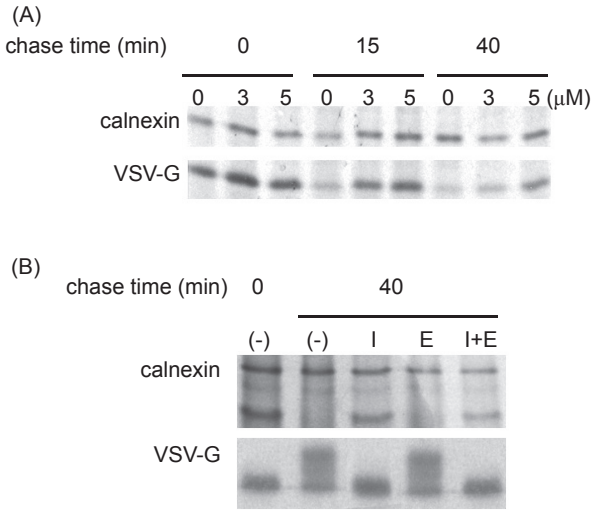

Fig. 6 Ionomycin concentration-dependent interaction of VSV-G and calnexin. **(A)** Transfection and labeling was performed as in Fig. 1A for HeLa cells and chased with 0, 3, or 5 μM of ionomycin for 0, 15, or 40 min. The cell lysates were harvested and subjected to IP with anti-calnexin Ab followed by protein G-sepharose. The samples were loaded to 8% SDS-PAGE and exposed to X-ray film. **(B)** After transfection of HeLa cells with pAR-VSV-G, the cells were labeled and chased in the presence of 5 mM EGTA alone or in combination with 5 μM ionomycin for 40 min. Transport efficiency is shown in the lower panel.

cycle" and contributes to ER quality control (8,9). In the present study, the calcium ionophore ionomycin inhibited ER-Golgi transport of VSV-G in BHK and HeLa cells. As binding between chaperone molecules and their cargo is dependent on the calcium ion concentration in the ER (15), we initially speculated that the interaction of VSV-G and calnexin would be affected by ionomycin. To explore the inhibitory mechanisms of ionomycin, we examined the kinetics of VSV-G binding with calnexin. Although ionomycin clearly inhibited ER-Golgi transport of VSV-G in BHK cells, binding between VSV-G and calnexin was not affected. Thus, in BHK cells, ionomycin might disrupt other steps in VSV-G export. More surprisingly, however, when the interaction of VSV-G and calnexin in HeLa cells was examined, ionomycin treatment prolonged the interaction of the two molecules. These results indicate that the processes controlling VSV-G export might differ between these two cell lines.

In an attempt to explain these differences, we noted that BHK cells had much higher transport kinetics than did HeLa cells and that VSV-G transport was completed after 40 min of chase in BHK cells. Although transport kinetics differed, VSV-G binding to and release from calnexin was observed in both cells. These results suggest that this difference is not attributable to differences in transport kinetics.

Calnexin binding to polypeptides or oligosaccharides is differentially regulated. In an *in vitro* experiment using purified proteins (11), removal of bound calcium from calnexin abrogated oligosaccharide binding. In contrast, calcium depletion resulted in formation of calnexin oligomers and enhanced the interaction with its polypeptide substrates in HeLa cells (16). The prolonged interaction of calnexin and VSV-G under ionomycin treatment was observed only in HeLa cells in our experiments. These results highlight the cell type-specific regulatory mechanisms of calnexin binding to its substrates.

A VSV-G mutant lacking the entire 29-amino acid cytoplasmic tail (Del 1-29) is defective in folding and oligomerization and exits the ER very slowly (7). Consistently, the transport rate of Del 1-29 was significantly reduced in our experiment. However, addition of ionomycin further reduced the transport rate in BHK cells, suggesting that the cytoplasmic portion was not the region responsible for the reduced transport rate caused by ionomycin.

VSV-G is a transmembrane protein with two N-linked oligosaccharides in its luminal domain. The composition of the N-linked oligosaccharide side chain is important in the interaction of VSV-G with calnexin (17). One oligosaccharide at either position was sufficient for cell surface expression of VSV-G (18). However, the contribution of each of these two oligosaccharides to binding with calnexin has never been examined in detail. The binding affinity of calnexin to these two N-linked oligosaccharides may differ and thus contribute to the differential dissociation of VSV-G and calnexin in BHK and HeLa cells.

Despite our extensive efforts, binding between BiP and VSV-G was not detected. VSV-G binds to BiP at a very early stage in its synthesis in the ER (10). Only after proper binding to and release from BiP can VSV-G be transferred to calnexin. Therefore, the difference between BHK and HeLa cells should not be attributed to a difference in the interaction of VSV-G with BiP. The mechanisms underlying this difference should be clarified in future studies.

Ionomycin releases calcium from the ER and can also enhance the influx of calcium ions from the outside of cells (19). To examine the contribution of calcium influx on reduced VSV-G transport, the calcium chelator EGTA was added to the culture media. Intriguingly, the addition of EGTA alone to culture media did not affect VSV-G transport; however, in combination with ionomycin, VSV-G transport was completely shut down. Depletion of intracellular calcium stores leads to activation of calcium

influx via so-called store-operated channels. This reaction is called store-operated calcium influx (SOCI) (19). The biological function of SOCI is to maintain proper calcium ion balance in cells. Our results suggest an important role for SOCI in maintaining ER-Golgi transport.

A previous report showed that the ER-resident immunoglobulin joining chain (J chain) was released from the ER retention mechanisms and exported to the outside of the cell (4). The J chain is a component of secretory immunoglobulins and can only be exported from the ER after correct incorporation with the immunoglobulins. Although the mechanisms that control the retention of the J chain in the ER are not well understood, ionomycin treatment exerted the opposite effect on VSV-G. Calcium ion deprivation elicits diverse effects on the transport of newly synthesized proteins.

Acknowledgments

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