Prodigiosin 25-C uncouples vacuolar type H+-ATPase, inhibits vacuolar acidification and affects glycoprotein processing

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Abstract Prodigiosin 25-C inhibited the accumulation of 3-(2,4-dinitroanilino)-3′-amino-N-methyldipropylamine and acridine orange in the acidic compartments of baby hamster kidney cells with little perturbation of cellular ATP levels. In rat liver lysosomes, prodigiosin 25-C inhibited the proton pump activity with an IC_{50} of approximately 30 nM, but did not affect ATPase activity up to 1 µM. It also delayed the transport of vesicular stomatitis virus G protein and induced a drastic swelling of Golgi apparatus and mitochondria. These results indicate that prodigiosin 25-C raises the pH of acidic compartments through inhibition of the proton pump activity of vacuolar type H+-ATPase, thereby causing the functional and morphological changes to the Golgi apparatus.

Key words: Acidification; Glycoprotein processing; Prodigiosin 25-C; Vacuolar type H+-ATPase

1. Introduction

Vacuolar organelles of eukaryotic cells such as Golgi apparatus, endosomes, lysosomes, and secretory vesicles, are acidified by the proton pump activity of vacuolar type H+-ATPase (V-ATPase), and their internal acidification plays an essential role in endocytic and exocytic pathways [1]. V-ATPases have been identified and purified from a variety of organelles of eukaryotic cells and are clearly distinguished from other H+-ATPases by the inhibitor specificity [1,2]. Recently, bafilomycins [3] and concanamycins [4], originally found in microbial metabolites, have been shown to be specific inhibitors of V-ATPase and have become the most useful probes to study vacuolar acidification by V-ATPase [5–11].

Prodigiosin 25-C is one of the red pigments produced by microorganisms including *Streptomyces* and *Serratia* [12,13]. We found that prodigiosin 25-C isolated from the culture broth of *Streptomyces hiroshimensis* [14] acts as a selective inhibitor of T cell proliferation induced by concanavalin A (Con A) and exerts immunomodulating activities in immunized mice [15–18]. However, the molecular target of prodigiosin 25-C at the cellu-

lar level has yet to be determined, although it is known to inhibit the mitogen response of murine splenocytes and growth of T cell lymphoma more strongly at a higher concentration of Con A [16,19]. Among a panel of inhibitors we tested, acidic monovalent ionophores (e.g. monensin and nigericin), V-ATPase inhibitors (e.g. bafilomycin B_1 and concanamycin A), and Golgi mannosidase II inhibitor (swainsonine), were also more cytotoxic to those cells after addition of Con A [20]. These reagents, except for swainsonine, raise the pH in the acidic compartments and affect intracellular transport of glycoprotein [9,10,21–23]. In this paper, we have studied the effect of prodigiosin 25-C on vacuolar acidification, intracellular transport of glycoprotein, and morphology of BHK cells, and we have found that prodigiosin 25-C uncouples V-ATPase and raises the pH of vacuolar organelles in intact cells.

2. Experimental

2.1. Materials
Baby hamster kidney (BHK) cells were maintained in Eagle's minimal essential medium (Gibco Laboratories, Gland Island, NY) supplemented with 10% (v/v) fetal calf serum (Bioserum, Victoria, Australia) and 50 µg/ml kanamycin. Rats (Wistar, male) were obtained from Sankyo Labo Service (Tokyo, Japan). Prodigiosin 25-C (molecular weight 393) was prepared from the culture broth of *Streptomyces hiroshimensis* as described previously [14]. Bafilomycin A_1 and concanamycin A were kindly provided by Prof. K. Altendorf, University of Osnabrück (Osnabrück, Germany) and Dr. K. Mizoue, Taisho Pharmaceutical Co. (Tokyo, Japan), respectively. Other reagents were purchased as commercial products.

2.2. Visualization of vacuolar acidification
3-(2,4-Dinitroanilino)-3′-amino-N-methyldipropylamine (DAMP) and acridine orange were used for the visualization of vacuolar acidification. DAMP stain was basically performed according to the methods described previously [24]. Briefly, BHK cells on coverslips were treated with prodigiosin 25-C for 30 min and further incubated with 30 µM DAMP (Oxford Biomedical Research Inc., Oxford, MI) for 30 min. Then the cells were fixed with paraformaldehyde, then incubated with anti-dinitrophenol monoclonal antibody (mAb) (Oxford Biomedical Research) and reacted with phycoerythrin-conjugated anti-mouse IgG mAb (Tago Inc., Burlingame, CA). For acridine orange stain, BHK cells on coverslips were treated with prodigiosin 25-C for 30 min and further incubated with 5 µg/ml of acridine orange (Polyscience Inc., Warrington, PA) for 30 min.

2.3. Measurement of cellular ATP content
Immediately after removal of culture medium, BHK cells were frozen on dry ice, suspended in 1 ml of water and sonicated for 30 s in a water bath. 100 µl of the samples were used for measuring ATP content using lucifer-LU plus kit (Kikkoman, Chiba, Japan).

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2.4. ATPase assay

Preparation of membrane ghosts from Triton-filled lysosomes (TMG) of rat liver, solubilization of TMG, and ATPase assay were essentially based on the methods described previously [25,26].

2.5. Proton pump assay

Preparation of fluorescein isothiocyanate (FITC)–dextran loaded lysosomes (dextranosomes) from rat liver and proton pump assay (acidification) were performed according to the methods described previously [27–29].

2.6. Labeling of VSV G protein

Preparation of VSV (New Jersey serotype) and labeling of VSV G protein were basically performed according to the methods described previously [9]. After BHK cells were absorbed with VSV (MOI 10) for

2.5 h in a microtiter plate, the medium was replaced with methionine deficient medium. BHK cells were incubated for 1.5 h in the presence or absence of prodigiosin 25-C. The culture medium contained the designated concentrations of prodigiosin 25-C throughout the experiment. ^{35}S protein labeling mixture (370 kBq/ml; NEN Research Product, Boston, MA) was added to the culture, and the cells were labeled for 2 h and chased for 2 h in the presence of unlabeled methionine (150 μg/ml) and cycloheximide (1 μg/ml). The culture supernatant was centrifuged at 150 $\times$ g for 5 min and the resultant precipitates and the cells were combined (the cell fraction).

2.7. Transmission electron microscopy

BHK cells removed from culture dishes using trypsin were prefixed with glutaraldehyde, postfixed with osmium tetroxide and embedded in Epon 812 after dehydration in a series of ethanol concentrations.
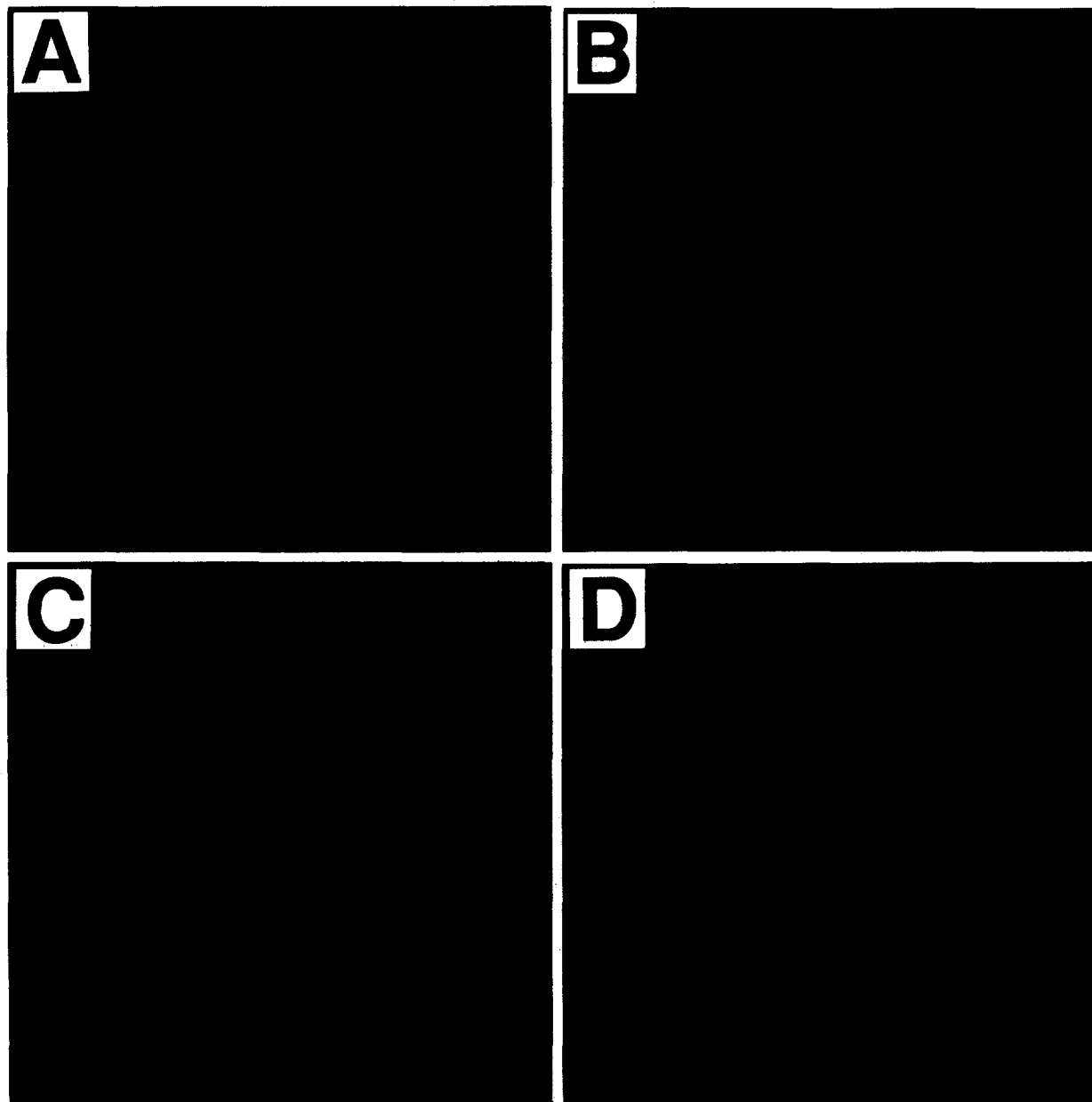

Fig. 1. Visualization of vacuolar acidification in BHK cells. (A–D) BHK cells were incubated in the absence (A) or presence (B, 25 nM; C, 250 nM; and D, 2.5 μM) of prodigiosin 25-C for 30 min, and further incubated with DAMP for 30 min. DAMP was visualized by the method described in section 2. The cells were observed under fluorescent microscopy. (E and F) BHK cells were incubated in the presence of prodigiosin 25-C alone (E, 2.5 μM; and F, 25 μM) for 1 h, and the autofluorescence of prodigiosin 25-C was viewed under fluorescent microscopy. (G and H) BHK cells were incubated in the absence (G) or presence of 250 nM prodigiosin 25-C (H) for 30 min, and further incubated with acridine orange for 30 min. The cells were observed under fluorescent microscopy.
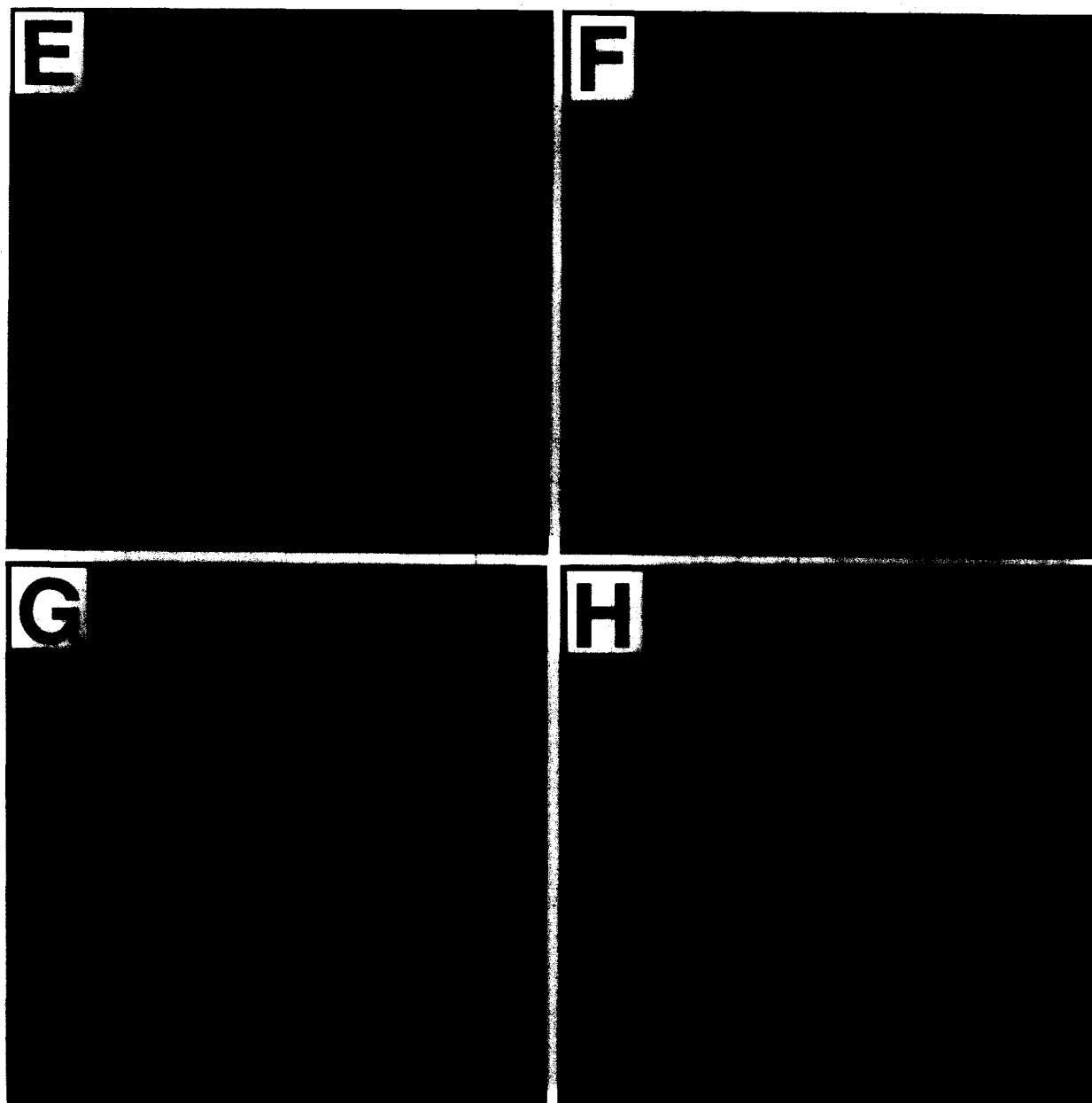
Fig. 1. (continued).

Ultrathin sections were stained with uranyl acetate and lead citrate, and observed under 1200EX transmission electron microscopy (JEOL, Tokyo, Japan).

3. Results

In order to visualize vacuolar acidification, DAMP was used as a pH probe (Fig. 1A–1D). Yellow granules were frequently observed in the cytoplasm of control BHK cells, showing acidification of vacuolar organelles (Fig. 1A). On the other hand, they were significantly decreased in cells treated with prodigiosin 25-C at 250 nM (Fig. 1C) and disappeared almost completely at 2.5 μM (Fig. 1D), suggesting an increase in vacuolar pH. Granular green fluorescence could also be seen in BHK cells treated with 2.5 μM prodigiosin 25-C (Fig. 1D), which is most probably due to autofluorescence of prodigiosin 25-C

because BHK cells treated with prodigiosin 25-C alone showed similar green fluorescent areas at 2.5 μM (Fig. 1E) and 25 μM (Fig. 1F). Interestingly, prodigiosin 25-C appears to be selectively localized in cytoplasmic granules and accumulated on one side of the cytoplasm near the nucleus but not inside the nucleus or by the plasma membrane (Fig. 1F).

To have additional support for the pH increase produced by prodigiosin 25-C in intact cells, acridine orange stain was performed. Granular orange dots were observed in the green background in control BHK cells (Fig. 1G), whereas they were significantly smaller and decreased in number in cells treated with 250 nM prodigiosin 25-C (Fig. 1H). These results clearly demonstrate that prodigiosin 25-C raises the pH in acidic compartments of intact cells.

To see if this effect of prodigiosin 25-C is a consequence of its general degenerative effect, cellular ATP content was esti-

mated in cells treated with the drug (Table 1). Like other pH perturbing agents, prodigiosin 25-C had no inhibitory effect on the cellular ATP level at 250 nM and decreased it by only 10% at 2.5 μM. In contrast, oligomycin, an inhibitor of mitochondrial F-type ATPase, decreased the ATP level by approximately 50%. This result indicates that prodigiosin 25-C does not significantly affect cellular ATP production.

Prodigiosin 25-C is a lipophilic weak base [12], however, it hardly affected the internal pH (~5.7) of lysosomes isolated from rat liver in neutral buffer at the low concentrations (up to 1 μM) used in this study (data not shown). The internal pH of acidic organelles is maintained by the activity of V-ATPase. Accordingly, we next examined the effect of prodigiosin 25-C on the proton pump (Fig. 2A) and ATPase (Fig. 2B) activities of V-ATPase in isolated lysosomes from rat liver. Prodigiosin 25-C inhibited the proton pump activity (acidification) with an IC$_{50}$ of approximately 30 nM (Fig. 2A). However, it hardly affected the ATPase activity up to 1 μM (Fig. 2B). In contrast, bafilomycin A$_1$ inhibited both proton pump and ATPase activ-

Fig. 3. Intracellular transport of VSV G protein. VSV-infected BHK cells were incubated at 37°C with prodigiosin 25-C (lanes 1 and 6, 0; anes 2 and 7, 2.5 nM; lanes 3 and 8, 125 nM; lanes 4 and 9, 250 nM; lanes 5 and 10, 2.5 μM;). The labeled proteins in the cell fraction (lanes 1–5) and the medium fraction (lanes 6–10) were separated by 10% SDS-PAGE and autoradiographed. L, large protein; G, glycoprotein; N, nucleocapsid protein; NS, non-structural protein; M, matrix protein.
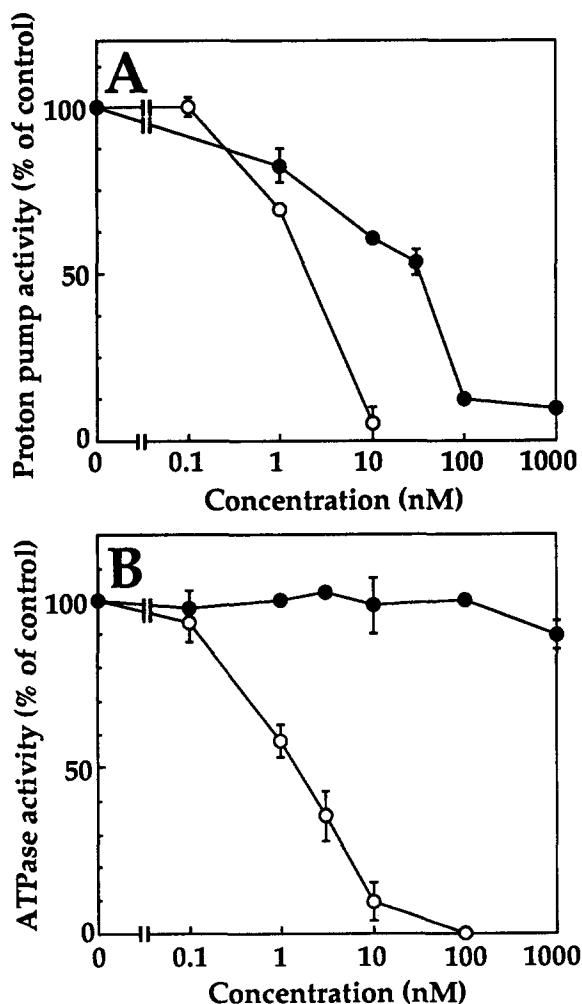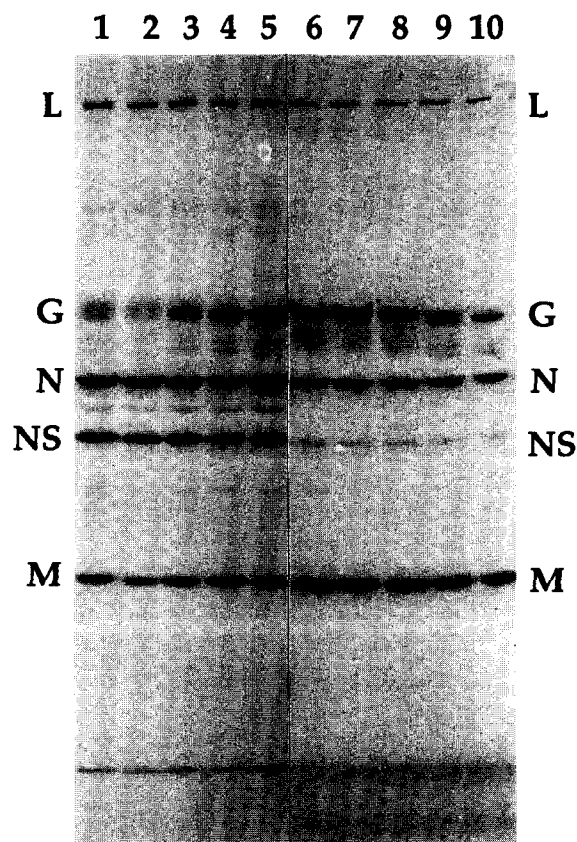

Fig. 2. Proton pump and ATPase activities in rat liver lysosomes. (A) FITC–dextran containing lysosomes were incubated with prodigiosin 25-C (●) or bafilomycin A$_1$ (○), and fluorescence quenching was measured after the addition of ATP. The plots were the average of duplicate samples. (B) V-ATPase activity in solubilized TMG was measured by the release of inorganic phosphate from ATP. The reaction mixture was incubated with prodigiosin 25-C (●) or bafilomycin A$_1$ (○). The plots are the average of duplicate samples.

ities strongly at the same concentration. Prodigiosin 25-C also inhibited the proton pump activity of lysosomal H$^+$-ATPase (IC$_{50}$ ≅ a few nM) in the reconstituted proteoliposomes which are free from anion channels (data not shown; S. Ohkuma et al., manuscript in preparation). These results suggest that prodigiosin 25-C inhibits the proton pump activity of V-ATPase by dissociating proton translocation from ATP hydrolysis.

VSV is a useful tool to study the intracellular transport of glycoproteins, since cellular macromolecular synthesis is shut off after the infection of VSV and only viral proteins, including

Table 1
Cellular ATP level in BHK cells

Treatments	Concentration (μM)	Cellular ATP (% of control) (pmol/10^3 cells)
Control		5.59 (100.0)
Prodigiosin 25-C	0.25	5.61 (100.4)
	2.5	5.06 (90.5)
Concanamycin A	1.0	5.80 (103.8)
Monensin	1.0	5.50 (98.4)
Oligomycin	0.1	2.77 (49.6)

BHK cells were treated with various inhibitors for 1 h and the cellular ATP content was measured. The data shown are averages of duplicate samples.
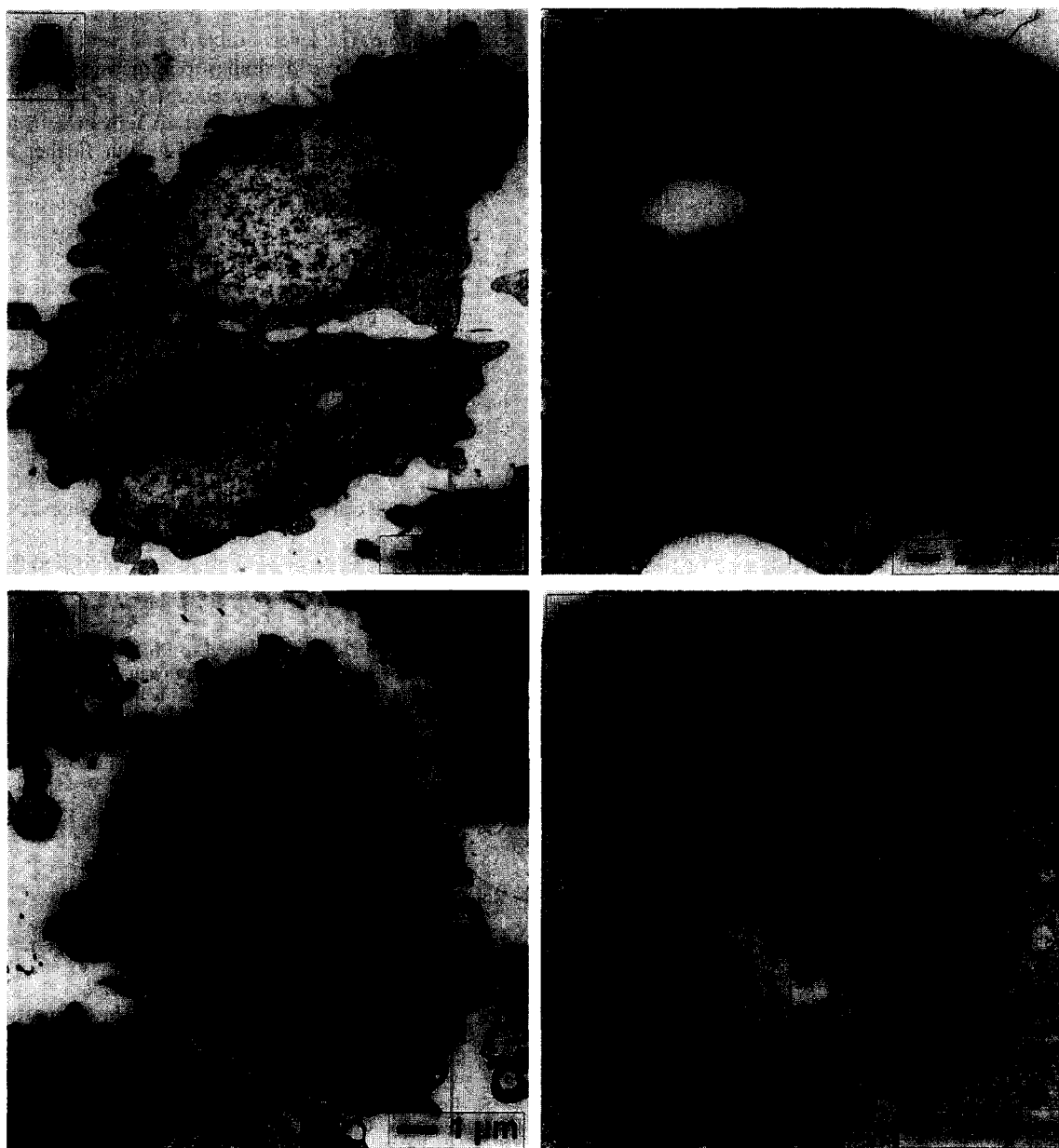
Fig. 4. Transmission electron micrographs of BHK cells. BHK cells were incubated in the absence (A and B) or presence of 250 nM prodigiosin 25-C (C and D) for 1 h. Panels B and D are magnified pictures of A and C, respectively.

G protein (glycoprotein), are synthesized. Fig. 3 shows the dose dependence of the effect of prodigiosin 25-C on the transport of VSV G protein. In control cells, G protein was secreted into the medium (lane 6) after 2 h chase in the presence of unlabeled methionine and cycloheximide, and only broad and faint bands were detected in the cell fraction (lane 1). In contrast, when cells were treated with prodigiosin 25-C at concentrations of 250 nM (lane 4) and 2.5 μM (lane 5), considerable amounts of G protein were clearly retained in the cell fraction. Under these conditions, G protein was also detected in the medium but its molecular weight was slightly decreased (lanes 9 and 10). Other proteins, such as L, N, NS and M, were not significantly influenced by prodigiosin 25-C. These results indicate that prodigiosin 25-C slows down the transport of G protein without decreasing protein synthesis.

We also observed the morphological changes in BHK cells treated with prodigiosin 25-C under transmission electron microscopy (Fig. 4). Golgi apparatus and mitochondria were clearly observed in control BHK cells (Fig. 4B), while Golgi apparatus were significantly dilated and mitochondria swollen in cells that were treated with 250 nM prodigiosin 25-C for 1 h (Fig. 4D). Prodigiosin 25-C did not induce any other significant changes in the endoplasmic reticulum or nucleus under these conditions.

4. Discussion

In this paper, we have shown that prodigiosin 25-C raises the internal pH of acidic organelles in intact cells through inhibition of the proton pump activity of V-ATPase, thereby causing

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the functional and morphological changes to Golgi apparatus. Prodigiosin 25-C inhibited the proton pump activity but not the ATP hydrolysis activity. This is consistent with our previous results on the plant V-ATPase showing inefficient inhibition of its ATPase activity by prodigiosin 25-C [20]. As anion channels do not seem to participate in the inhibition of acidification by prodigiosin 25-C, it is suggested that prodigiosin 25-C inhibits acidification (and increases vacuolar pH) by dissociating (or uncoupling) proton translocation from ATP hydrolysis on V-ATPase. Furthermore, prodigiosin 25-C does not show significant protonophore (S. Ohkuma, unpublished observation) or potassium ionophore [20] activities at the concentrations used, unlike general 'uncouplers of oxidative phosphorylation'. These observations indicate that prodigiosin 25-C may be a new type of inhibitor of H^+-ATPase with potential usefulness for the clarification of the proton translocation mechanism.

Probably as a consequence of increased vacuolar pH, prodigiosin 25-C delayed the transport of VSV G protein while slightly decreasing its molecular weight. Monensin blocks or slows down the intracellular transport of glycoprotein [21–23] and arrests the transport of VSV G protein prior to reaching the trans Golgi compartment. Bafilomycin A_1 and concanamycin A also effectively block the cell surface expression of VSV G protein [9,10] but concanamycin A stops the transport of G protein from the endoplasmic reticulum to the cis Golgi compartment [9]. Compared with these inhibitors, the action of prodigiosin 25-C on the transport of VSV G protein appears to be quite leaky. Moreover, prodigiosin 25-C up to 250 nM did not neutralize the toxicity of diphtheria toxin, but partially neutralized it at 2.5 μM in our system (data not shown), while bafilomycin A_1 neutralizes the toxicity of diphtheria toxin strongly [6]. These observations suggest that prodigiosin 25-C does not increase the pH of acidic compartments to the same level as the other inhibitors in intact cells.

The decrease in the molecular weight of G protein by prodigiosin 25-C might be due to a defect in the addition of terminal sugars such as sialic acids, because the decrease in the content of sialic acids on glycoproteins has been reported in acidification defective cells [30] and in cystic fibrosis which is defective of the chloride channel [31]. The internal pH of the trans Golgi/ trans Golgi network in cystic fibrosis is slightly raised in a range from pH 6.5 to 6.8 [31], since vacuolar acidification is dependent on chloride channels [32]. This pH increase is thought to reduce the activity of sialyltransferase which has an acidic pH optima and the content of sialic acids is decreased. A similar mechanism appears to occur in prodigiosin 25-C treated cells.

Prodigiosin 25-C induced significant dilation of Golgi apparatus. Monensin also induces a drastic dilation of Golgi apparatus, possibly due to the passive influx of water caused by ionic imbalance [22,23]. Although it is likely that the inhibition of the proton pump activity of V-ATPase by prodigiosin 25-C may trigger the ionic and/or osmotic imbalances, thereby resulting in the water influx, the precise mechanism leading to the dilation of Golgi remains to be clarified.

Although the morphological effects on Golgi apparatus are similar between prodigiosin 25-C and monensin, those on mitochondria are quite different from each other; prodigiosin 25-C also induced the swelling of mitochondria (Fig. 4), while monensin induces the contraction of mitochondria [22,23]. We have found that prodigiosin 25-C also inhibited the proton pump activity of mitochondrial ATPase (S. Ohkuma, unpub-

lished observation). However, prodigiosin 25-C did not decrease cellular ATP levels (Table 1) and viral protein synthesis in BHK cells at 2.5 μM, similar to monensin [23]. The swelling might be due to the accelerated uptake of potassium ions by energized mitochondria which retain active electron transport in the presence of prodigiosin 25-C, but further studies are required.

Prodigiosin 25-C accumulated in the cytoplasmic granules, as judged by its autofluorescence under microscopic observation. Since prodigiosin 25-C has an apparent pKa value of 7.62 [12], the drug appears to accumulate selectively as a weak base in acidic compartments, although a more specific mechanism might also participate in its perinuclear accumulation. On the other hand, the matrix of mitochondria in which F-ATPase is localized has a more alkaline pH than the cytosol when the electron transport system is active. Therefore, prodigiosin 25-C may accumulate inefficiently in the mitochondria and be unable to effectively inhibit the proton translocation of mitochondrial ATPase in live cells, and hence have little influence on cellular ATP levels.

Further studies are now in progress to clarify fully the mechanism of action of prodigiosin 25-C on H^+-ATPases.

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