In bafilomycin A_1-resistant cells, bafilomycin A_1 raised lysosomal pH and both prodigiosins and concanamycin A inhibited growth through apoptosis

Keiji Tanigaki[a], Satoshi Sasaki[b], Shoji Ohkuma[c,*]

[a] *Department of Dynamic Physiology, Division of Life Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Takara-machi 13-1, Kanazawa, Ishikawa 920-0934, Japan*
[b] *Department of Molecular Cellular Biology, Division of Pharmaceutical Science, Graduate School of Natural Science and Technology, Kanazawa University, Takara-machi 13-1, Kanazawa, Ishikawa 920-0934, Japan*
[c] *Laboratory of Biochemistry and Molecular Cell Biology, Department of Pharmaceutical Biology, Faculty of Pharmaceutical Sciences, Kanazawa University, Takara-machi 13-1, Kanazawa, Ishikawa 920-0934, Japan*

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Abstract In bafilomycin A_1-resistant cells (Vero-317 and MC-3T3-E1), bafilomycin A_1 neither inhibited cell growth, induced cell death, nor activated caspase-3. However, 100 nM bafilomycin A_1 did raise the lysosomal pH similar to 10 mM NH_4Cl. Prodigiosins, H^+/Cl^- symporters that raise the lysosomal pH, inhibited cell growth through apoptosis and caused the activation of caspase-3. Concanamycin A also inhibited the growth of these cells through apoptosis. 10 mM NH_4Cl inhibited the growth of these cells as well, but cytostatically. These results suggest that plecomacrolides inhibited cell growth apoptotically through specific site(s), in contrast to the cytostatic effect of 10 mM NH_4Cl, besides raising the lysosomal pH.

Key words: pH; Lysosome; Cell death; Apoptosis; Concanamycin

1. Introduction

Plecomacrolides, including bafilomycins and concanamycins, have been shown to inhibit vacuolar type H^+-ATPase (V-ATPase) more strongly than phosphorylated H^+-ATPase [1] and therefore are thought to be 'specific' inhibitors of V-ATPase [2,3]. However, their binding site is disputed: subunit c (16 kDa), subunit a (116 kDa), or other subunit proteins on V-ATPase [4–7]. Recent evidence using a photoaffinity labeling derivative of a concanamycin analogue suggested that plecomacrolides bind to subunit c [8]. The relationship of plecomacrolides with destruxins or enamides [9–11] (both inhibitors of V-ATPases) is not completely clear yet. Bafilomycin A_1 not only inhibited cell growth [12,13], but also induced cell differentiation [14,15] and apoptosis [2,3,16] by unknown mechanisms. Prodigiosins also inhibited the acidification of intracellular vesicles, including lysosomes, with little inhibition of ATP hydrolysis [17–20], and were found to act as H^+/Cl^- symporters [21]. In fact, prodigiosins inhibited proton pumps of respiration (Tanaka et al., in preparation) and H^+-pyrophosphatase [22]. Recently, cycloprodigiosin, like plecomacrolides, was found to induce cell differentiation and apoptosis in HL-60 cells [23]; the mechanism of its action remains to be clarified.

In the present paper, we report that (1) bafilomycin A_1 did not inhibit the growth of Vero-317 or MC-3T3-E1 cells, but that (2) 100 nM bafilomycin A_1, like 10 mM NH_4Cl, raised the lysosomal pH in each of these cell lines, and that (3) prodigiosins and concanamycin A inhibited the cell growth *apoptotically*. We also showed that (4) 10 mM NH_4Cl inhibited growth *cytostatically*.

2. Materials and methods

2.1. Materials

2′,7′-Bis(carboxyethyl)-5(6)-carboxyfluorescein-tetraacetoxymethyl ester (BCECF-AM) was purchased from Molecular Probes (Eugene, OR, USA). Hoechst 33258, propidium iodide (PI) and other chemicals were purchased mostly from Sigma (St. Louis, MO, USA).

2.2. Cell culture

CHO-K1 cells (a Chinese hamster ovary cell line) were cultured in F-12 Ham (Nissui, Tokyo, Japan) plus 10% fetal bovine serum (FBS; Wako, Tokyo, Japan) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). PC12 cells (a rat pheochromocytoma cell line) were cultured in Dulbecco's modified essential medium (Eagle) (DMEM; Nissui) plus 10% FBS (Wako), 5% horse serum (Wako) and antibiotics (see above). Vero-317 cells (a subclone of Vero cells from the African green monkey kidney, RCB-0272; RIKEN Cell Bank, Wako, Japan) were cultured in minimum essential medium (Eagle) (Nissui) plus 10% FBS and antibiotics. MC-3T3-E1 cells (C57BL/6 mouse fibroblast-like cells that can differentiate into osteoblasts, RCB-1126; RIKEN Cell Bank) were cultured in DMEM plus 10% FBS and antibiotics.

2.3. Determination of cell growth

Cell growth was measured based on the fluorescence increase of Hoechst 33258 due to the binding to DNA [12], according to [24].

2.4. Determination of caspase activity

Caspase-3 activity was measured using EnzChek® Caspase-3 Assay Kit #1 of Molecular Probes according to the manufacturer's instructions. The fluorescence of 7-amino-4-methylcoumarin (AMC) released

*Corresponding author. Fax: (81)-76-234 4462.
E-mail address: ohkuma@kenroku.kanazawa-u.ac.jp (S. Ohkuma).

Abbreviations: BCECF-AM, 2′,7′-bis(carboxyethyl)-5(6)-carboxy-fluorescein-tetraacetoxymethyl ester; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; DMEM, Dulbecco's modified essential medium (Eagle); DMSO, dimethylsulfoxide; FBS, fetal bovine serum; FD, fluorescein-labeled dextran; PBS(-), phosphate-buffered saline without Mg^{2+} and Ca^{2+}; PI, propidium iodide; V-ATPase, vacuolar type H^+-ATPase

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from Z-DEVD-AMC substrate (E-13183) was measured at λ_{ex} = 350 nm and λ_{em} = 450 nm in a spectrofluorometer (F-4500; Hitachi).

2.5. Estimation of lysosomal pH (pH_L) by fluorescein-labeled dextran (FD) fluorescence

Cells were cultured with 10 mg/ml of FD (MW ≈ 70 000) overnight and their fluorescence was observed under a confocal laser scanning fluorescence microscope (LSM-510; Karl Zeiss, Nagoya, Japan) with excitation at 488 nm (argon laser). The intensity of the light was kept constant during the measurement.

2.6. Estimation of cytoplasmic pH (pH_C)

Cultured cells (2×10^6) were treated with 0.05% trypsin–0.02% EDTA, centrifuged (1000 rpm for 5 min) and suspended in 2 ml of complete medium. The cells were incubated at 37°C for 30 min in 10 μM BCECF-AM, washed twice with phosphate-buffered saline without Mg^{2+} and Ca^{2+} (PBS(−)) and suspended in complete medium. Their fluorescence was measured in a spectrofluorometer (F-4500; Hitachi) with λ_{ex} = 490 nm and λ_{em} = 530 nm. The calibration of pH_C was done in situ using 1 μM nigericin-containing buffer that mimics cytoplasm according to [25].

2.7. Estimation of viability and apoptosis

The fluorescence of non-fixed cells was viewed under a fluorescence microscope (Karl Zeiss) using 50 nM PI (G-excitation with filter #20; red fluorescence for nuclei of dead cells) and/or 10 μg/ml of Hoechst 33258 (V-excitation with filter #1; blue fluorescence for nuclei); nuclear condensation or fragmentation is observed in apoptotic cells with either stain. FACS (fluorescence-activated cell sorter) using a FACSCalibur HG 3 Color type (Becton-Dickinson, San Jose, CA, USA) was also used to detect apoptosis (sub-G_0/G_1 cells) according to the manufacturer's instructions.

3. Results

3.1. Bafilomycin A_1 resistance of Vero-317 and MC-3T3-E1 cells

We found that bafilomycin A_1 did not inhibit the growth of Vero-317 cells or MC-3T3-E1 cells [2,3], whereas it inhibited the growth of CHO-K1 and PC12 cells [12]: IC_{50}s were > 500 nM–1 μM against Vero-317 and MC-3T3-E1 cells, versus 5–10 nM against CHO-K1 and PC12 cells (Fig. 1). In fact, in the bafilomycin A_1-resistant (Vero-317 and MC-3T3-E1) cells, 100 nM bafilomycin A_1 did not increase the caspase-3 activity required for apoptosis: 0.8 ± 0.2–1.0 ± 0.0 and 1.0 ± 0–1.0 ± 0 times that of the control level, respectively (mean ± S.D., n = 3), after 24–48 h. In contrast, 100 nM bafilomycin A_1 activated caspase-3 in PC12 and HeLa cells after 40 h: 3.6 ± 0.1 and 4.2 ± 0.2 times the control level, respectively (mean ± S.D., n = 3), consistent with the data on concanamycin A [26].

3.2. pH_L was increased by bafilomycin A_1 in the bafilomycin A_1-resistant cells

The fluorescence intensity of FD increases as the pH increases from 3 to 8 [27]. Fig. 2 shows views under a confocal fluorescence microscope of FD-containing cells. PC12 cells are sensitive to bafilomycin A_1 while Vero-317 and MC-3T3-E1 cells are both resistant to bafilomycin A_1. To our surprise, 100 nM bafilomycin A_1, like 10 mM NH_4Cl, increased the fluorescence intensity of all cells after 30 min, indicating arise in pH_L. These results show that the pH_L was increased by bafilomycin A_1 even in the bafilomycin A_1-resistant cells.

The pH_C of both Vero-317 and MC-3T3-E1 cells was about 7.6, which was slightly higher than that of PC12 cells (7.2). This is again contrary to expectation. The slightly higher pH_C decreased slightly, to 7.2, after treatment with either bafilomycin A_1, amiloride or 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) (data not shown).

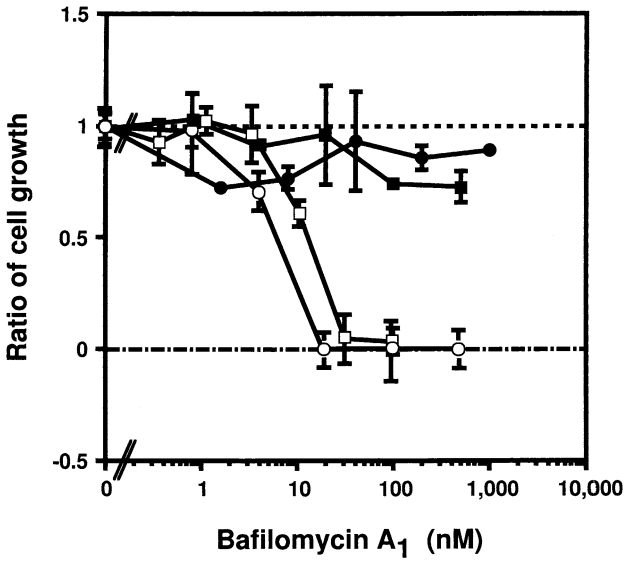

Fig. 1. Effect of bafilomycin A_1 on the growth of CHO-K1 (□), PC12 (○), Vero-317 (■) and MC-3T3-E1 (●) cells. Cell growth was estimated by the determination of cellular DNA content using Hoechst 33258 and a spectrofluorometer (Hitachi model F-4500, λ_{ex} = 356 nm and λ_{em} = 458 nm). Data represent the mean ± S.D. (n = 4).

3.3. Inhibition of the growth of bafilomycin A_1-resistant cells through apoptosis by prodigiosins

It is possible that bafilomycin A_1-resistant cells are defective in apoptosis in general. Recently, cycloprodigiosin was found to induce cell differentiation and apoptosis in HL-60 cells [23] similar to bafilomycin A_1. As shown in Fig. 3, the growth of MC-3T3-E1 cells was inhibited by prodigiosins. The growth of Vero-317 cells was also inhibited by prodigiosins. The IC_{50}s of prodigiosin and metacycloprodigiosin against the growth of Vero-317 and MC-3T3-E1 cells were both about 10 nM, in contrast to values of 800 nM and 600 nM, respectively, against the growth of PC12 cells. The apoptosis of these cells by prodigiosins was evident from the nuclear condensation and fragmentation (Fig. 4 for MC-3T3-E1 cells; Vero-317 cells showed similar results). FACS also indicated an increase in sub-G_0/G_1 cells in both populations (data not shown). Prodigiosins, as well as cisplatin (15 μg/ml for 40 h) [28], activated the caspase-3 of these cells (2–3.5 times; data not shown).

3.4. Inhibition of the growth of bafilomycin A_1-resistant cells through apoptosis by concanamycin A

Do other plecomacrolides affect the growth of bafilomycin A_1-resistant cells? As shown in Fig. 5, the growth of the bafilomycin A_1-resistant cells was inhibited by concanamycin A. The IC_{50}s of concanamycin A against the growth of Vero-317 and MC-3T3-E1 cells were about 0.3 nM and 10 nM, respectively. The IC_{50} of concanamycin A against the growth of PC12 cells was about 10 nM. The apoptosis of these cells by concanamycin A was evident from their nuclear condensation and fragmentation (as detected with Hoechst 33258 staining) as well as the increase of sub-G_0/G_1 cells in FACS analyses (data not shown).

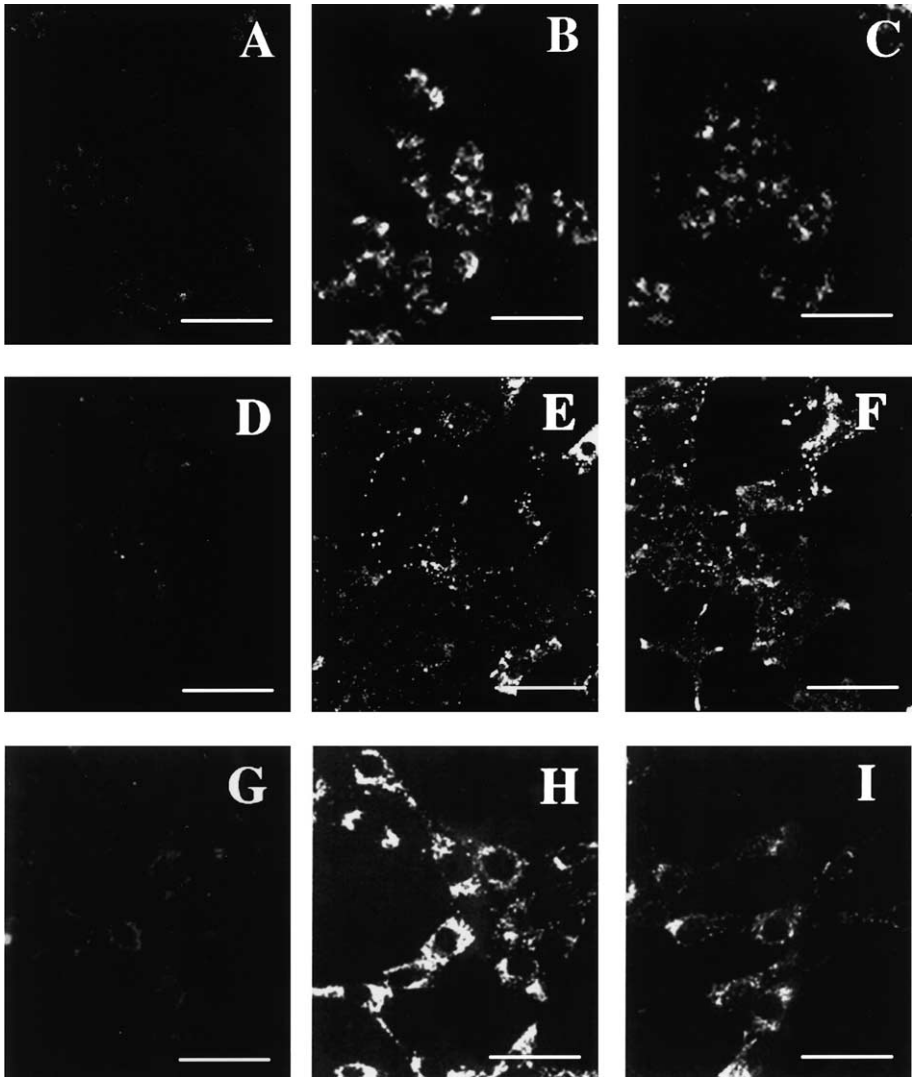

Fig. 2. Effects of bafilomycin A_1 on the pH_L of MC-3T3-E1 cells. Cells were cultured in 10 mg/ml FD-containing medium overnight (about 18 h) before being washed twice with PBS(−) and cultured in a drug-containing medium at 37°C for 30 min. The bars represent 50 μm. A–C: PC12 cells. D–F: Vero 317 cells. G–I: MC-3T3-E1 cells. A,D,G: 1% dimethylsulfoxide (DMSO). B,E,H: 10 mM NH_4Cl. C,F,I: 100 nM bafilomycin A_1.

3.5. *Cytostatic inhibition of the growth of cells by 10 mM NH_4Cl*

At 10 mM, NH_4Cl increased the pH_L of the bafilomycin A_1-resistant Vero-317 and MC-3T3-E1 cells as well as the bafilomycin A_1-sensitive CHO-K1 and PC12 cells (Fig. 2). At the same time, NH_4Cl inhibited the growth of all of these cells, with almost the same IC_{50} (~2 mM) (Fig. 6). However, all of these cells were viable in the presence of 10 mM NH_4Cl according to the viability test using PI (Fig. 7). This is in contrast to the apoptotic death of PC12 cells caused by bafilomycin A_1, as detected with PI and Hoechst 33258 (Fig. 7). However, arrest at a specific stage of the cell cycle was not evident at 10 mM NH_4Cl. PC12 cells were dead after treatment with 100 mM NH_4Cl (or 25 μM chloroquine), but they were not apoptotic, in contrast to cells treated with 100 μM bafilomycin A_1 (Fig. 7).

4. Discussion

The mechanism of growth inhibition by bafilomycin A_1 is not known [2,3,12,13]. The results in this paper indicate that bafilomycin A_1 increased the pH_L even in the bafilomycin A_1-resistant Vero-317 and MC-3T3-E1 cells. These cells still show growth inhibition through apoptosis in response to prodigiosins or cisplatin. This indicates that the sole increase in pH_L is actually a minor factor in growth inhibition induced by bafilomycin treatment. This is in accordance with previous findings that bafilomycin A_1-induced apoptosis of PC12 cells occurs independently of intracellular pH [15]. This also suggests that bafilomycin A_1 had no effect on the growth of Vero-317 or MC-3T3-E1 cells probably because it did not produce apoptosis in these cells even though it raised their pH_L.

Recently, the participation of caspase(s) has been suggested in bafilomycin A_1-induced apoptosis, consistent with the finding with concanamycin A [26]. Concanamycin A induced growth inhibition and apoptosis of Vero-317 and MC-3T3-E1 cells, suggesting that it induced apoptosis through specific apoptosis receptor site(s) different from bafilomycin A_1. Recently, evidence was presented suggesting that the site in V-ATPase for the binding of concanamycin in *Neurospora*

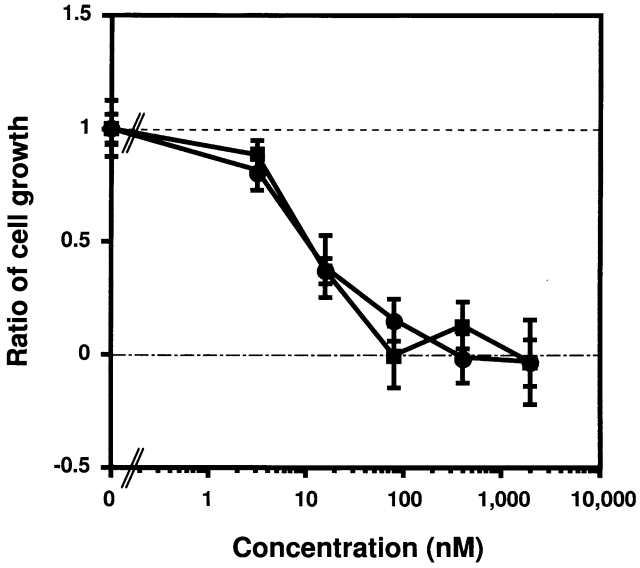

Fig. 3. Effects of prodigiosins on the growth of MC-3T3-E1 cells. The growth of MC-3T3-E1 was estimated as in Fig. 1. Data represent the mean ± S.D. ($n = 4$). ■, metacycloprodigiosin; ●, prodigiosin.

crassa is different from that for binding of bafilomycin A_1 [7]. Concanamycin A induced caspase-3-dependent cleavage of retinoblastoma protein, poly(ADP-ribose) polymerization [26] and intracellular apoptotic factor (33 kDa) in B-cell hybridoma HS-72 [29]. It also induced release of cytochrome *c* and decreased heat shock protein of 27 kDa in a human submandibular gland ductal cancer cell line (HGS) [30]. It would be interesting to see if bafilomycin A_1 and other V-ATPase inhibitors (including prodigiosins) induce similar effects.

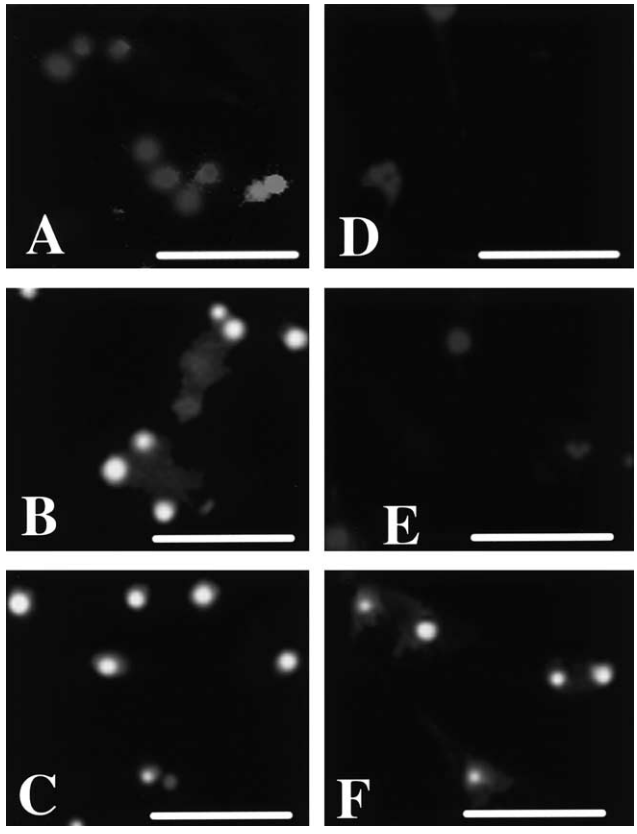

Fig. 4. Metacycloprodigiosin caused apoptosis in MC-3T3-E1 cells. The fluorescence of non-fixed cells was viewed after staining with Hoechst 33258 at 37°C for 30 min. The bars represent 50 μm. A–C: PC12 cells. D–F: MC-3T3-E1 cells. A,D: 1% DMSO. B,E: 100 nM bafilomycin A_1. C,F: 2 μM metacycloprodigiosin.

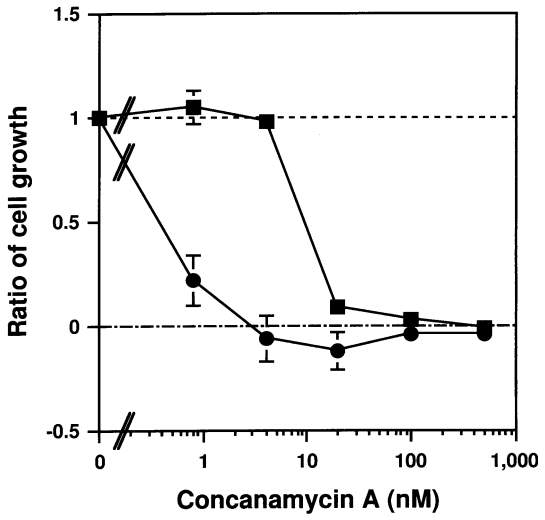

Fig. 5. Effects of concanamycin A on the growth of Vero-317 (■) and MC-3T3-E1 (●) cells. Cell growth was estimated as in Fig. 1. Data represent the mean ± S.D. ($n = 4$).

The mechanism of induction of apoptosis by prodigiosins is not clear yet. It might be similar to that of plecomacrolides because both types of compounds have similar effects on cells (cell differentiation and apoptosis). On the other hand, bafilomycin A_1 selectively inhibits the ATP hydrolysis of V-ATPase, while prodigiosins act as H^+/Cl^- symporters and inhibit proton translocation non-specifically with little effect on substrate catalysis (ATP/inorganic phosphate hydrolysis or respiration). The decrease in pH_C has been implicated in the apoptosis of liver cancer cell lines, HL-60 cells and breast cancer by cycloprodigiosin because 10 mM imidazole concomitantly

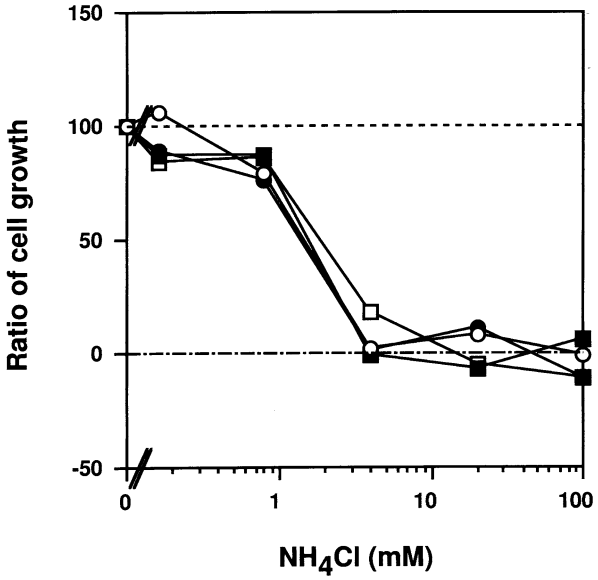

Fig. 6. Effects of NH_4Cl on the growth of CHO-K1 (□), PC12 (○), Vero-317 (■) and MC-3T3-E1 (●) cells. Cell growth was estimated as in Fig. 1. Data represent the mean ± S.D. ($n = 4$).

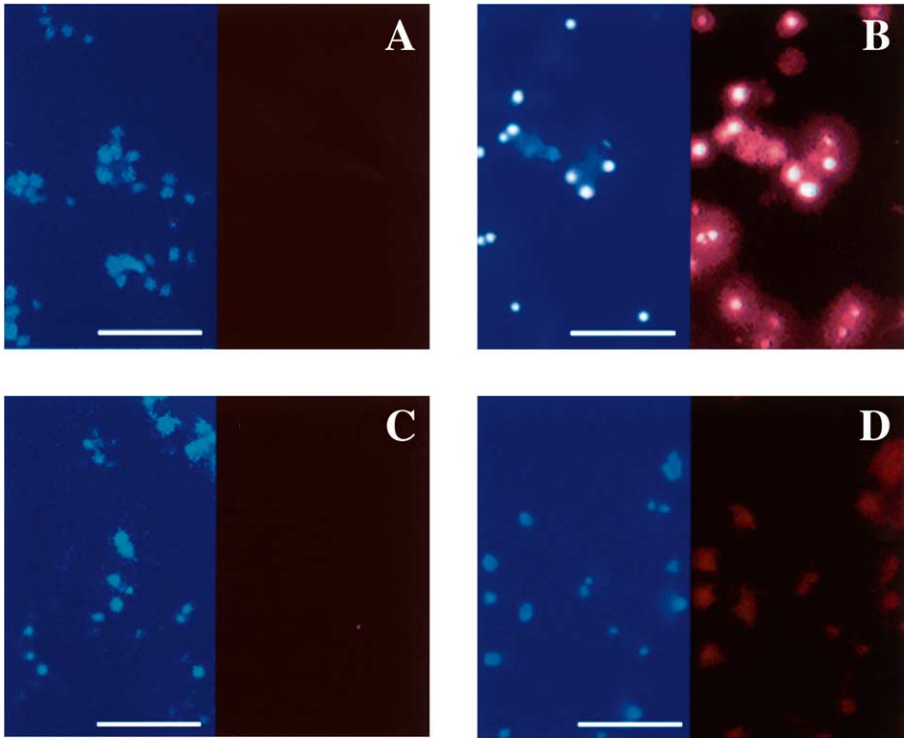

Fig. 7. Effect of NH_4Cl on viability and nuclear morphology of PC12 cells. PC12 cells were cultured in drug-containing medium for 72 h before treatment with 10 μg/ml of Hoechst 33258 and 10 μM PI. The same non-fixed cells were observed under a fluorescence microscope using V- or G-excitation at the same magnification. A: 1% (v/v) DMSO. B: 100 nM bafilomycin A_1. C: 10 mM NH_4Cl. D: 100 mM NH_4Cl. Red fluorescence indicates nuclei of dead cells stained with PI, and blue fluorescence indicates nuclei (live or dead) stained with Hoechst 33258. Nuclear condensation (bright) or fragmentation is observed in apoptotic cells (B) with either stain.

inhibited apoptosis and the decrease in pH_C [23,31,32]. In PC12 cells, however, 10 mM NH_4Cl or imidazole inhibited neither the neurite outgrowth nor the apoptosis caused by prodigiosins (manuscript in preparation). Furthermore, there is a paper insisting that the copper-promoted nuclease activity of prodigiosins correlated well with their death-inducing effect [33].

In bafilomycin A_1-resistant cells, the pH_C (7.6) was slightly higher than that in the PC12 cells (7.2). The reason for the more alkaline pH_C in the bafilomycin A_1-resistant cells is not clear at the moment. The higher pH_C was reduced only slightly to 7.2 by either bafilomycin A_1, amiloride or DIDS, suggesting little participation of V-ATPase, Na^+/H^+ antiporters or Cl^-/HCO_3^- antiporters [34,35]. Also, concanamycin A had only a minor effect on the pH_C in the bafilomycin A_1-resistant cells. In fact, functional V-ATPase was hardly detectable on the plasma membranes of Vero-317 or MC-3T3-E1 cells, as judged by the recovery from acidic pH_C caused by rapid removal of NH_4Cl from the medium [36] (data not shown).

In acidic vesicles, the pH clearly affects cellular functions, but the functional role(s) of vacuolation (swelling of acidic vesicles) is not clear [37]. This is the reason why researchers prefer bafilomycin A_1 to NH_4Cl: bafilomycin A_1 only raises the pH, while NH_4Cl both raises the pH and causes vacuolation, in acidic vesicles. Furthermore, bafilomycin A_1 has been thought to inhibit cell growth by increasing the pH (maintained by V-ATPase) in acidic vesicles [12,13]. However, the present results suggest that the sole increase in pH of acidic vesicles did not inhibit cell growth. Furthermore, we found that 10 mM NH_4Cl inhibited cell growth *cytostatically*, while 100 μM bafilomycin A_1 inhibited it *apoptotically*. The paper examining the effect of basic substances on the growth of 3T3 fibroblasts [37] and the one examining the parallel increase of pH_L between NH_4Cl- and bafilomycin A_1-treated cells [12] contain significant divergences. The mechanism of growth inhibition by 10 mM NH_4Cl, as well by bafilomycin A_1, is still to be clarified.

It must be emphasized that it was impossible to see the combined effect of alkalinization and vacuolation in acidic vesicles, before the discovery of bafilomycin A_1-resistant cells. Furthermore, the properties and relationships of the site(s) of various inhibitors against V-ATPase (including prodigiosins) for differentiation and for death remain to be clarified. Studies on their clarification are now in progress in our laboratory.

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