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MARINE BIOTECHNOLOGY

Accumulation of H^+ in Vacuoles Induced by a Marine Peptide Toxin, Theonellamide F, in Rat Embryonic 3Y1 Fibroblasts

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Abstract: The effects of theonellamide F, a marine bicyclic peptide, on vacuolar formation in cultured cells were studied. Theonellamide F induced large vacuoles in 6 types of mammalian cells. The vacuoles induced by theonellamide F in 3Y1 cells accumulated acridine orange, a fluorescent probe indicating the presence of an acidic organelle. Their disappearance following treatment with bafilomycin A1 suggests that these vacuoles contain vacuolar ATPase to maintain an acidic internal milieu, and this is similar to those induced by *Helicobacter pylori* toxin VacA. The vacuoles induced by theonellamide F were not significantly decreased in size or number by nocodazole treatment, and the localization of a small GTPase, rab7, did not always correspond to the outline of the vacuoles. These results suggest that the molecular mode of action of vacuolar formation by theonellamides may differ from that by VacA and can be considered unique.

Key words: theonellamide, vacuole, acridine orange, bafilomycin A1, bicyclic peptide, marine toxin.

INTRODUCTION

Marine sponges of the genus *Theonella* are prominent sources of metabolites exhibiting unusual chemical structures and biological activities of interest (Fusetani and Matsunaga, 1993). Some compounds isolated from these sponges have attracted the attention of cell biologists as potential molecular probes, and their physiological functions in the ecological system are also intriguing (Faulkner, 2000). For example, swinholide A and bistheonellide A were developed as actin-depolymerizing agents for use in functional studies of microfilaments in cellular morphology and movement (Bubb et al., 1995; Watabe et al., 1996; Terry et al., 1997; Saito et al., 1998). Cyclotheonamides are potent inhibitors of thrombin and thus are considered promising as antithrombotic agents (Lewis et al., 1993). Derivatives of calyculins, known to be potent inhibitors of protein phosphatases, were also isolated from a *Theonella* sponge (Steube et al., 1998).

Theonellamides isolated from *Theonella* sp. also show unique features of note. Theonellamide F (Figure 1) was initially isolated as an antifungal and cytotoxic constituent (Matsunaga et al., 1989), and isolation of its analogs followed (Bewley and Faulkner, 1994; Matsunaga and Fuse-

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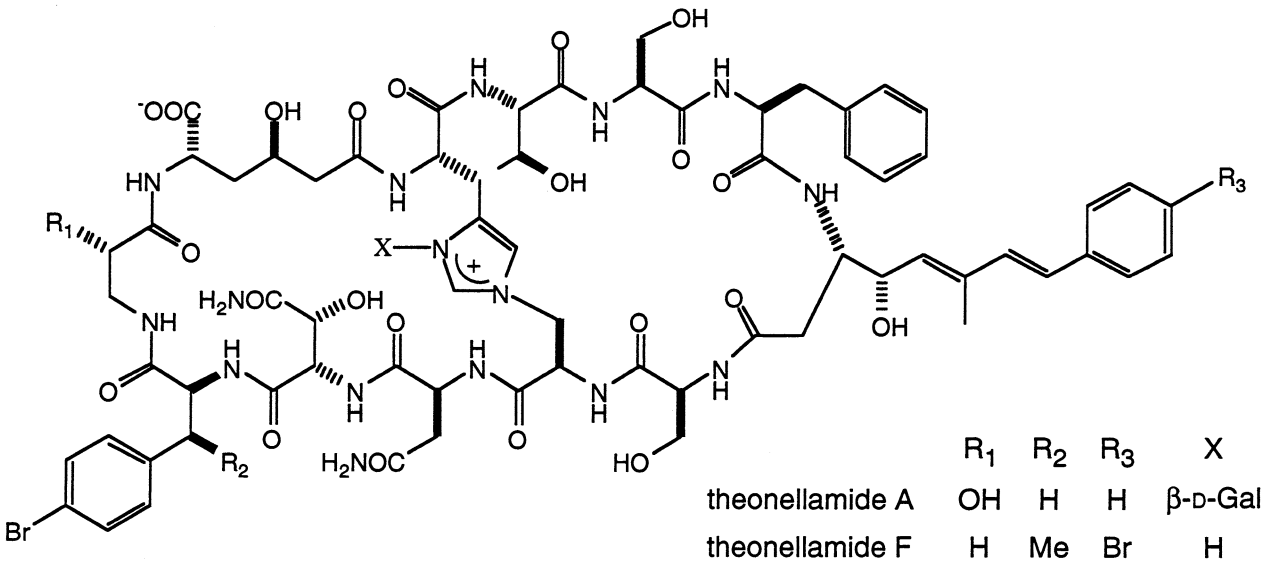

Figure 1. Structures of theonellamides A and F. Elements or groups representing positions R_1, R_2, R_3, and X in the structure are indicated.

tani, 1995; Schmidt et al., 1998). Theonellamide F showed moderate cytotoxicity against P388 murine leukemia cells (Matsunaga et al., 1989), and our previous study revealed that theonellamide F induced formation of extraordinarily large vacuoles in 3Y1 rat embryonic fibroblasts (Wada et al., 1999).

Monensin, a Na^+ ionophore currently used as a Golgi-disturbing agent, induces a similar type of vacuole formation in the cell (Tartakoff and Vassalli, 1977; Ledger et al., 1980; Mollenhauer et al., 1990; Dinter and Berger, 1998). However, we found that its toxicity was stronger than that of theonellamide F, since the cells treated with monensin at higher concentrations or for longer periods died without forming extraordinarily large vacuoles (Wada et al., 1999). Monensin exchanges H^+ in the intracellular membrane system with external Na^+ at a molar ratio of 1:1 (Mollenhauer et al., 1990). Some organelles have an active vacuolar ATPase (V-ATPase), which supplies H^+ to the organelles constantly and maintains their acidic internal environment (Pedersen and Carafoli, 1987; Futai et al., 1998; Nelson and Harvey, 1999; Forgac, 1999; Wieczorek et al., 1999). When cells are treated with monensin, a large amount of Na^+ flows into organelles with simultaneous outflow of an equal amount of H^+. However, some acidic organelles possessing V-ATPase are supplied with a high concentration of H^+, leading to a higher osmotic pressure within them than within other organelles, and thus resulting in the influx of water molecules (Mollenhauer et al., 1990). These organelles swell to become large vacuoles, visible even with a light microscope. The inner pH of these vacuoles is higher than that of the original organelles, since the H^+ concentration is reduced as much as the Na^+ inflow by the action of monensin (Basu et al., 1981; Marnell et al., 1982; Marsh et al., 1982; Maxfield, 1982; Tycko et al., 1983; Pohlmann et al., 1984; Anderson and Pathak, 1985; Yanagishita and Hascall, 1985).

The cytotoxin VacA, produced by pathogenic strains of *Helicobacter pylori*, is another well-studied agent known to induce formation of large vacuoles in cells (Leunk et al., 1988; Catrenich and Chestnut, 1992; Cover and Blaser, 1992; Cover, 1996; Papini et al., 1998). Although the mechanism of vacuolar formation by this toxin is still ambiguous, some aspects of its action have been reported. Vacuoles induced by this toxin in cells originate from late endosomal compartments (Papini et al., 1994) and possess V-ATPase activity. The interior of the vacuoles is acidic, in contrast to those induced by monensin (Cover et al., 1993; Papini et al., 1993, 1994, 1996). The increase in vacuolar size is promoted by the function of the small GTP-binding protein rab7 (Papini et al., 1994, 1997).

The fact that theonellamide F induces larger, easily observable vacuoles with low fatality in fibroblasts augurs its potential utility as a molecular probe for studies on intracellular membrane structures (Wada et al., 1999). Thus how theonellamides initiate vacuolar formation on 3Y1 fibroblasts is of interest. Toward this aim, in this study we examined the features of the vacuoles induced by theonellamide F for comparison with those induced by other vacuole-forming agents. Vacuoles induced by theonellamide F absorbed acridine orange and disappeared

when treated with bafilomycin A1, suggesting that the V-ATPase–mediated accumulation of protons in these vacuoles was similar to the case of VacA. However, these vacuoles' formation processes were somewhat distinct in terms of the functions of microtubules and rab7.

Materials and Methods

Chemicals

Theonellamides A and F were isolated from the marine sponge *Theonella* sp. as previously described (Matsunaga et al., 1989; Matsunaga and Fusetani, 1995). The bicyclic peptides were dissolved in deionized water at 1 mg/ml as stock solutions and stored at 4°C. Other reagents, unless indicated otherwise, were of analytical grade and obtained from either Sigma Chemical (St. Louis, Mo.) or Wako Pure Chemicals (Osaka, Japan). Stock solutions of other compounds such as lasalocid, bafilomycin A1, 2′,7′-*bis*(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM), and nocodazole were prepared in dimethyl sulfoxide at 1 mM, 2 μM, 1 mM, and 100 μg/ml, respectively. Monensin, TN-16, and nicotine were dissolved in methanol at 500 μg/ml, 1 mM, and 3 M, respectively. Nigericin was dissolved in ethanol at 5 mM. Acridine orange was dissolved in the cell culture medium described below at 1 mg/ml immediately before use.

Cell Culture and Treatment with Reagents

The Japanese Cancer Research Resources Bank provided rat embryonic fibroblasts, 3Y1 B clone 1-6 (Kimura et al., 1975); mouse embryonic fibroblasts, NIH3T3 cells (Jainchill et al., 1969); hamster kidney-derived cells, BHK21 (Macpherson and Montagnier, 1964); Chinese hamster ovary fibroblasts, CHO cells (Puck et al., 1958); and human cervical cancer cells, HeLa cells (Gey et al., 1952). These cells, with the exception of HeLa cells, were cultured as described previously (Wada et al., 1998) in 5% CO_2 at 37°C and low glucose concentration (1 g/L) of Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Rockville, Md.) containing 10% fetal calf serum (BioWhittaker, Walkersville, Md.) and an antibacterial-antimycotic cocktail (Gibco BRL) consisting of 100 U/ml penicillin G sodium, 100 μg/ml streptomycin, 250 ng/ml amphotericin B, and 10 μg/ml gentamicin. HeLa cells and human endothelial cells EA.hy 926 (Edgell et al., 1983), provided courtesy of Dr. C.-J.S. Edgell (University of North Carolina), were cultured under the same conditions except for the use of Earle-modified minimal essential medium (MEM) (Gibco BRL) and high glucose concentration (4.5 g/L) of DMEM, respectively. The cell morphology was observed with an Olympus IX-70 phase-contrast microscope equipped with an IX-FLA fluorescent apparatus and an SC35 camera. In experiments of fluorescence staining of cells, filter cubes U-MNIBA and U-MWIG were used for observation of green and red fluorescence, respectively. The number and size of the vacuoles induced by theonellamide F were determined from micrographs.

When cells were treated with the reagents, final concentrations of the solvents were limited to less than 1%, which showed no significant, augmentable, or detrimental effects. The cells for microscopic observation were plated in each well of 24-well microplates at 1×10^4 cells per 500 μl of medium, and cultured for 24 hours before treatment with reagents.

Uptake of Acridine Orange into 3Y1 Cells

The localization of acridine orange was observed in 3Y1 cells by addition of 20 μl of 1 mg/ml acridine orange solution to each well of the cells treated with respective agents and incubated at 37°C for 10 minutes. After washing 4 times with phosphate-buffered saline (PBS) containing 138 mM NaCl, 2.7 mM KCl, 7.5 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 (pH 7.4), fluorescence images were observed with the fluorescence microscopic system.

In the experiments to determine uptake of acridine orange, cells were plated in each well of the microplate at 1.0×10^5 cells per 500 μl of medium and incubated for 24 hours. The cells in a quarter of the wells were treated with theonellamide F and other agents under the same conditions, and stained with acridine orange followed by washing as described above. The nuclei of the cells in one well of each treatment were observed with the green fluorescence of the dye, and photographed. The nuclei in 6 randomly selected squares of 9×10^{-2} mm^2 were counted from the photograph to estimate the number of cells in a well after a series of treatments with the reagents. Then 500 μl of 10% sodium dodecyl sulfate (SDS) solution containing 10 mM HCl was added to the other 3 wells and incubated at 37°C for 30 minutes to dissolve acridine orange in the cells. The fluorescence of the solution was measured with a SPECTRA MAX microplate spectrofluorometer (Molecular Devices, Sunnyvale, Calif.) at λex. 492 nm and λem. 533 nm and

compared with a standard curve prepared from various concentrations of acridine orange, subjected to the same treatment.

Flow Cytometry

The average intracellular pH (pH_i) in whole cells of 3Y1 fibroblasts was determined by the method of Franck et al. (1996) using BCECF-AM. The cells suspended in 5.25 ml of DMEM at 2×10^4 cells/ml were plated on cell culture dishes 6 cm in diameter and incubated at 37°C for 24 hours. After treatment with theonellamide F for 24 hours, cells were washed with PBS and incubated in 5 ml of 0.1% trypsin-containing PBS at 37°C for 5 minutes. The same volume of the culture medium was added to terminate the reaction, and after centrifugation of the suspension at 250 *g* for 5 minutes, the supernatant was removed. The cell pellet was washed with Earl's balanced salt solution (EBSS) containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, and 25 mM HEPES (pH 7.3). Cells were suspended in 90 μl of EBSS, BCECF-AM was added at 10 μM, and cells were incubated at 37°C for 25 minutes. The cells were centrifuged at 250 *g* for 5 minutes, stored in ice, and resuspended in 3 ml of fresh EBSS immediately before cytometric analysis.

The standards of pH_i were measured using high $[K^+]$ buffers of known pH. These buffers were prepared by mixing appropriate proportions of mixtures A (135 mM KH_2PO_4, 20 mM NaCl) and B (110 mM K_2HPO_4, 20 mM NaCl), containing 10 μM nigericin. The control cells, after being stained with BCECF-AM as described above, were suspended in 3 ml of each buffer and incubated for 5 minutes before the analysis. The cells were analyzed using an EPICS Elite ESP flow cytometer (Coulter, Miami, Fla.). BCECF generated in the cells was excited by argon laser with λex. 488 nm, and the green and red fluorescence emissions were selectively detected using 525-nm and 610-nm bandpass filters, respectively. Green to red fluorescence ratios were calculated from the cytometer data analysis system.

Localization of rab7

The localization of rab7 in cells treated with theonellamide F was observed according to the method of Papini et al. (1997). Briefly, cells were fixed with 3% paraformaldehyde containing PBS for 20 minutes, treated with a solution containing 0.27% NH_4Cl and 0.38% glycine for 10 minutes, and permeabilized with a solution containing 0.2% saponin and 0.5% bovine serum albumin (BSA) in PBS for 30 minutes. Anti-rab7 primary antibody, sc-6563, obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.) was diluted in the permeabilization buffer to 1:5 and incubated with the cells for 2 hours. After several washes with the buffer, the secondary antibody of rhodamine-conjugated antigoat IgG (Chemicon, Temecula, Calif.) diluted to 1:50 in the buffer, was reacted with the cells for 1 hour. Then the cells were washed 3 times for 5 minutes with the buffer and observed with the fluorescence microscopic system described previously.

Results

Vacuolar Induction in Cells by Theonellamides

Results shown in Figure 2 indicate that theonellamides induced the formation of vacuoles tin 3Y1 fibroblasts, as in our previous report (Wada et al., 1999). The cells spread out on the substratum with well-developed lamellipodia at interphase (Figure 2 A). Many large vacuoles were induced around the nuclei of the cells treated with 2 μM theonellamide F for 24 hours (Figure 2 B). This concentration was 3-fold lower than that in our previous report (Wada et al., 1999). Instead of dimethyl sulfoxide, which was previously used, theonellamide F was dissolved in water and stored at 4°C to avoid repeated freezing and thawing, so the stability of the agent would be increased. Theonellamide A, though less effective, also induced such vacuoles at 4 μM for 24 hours (Figure 2 C). The outline of these cells hardly changed and showed lamellipodia at 24 hours (Figure 2 B, and C, arrows). Extraordinarily large vacuoles exceeding 30 μm in diameter, almost as large as the original 3Y1 cells, were induced in the cells at higher concentrations and prolonged treatments with the agents (Figure 2 D, arrow; Table 1). These cells remained alive under such conditions, continued to attach to the substratum, and grew to confluence.

When treated with theonellamides, similar effects were also observed in the other strains of cultured cells NIH3T3 (Figure 2 E), HeLa (Figure 2 F), BHK21, CHO, and EA.hy 926 (data not shown). HeLa and BHK21 cells showed similar sensitivities to theonellamides A and F as 3Y1 cells. EA.hy 926 cells were more sensitive to these agents than 3Y1 cells, while smaller and fewer vacuoles were induced in NIH3T3 cells in the same treatment. Although vacuolation

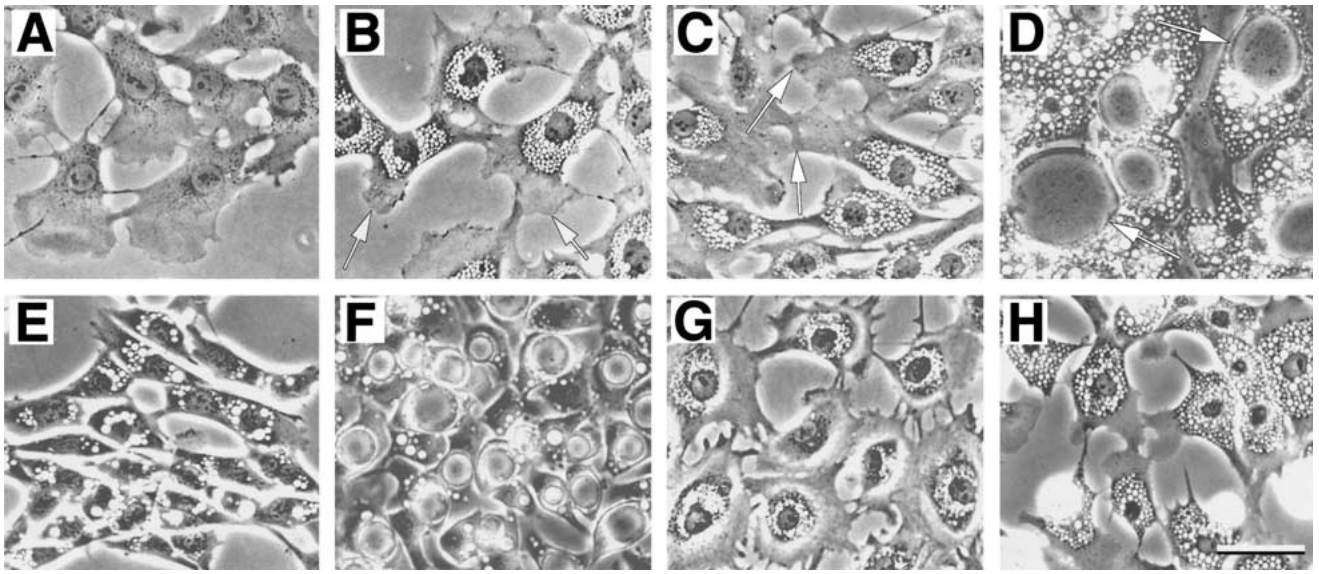

Figure 2. Induction of vacuolar formation in cells by theonellamides A, F, and other low molecular weight compounds. Rat embryonic 3Y1 fibroblasts (**A**) were treated with 2 μM theonellamide F for 24 hours (**B**), 4 μM theonellamide A for 24 hours (**C**), or 10 μM theonellamide A for 72 hours (**D**). Mouse embryonic 3T3 fibroblasts were treated with 6 μM theonellamide F for 24 hours (**E**), and human cervical carcinoma HeLa cells were treated with 4 μM theonellamide F for 72 hours (**F**). Rat embryonic 3Y1 fibroblasts were treated with 10 μM lasalocid for 3 hours (**G**), or 6 mM nicotine for 24 hours. (**H**). Arrows indicate lamellipodia in panels B and C, and indicate extraordinarily large vacuoles in panel D. Magnification is the same in all panels, and the bar in panel H represents 50 μm.

Table 1. Effect of Theonellamide F and Monensin on Vacuole Production in 3Y1 Cells*

	Number of vacuoles by size		
Treatment	0.5–5 μm	5–30 μm	>30 μm
Control (TF 0), 24 h	0.27 ± 0.83	0	0
TF 2 μM, 24 h	50.77 ± 21.56	1.50 ± 1.70	0
TF 6 μM, 72 h	43.77 ± 14.97	26.17 ± 11.99	0.77 ± 0.43
TF 2 μM + BA 10 nM, 24 h	0.13 ± 0.43	0	0
TF 2 μM, 24 h + BA 10 nM, 3 h	3.57 ± 6.64	0.07 ± 0.25	0
MN 3 μM, 24 h	15.00 ± 11.36	0.50 ± 0.78	0
MN 3 μM + BA 10 nM, 24 h	28.40 ± 15.03	0.13 ± 0.51	0

*Number of vacuoles produced is given as mean ± SD. Cells after each treatment were photographed, and the number and size of the vacuoles were counted in 30 randomly selected cells. TF indicates theonellamide F; BA, bafilomycin A1; and MN, monensin.

was also observed in CHO cells, these cells easily died without forming large vacuoles (data not shown).

Among low molecular weight compounds, monensin, a Na^+ ionophore, induced a similar type of vacuole in 3Y1 cells at 2 to 10 μM for 24 hours (Wada et al., 1999). A Ca^{2+} ionophore, lasalocid, has been reported to induce vacuoles in cells (Somlyo et al., 1975) and was therefore tested on 3Y1 fibroblasts in this study. Lasalocid induced vacuoles of similar shape and size in the cells at 10 μM for 3 hours (Figure 2 G). Nigericin, another ionophore, also induced such vacuoles at concentrations similar to the theonellamides (data not shown). Among the weak bases that induce the formation of vacuoles in cells (Ohkuma and Poole, 1981; Cover et al., 1992), nicotine induced similar vacuoles, although the effective concentration was higher than 3 mM (Figure 2 H). However, these ionophores and a weak base, nicotine, did not induce extraordinarily large vacuoles exceeding 15 μm in diameter, but did induce cell death when applied at higher concentrations or for longer time periods.

Acidity in Vacuoles Induced by Theonellamide F

The pH of vacuoles in cells treated with monensin was higher than that of their original organelles, since intravacuolar H^+ is replaced by extravacuolar Na^+ (Mollenhauer et al., 1990). Thus the pH of vacuoles induced by theonellamide F was compared with the intravacuolar pH of monensin-treated cells, with the aid of acridine orange, a common dye used to visualize acidic organelles in cells (Canonico and Bird, 1969; Moriyama et al., 1982). Fluorescence of this acidotropic dye is green at low concentrations and changes to orange at high concentrations; thus nuclei appear green and acidic organelles appear orange (Yoshimori et al., 1991).

Particles emitting weak red fluorescence were randomly seen in 3Y1 cells treated with acridine orange (Figure 3, A and B); in the cells treated with 3 μM monensin for 24 hours, acridine orange did not localize in vacuoles (Figure 3, C and D, arrows). Similar results were observed in the vacuolated cells induced by lasalocid and nicotine, although the cells treated with nicotine showed severe damage after acridine orange treatment (data not shown). The cells treated with theonellamide F showed contrasting results. Theonellamide F–induced vacuoles less than 5 μm in diameter disappeared under treated with 40 μg/ml of acridine orange (Figure 3 E), and the dye was localized where vacuoles had previously been formed by treatment with theonellamide F at 2 μM for 24 hours (Figure 2 B; Figure 3 E and F). Although large vacuoles remained during accumulation of acridine orange, they also highly absorbed the dye (Figure 3, G and H). An increase in the concentration of theonellamide F caused a proportional uptake of acridine orange in living cells (Figure 4). These results suggest that the vacuoles induced by theonellamide F are acidic.

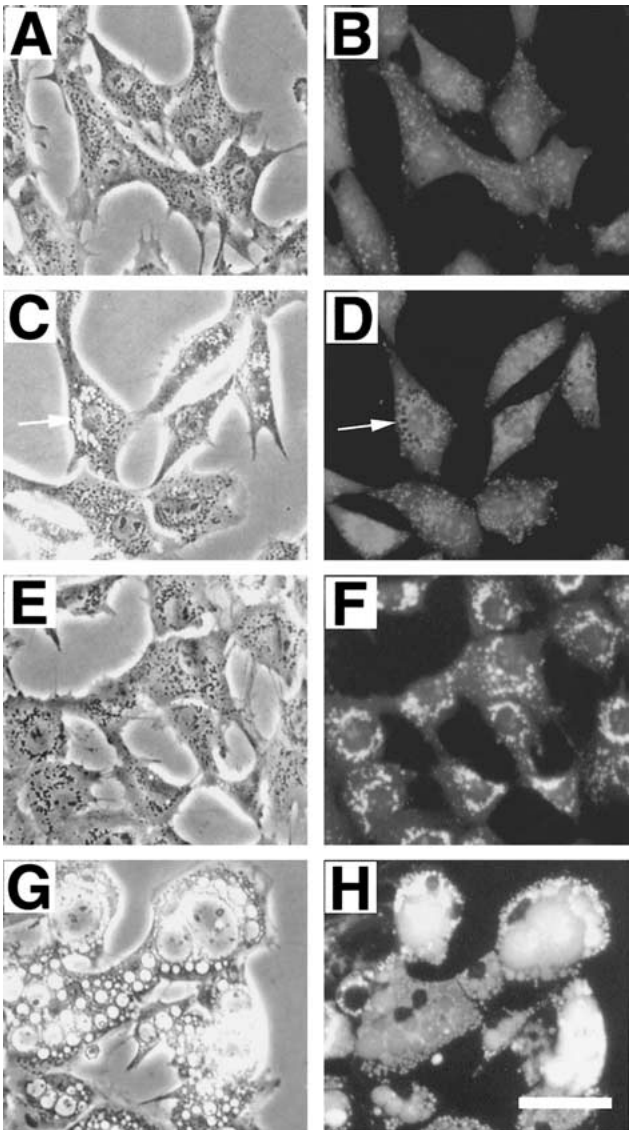

Figure 3. Accumulation of acridine orange in vacuoles induced by theonellamide F. 3Y1 cells without any treatment **(A, B)**, treated, with 3 μM monensin for 24 hours **(C, D)**, 2 μM theonellamide F for 24 hours **(E, F)**. and 5 μM theonellamide F for 24 hours **(G, H)**, were stained with 40 μg/ml acridine orange for 10 minutes. Panels A, C, E, and G are phase-contrast microscopic images, and panels B, D, E, and H are corresponding images of the red fluorescence of acridine orange. Arrows in panels C and D indicate the vacuoles in which dye did not accumulate. Magnification is the same in all panels, and the bar in panel H represents 50 μm.

Involvement of V-ATPase In Formation of Vacuoles

Several organelles such as lysosome have V-ATPase, which pumps H^+ into the organelles and maintains the acidic internal environment (Pedersen and Carafoli, 1987; Futai et al., 1998; Nelson and Harvey, 1999; Forgac, 1999; Wieczorek et al., 1999). Bafilomycin A1 is a specific inhibitor of this enzyme (Bowman et al., 1988). When treated with 10 nM bafilomycin A1 for 24 hours, 3Y1 fibroblasts became spindle-shaped (Figure 5, A). The vacuolating effect of 2 μM theonellamide F was completely inhibited by concomitant treatment with 10 nM bafilomycin A1 for 24 hours (Table 1), and the cells showed morphology characteristic of bafilomycin A1 treatment only (Figure 5, B). Vacuoles smaller than 5 μm in diameter formed upon treatment with theonellamide F, then disappeared with the

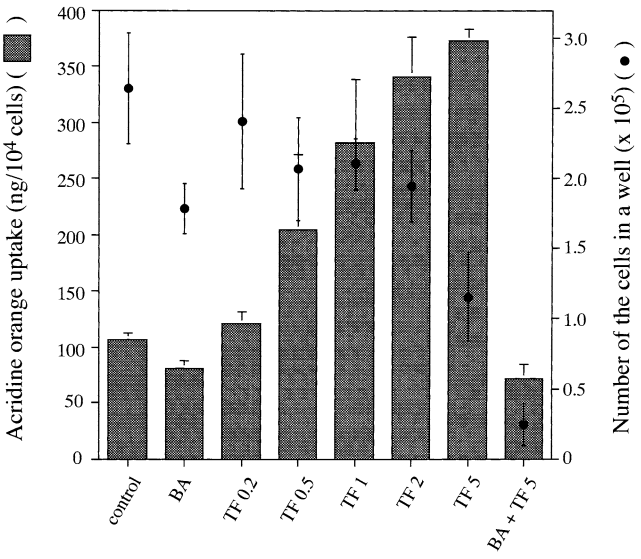

Figure 4. Acridine orange uptake into cells treated with theonellamide F. Cells after treatments with theonellamides at each concentration for 24 hours were stained with 40 μg/ml acridine orange for 10 minutes, then homogenized with 10% SDS solution containing 10 mM HCl, and the extracted dye was measured for the intensity of the fluorescence. After staining with the dye, living cells were counted in 6 randomly selected squares 9×10^{-2} mm^2 on the photographs, and the number of cells per well was estimated. TF represents theonellamide F; BA, bafilomycin A1. Numbers after TF indicate the micromolar concentration of theonellamide F used. Bafilomycin A1 was used at 10 nM, and theonellamide F and bafilomycin A1 coexisted in one treatment. Data are presented as mean ± SD (n = 3).

addition of 10 nM bafilomycin A for 3 hours (Figure 5, C; Table 1); in contrast, the number of vacuoles increased in the cells treated with both monensin and bafilomycin A1 (Figure 5, D; Table 1).

The localization of acridine orange in the vacuoles induced by theonellamide F disappeared, and fluorescence of the dye became weak after treatment with 10 nM bafilomycin A1 for 3 hours (Figure 5 E). As shown in Figure 4, cells treated with 5 μM theonellamide F for 24 hours absorbed a higher quantity of acridine orange than control cells. This effect was drastically inhibited by the presence of 10 nM bafilomycin A1 (Figure 4), although cell death increased under such severe conditions. These results suggest that vacuoles induced by theonellamide F contain V-ATPase, which is responsible for the vacuolar increment in size and number.

The acidic vacuolar formation induced by theonellamide F was accompanied by a gross decrease of pH_i. When treated with 2 μM theonellamide F for 24 hours, the pH_i of the cells was lowered by more than 0.3 from the mean value of the control (Figure 6).

Comparison of Theonellamide F–Induced and VacA-Induced Vacuoles

Induction of acidified vacuoles has also been reported in cells treated with a toxic protein of *H. pylori*, VacA (Leunk et al., 1988; Catrenich and Chestnut, 1992; Cover and Blaser, 1992; Cover, 1996, Papini et al., 1998). While Cover et al. (1992, 1993) and Papini et al. (1993, 1994, 1996, 1997) had previously examined the process of vacuole formation in cells treated with VacA, we compared the features of vacuole induction by theonellamide F to those of VacA. In the case of VacA, vacuolar formation is inhibited by monensin (Cover et al., 1992) and tubulin-depolymerizing agents (Papini et al., 1994) in HeLa cells. Cells treated with 2 μM theonellamide F for 24 hours in the presence of 6 μM monensin became spindle-shaped and vacuoles were reduced in number and size (Figure 7 A); however, up to 1 μg/ml nocodazole did not significantly inhibit vacuolar induction by 2 μM theonellamide F (Figure 7 B). The same result was obtained when another tubulin-depolymerizing agent, TN-16 (Arai, 1983), was used (data not shown). The effects of monensin and tubulin-depolymerizing agents on theonellamide-treated cells were confirmed by the uptake of acridine orange into respective cells. Acridine orange uptake of theonellamide F–treated cells decreased drastically in the presence of monensin but not noticeably in the presence of nocodazole (Figure 7 C).

A prominent feature of vacuoles induced by VacA in HeLa cells is the localization of a small GTPase, rab7, around them (Papini et al., 1994, 1997). The localization of rab7 was clearly observed in areas around the nuclei in 3Y1 cells (Figure 8, A and B). Intense localization of rab7 was seen around certain vacuoles induced by theonellamide F (Figure 8, C–F, arrowheads). Fluorescence signals were faintly observed around the extremely large vacuoles (Figure 8, E and F). However, other vacuoles of similar size in the same cells did not contain rab7, and some intense localization of these was observed in areas distinct from the vacuolar outline (Figure 8, D and F, arrows). These results suggest that rab7 may be required at most only in certain vacuoles or steps of vacuolar formation in theonellamide F–treated 3Y1 cells.

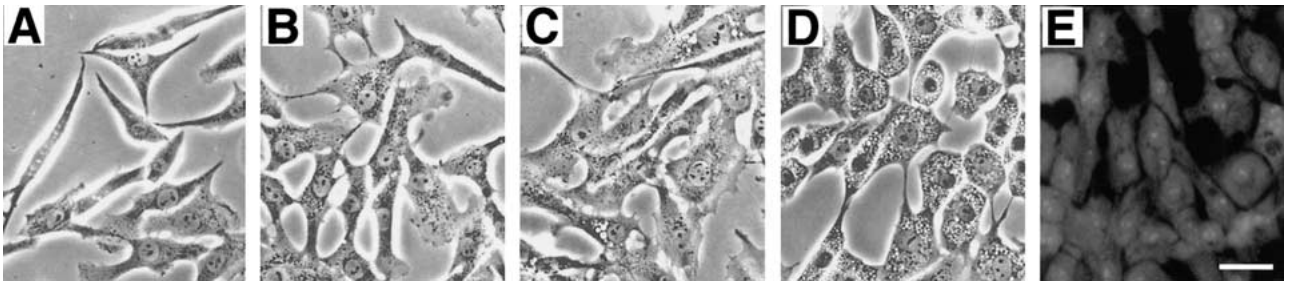

Figure 5. Inhibition of theonellamide F–induced vacuole formation by bafilomycin A1. 3Y1 cells were treated with 10 nM bafilomycin A1 for 24 hours (**A**), 2 μM theonellamide F and 10 nM bafilomycin A1 for 24 hours (**B**), 2 μM theonellamide F for 24 hours, and then with added 10 nM bafilomycin A1 for 3 hours (**C**), and 3 μM monensin and 10 nM bafilomycin A1 for 24 hours (**D**). Cells after the treatment in panel C were stained with 40 μg/ml acridine orange for 10 minutes. (**E**). The bar in panel E represents 50 μm.

Discussion

The vacuolar formation effect of theonellamides was demonstrated in all 6 mammalian culture cells examined in this study. Extraordinarily large vacuoles more than 30 μm in diameter were induced in 3Y1 fibroblasts. Monensin, lasalocid, nigericin, and nicotine also induced large vacuoles, which did not exceed 15 μm in diameter or contain an acidic internal environment. VacA and aerolysin (Abrami et al., 1998) are high molecular weight proteins known to induce large vacuoles in mammalian cells. Thus, to the best of our knowledge, theonellamides are the first low molecular weight compounds shown to induce extraordinarily large vacuoles in mammalian cells. As seen in Figure 2 D, cells grew to confluence in the dish even when highly vacuolated. The ability of theonellamide F to induce large vacuoles and cause minimal toxicity to cells suggests its potential as a good molecular probe for research on intracellular membrane structures.

The primary target molecule and mode of action of theonellamides remain unclear. We previously showed that glutamate dehydrogenase and 17β-hydroxysteroid dehydrogenase IV specifically bound to theonellamide A–conjugated affinity beads, and the binding was abolished by an excess amount of theonellamide F (Wada et al., 2000). However, whether these proteins are involved with vacuolar formation in culture cells by theonellamides A and F is unclear. Vacuoles induced by theonellamides are considered to be swollen organelles that accumulate a high concentration of certain ions and water, as in the case of other large vacuoles (Dinter and Berger, 1998).

In this study, we showed that the internal milieu of vacuoles induced by theonellamide F in 3Y1 cells was acidic, similar to VacA. Although the principle behind the action of VacA is still ambiguous, it has been reported that a hexameric complex of VacA forms a transmembrane pore

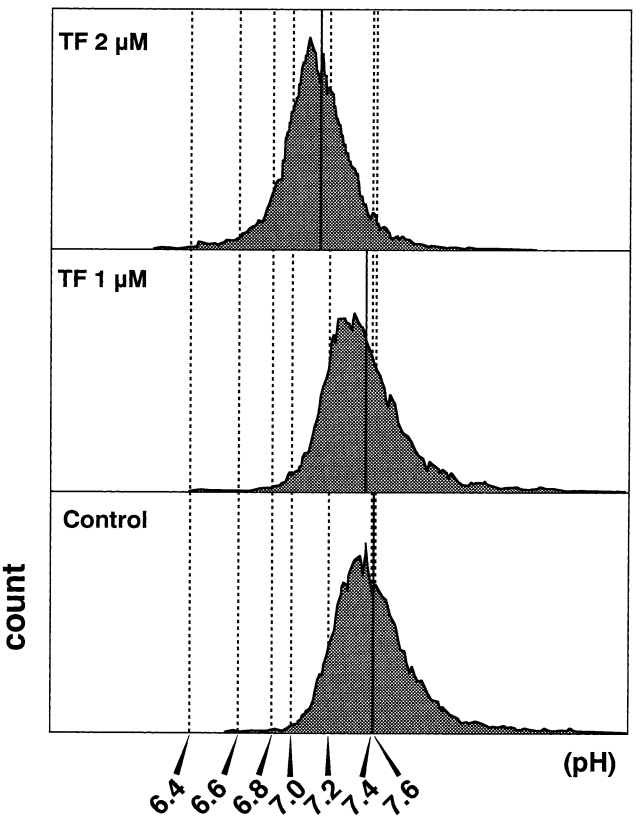

Figure 6. Flow cytometric analysis of intracellular pH of theonellamide F–treated 3Y1 fibroblasts. A typical result of 3 experiments is presented. Cells after treatment with theonellamide F for 24 hours were stained with 10 μM BCECF-AM. The ratios of 525-nm fluorescence to 620-nm fluorescence are presented as gray histograms, and the mean values are indicated as solid lines. The standard pH values were measured at the same time using high $[K^+]$ buffers containing 10 μM nigericin prepared at each pH value. Mean values for standard are indicated as dotted lines.

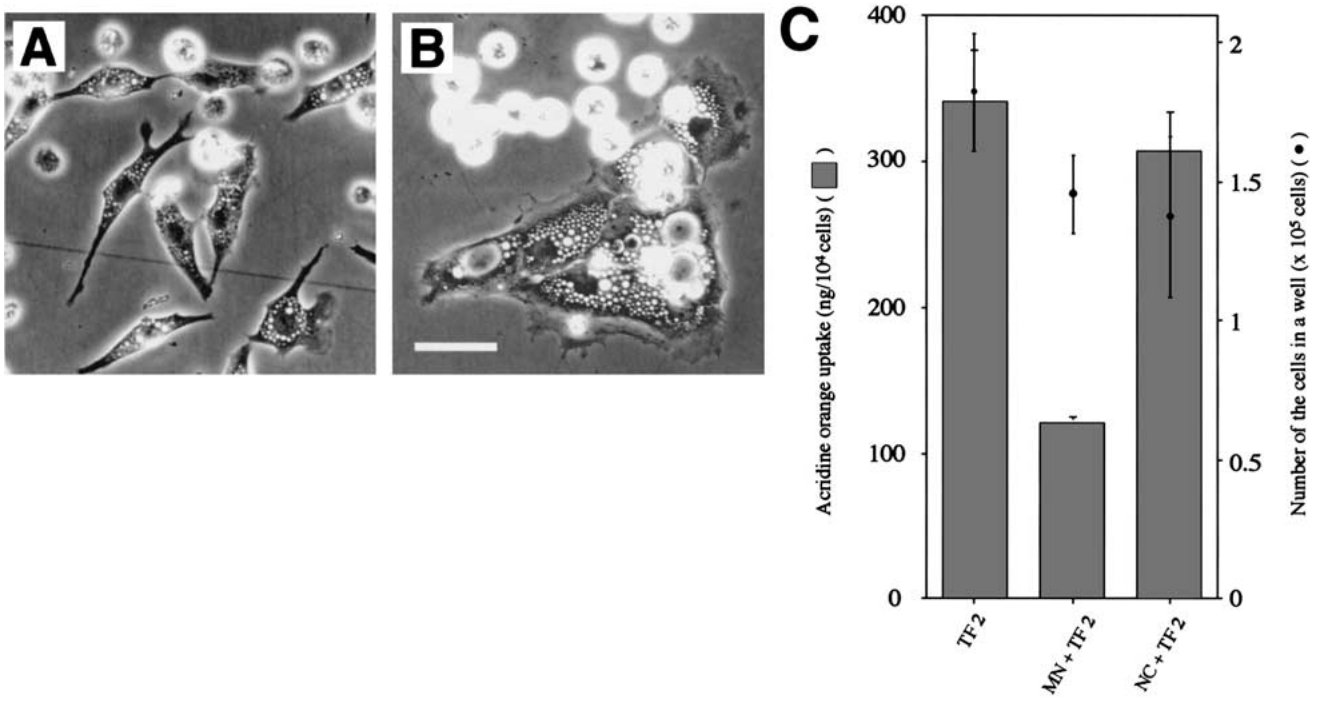

Figure 7. Effects of monensin and nocodazole on the formation of vacuoles by theonellamide F. 3Y1 cells were treated with 2 μM theonellamide F and 6 μM monensin for 24 hours (**A**) and 2 μM theonellamide F and 1 μg/ml nocodazole for 24 hours (**B**) The bar in panel B represents 50 μm. Acridine orange uptake and number of living cells (**C**) were deduced similarly to those in Figure 4. TF 2 indicates treatment with 2 μM theonellamide F; MN, 6 μM monensin; and NC, 1 μg/ml nocodazole, for 24 hours. Each of the latter 2 compounds was used at the same time as the treatment with theonellamide F. The difference was statistically significant between cells treated with TF 2 and those treated with MN plus TF 2 (Student's t test, $P < 0.01$), but not between cells treated with TF 2 and those treated with NC plus TF 2 ($P < 0.05$). Data are represented as mean ± SD ($n = 3$).

(Cover et al., 1997; Czajkowsky et al., 1999; Reyrat et al., 1999), which probably acts as an anion-selective channel (Iwamoto et al., 1999; Szabo et al., 1999; Tombola et al., 1999a, 1999b). Thus it is possible that theonellamide F also acts to promote transportation of anions into the vacuoles. Since most of the amino acids contained in theonellamides are hydrophilic (see Figure 1) and water soluble, it would be difficult for theonellamide F to remain in or pass through the vacuolar membrane as ionophores and to directly regulate transportation of ions. Therefore, theonellamide F possibly affects some regulators of intracellular ion transportation.

Whether theonellamides activate V-ATPase remains a matter of conjecture, though the function of this enzyme seems to influence the toxicity or activity of theonellamides on eukaryotic cells. Internal acidity of the vacuoles and inhibition of formation of the vacuoles by bafilomycin A1 suggest that V-ATPase were required in the formation of large vacuoles induced by theonellamide F in the mammalian cells we studied.

In the case of VacA, integrity of microtubules and function of rab7 are essential for the formation of vacuoles (Papini et al., 1997; Tombola et al., 1999). Microtubules function as cables on which membrane components are transported to large vacuoles in HeLa cells treated with VacA. Rab7 regulates transportation and membrane fusion in the cell (Papini et al., 1994, 1997). But the integrity of microtubules was not essential in the case of vacuolar formation induced by theonellamide F in 3Y1 cells (Figure 7). The role of rab7 in vacuolar formation may be limited to certain types of vacuoles or processes of vacuolar formation in the cell (Figure 8). These facts suggest that VacA and theonellamide F induce different processes of vacuolar formation although the vacuoles formed by the 2 agents are similar in size and acidity.

We previously suggested that theonellamide F inhibited cellular autophagic process because 3Y1 cells became susceptible to theonellamide F in an amino-acid-incomplete medium (Wada et al., 1999). Small particles showing Brownian movement accumulated in extraordinarily large

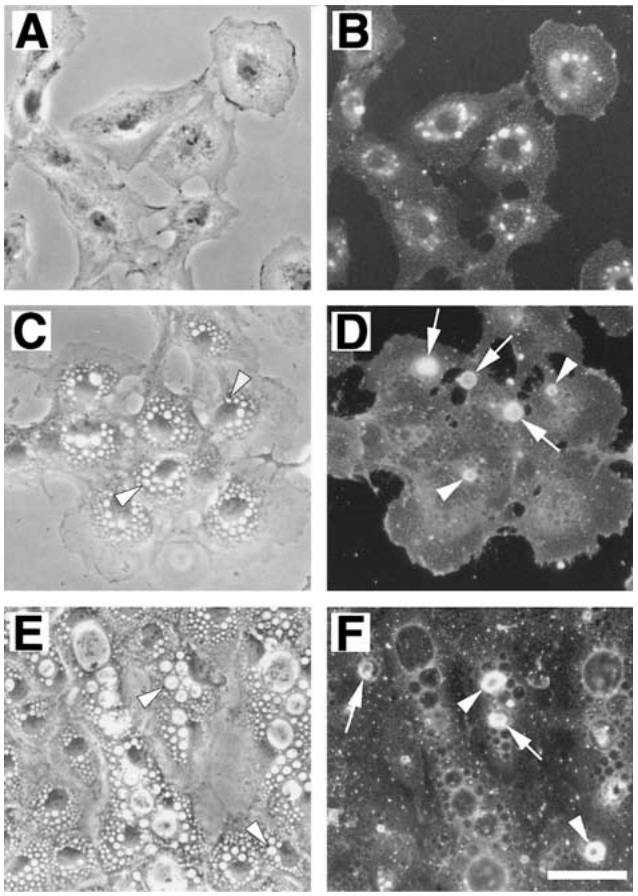

Figure 8. Localization of rab7 in 3Y1 cells treated with theonellamide F. 3Y1 cells were without any treatment **(A, B)**, treated with 2 μM theonellamide F for 24 hours **(C, D)**, or treated with 2 μM theonellamide F for 72 hours **(E, F)**. Rab7 was immunohistochemically stained with anti–carboxyl terminus peptide of rab7 antibody and rhodamine-conjugated secondary antibody **(B, D,** and **F)**. Arrowheads in panels **C** through **F** represent the vacuoles that correspond to the region of localization of rab7. Arrows in panels **D** and **F** indicate the localization of rab7 that does not correspond to or deviate from a particular vacuole. The bar in **F** represents 50 μm.

vacuoles induced by theonellamide F (Figure 2 D), which appeared to be undigested organelles derived from the inhibition of autophagy. Currently, which step of autophagy is affected by theonellamide F is unclear. The disturbance of intralysosomal pH by theonellamide F may affect the function of lysosomal proteases that are responsible for autophagic degradation of organelles. Alternatively, membrane traffic or fusion in the autophagic process may be disturbed by the agent.

In conclusion, we have shown that theonellamides A and F induced large vacuoles in culture cells, with minimal side effects. The intravacuolar milieu was acidic and highly associated with the function of V-ATPase; thus it was similar to the previously reported case of vacuole induction by VacA. However, the involvement of microtubules and rab7 in the formation and growth of the vacuoles in theonellamide-treated 3Y1 cells was not pronounced.

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