The role of vacuolar-type H\(^+\)-ATPase (V-ATPase) in the cytotoxic action of diphtheria toxin (DT) was studied by using bafilomycin A1, a specific inhibitor of V-ATPase. Studies with acridine orange showed that the acidification of intracellular acidic compartments was inhibited strongly when Vero cells were treated with 500 nM bafilomycin A1, indicating that bafilomycin effectively inhibits V-ATPase when it is added to the culture medium. The toxicity of DT to Vero cells, which was determined by the inhibition of protein synthesis by DT, was inhibited partially by bafilomycin at 10 nM and inhibited completely at 500 nM. Therefore, V-ATPase is involved in the expression of the toxicity of DT. Studies using \(^{125}\)I-labeled DT showed that bafilomycin inhibited the degradation of internalized DT, indicating that V-ATPase is also involved in this step. Subcellular fractionation revealed that \(^{125}\)I-DT accumulated mainly in the endosome fraction, and not in the lysosome fraction, when the cells were incubated with \(^{125}\)I-DT in the presence of bafilomycin. Under the cell fractionation conditions similar to those used for the DT-treated cells, we determined the location of \(^{125}\)I-labeled epidermal growth factor in the degradation pathway. The result suggests that bafilomycin A1 does not inhibit the transport of epidermal growth factor to lysosome.

Diphtheria toxin (DT) is a cytotoxic protein that inhibits cellular protein synthesis in eukaryotes by inactivating EF-2 through ADP-ribosylation (1-4). Entry of the toxin molecule(s), or at least its fragment(s), into the cytoplasm is a requisite process for the cytotoxic action (5). The generally accepted mechanism for toxin entry into the cell is as follows: DT binds to a specific receptor on the cell surface of DT-susceptible cells (6, 7), it is concentrated to coated pits and then internalized by endocytosis (8, 9). A conformational change of the toxin molecule takes place in an acidic compartment, resulting in the exposure of hydrophobic domains, which are mainly in the B fragment (10, 11). Finally, the enzymatically active A-fragment is translocated to the cytosol where it exerts its toxicity. Several lines of evidence have indicated that the exposure of DT to low pH is essential for its translocation to the cytosol (12-23). However, the enzyme responsible for acidification of the vesicle in which DT is trapped has not been characterized.

It is known that the interior of several intracellular compartments is acidic. They include the Golgi complex, secretory vesicles, phagosomes, endosomes, and lysosomes (for review, see Ref. 24). A specific class of ATPase, referred to as the vacuolar-type H\(^+\)-ATPase (V-ATPase), capable of forming a proton gradient has been identified and purified from the membranes of fungal and plant vacuoles (25), coated vesicles (26-28), chromaffin granules (29, 30), and the Golgi complex (31). Although this type of ATPase is distinguishable from mitochondrial F\(_{0}\)F\(_{1}\)-ATPase or from E\(_{1}\)E\(_{2}\)-ATPase in the plasma membrane of fungi (32), no specific inhibitor of the V-ATPase has been known. Recently, bafilomycin A1, a macrolide antibiotic isolated from Streptomyces sp. (33), was shown to be a highly specific inhibitor of the V-ATPase (32).

Vero cells, derived from monkey kidney, are highly sensitive to DT because of a large number of DT receptors (39). In this study, we used bafilomycin A1 in intact Vero cells and studied its effect on the cytotoxic action of DT. Bafilomycin A1 strongly inhibited the cytotoxic action of DT and the degradation of DT when this drug was added to the medium.

**EXPERIMENTAL PROCEDURES**

**Materials**—DT was produced and purified as described previously (34). Bafilomycin A1 was kindly given by Prof. K. Altendorf (Universität Osnabrück, West Germany). Trypsin (Type XIII, TPCK-treated) and \(\alpha\)-chymotrypsin (Type II, \(\times\) crystallized) were purchased from Sigma.

**Cells**—Vero cells were grown in Eagle’s minimum essential medium supplemented with nonessential amino acids, 100 units/ml penicillin G, 100 \(\mu\)g/ml streptomycin, and 10% calf serum. In all experiments, cells were seeded in tissue culture dishes at the density of 4 \(\times\) 10\(^5\) cells/cm\(^2\) at 16 h before use.

**Assay of the Rate of Protein Synthesis in Cells Cultured with Toxins**—Toxicity of DT was measured by assaying the rate of protein synthesis. Vero cells (1 \(\times\) 10\(^5\) cells/well) on a 24-well tissue culture plate were washed twice with ice-cold PBS (150 mm NaCl, 2.7 mm KCl, 10 mm phosphate buffer, pH 7.2), and then 0.4 ml of toxicity assay medium (Ham’s F-12 containing 10% calf serum and 20 mm HEPES buffer, pH 7.2, 100 units/ml of penicillin, and 0.1 mg/ml of streptomycin) was added. Various concentrations of bafilomycin A1 were added to the culture medium, and the cells were incubated at 37° C for 30 min. Then the cells were incubated with 1 nCi/ml of DT for 2 h at 37° C, followed by further incubation with 0.5 \(\mu\)Ci/ml of \({}^{3}H\)leucine for 1 h. The radioactivity incorporated into protein was measured (35). The rate of protein synthesis in each culture was

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**The Cytotoxic Action of Diphtheria Toxin and Its Degradation in Intact Vero Cells Are Inhibited by Bafilomycin A1, a Specific Inhibitor of Vacuolar-type H\(^+\)-ATPase**

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Association of 125I-Labeled DT with Cells—Purified diphtheria toxin was labeled with Na125I using Enzymobeads (Bio-Rad) as reported previously (36). The labeled toxin had a specific activity of 2-3 x 107 cpm/pg. Vero cells were incubated with 125I-DT in binding assay medium (Eagle's minimal essential medium containing nonessential amino acids, 10% calf serum, and 20 mM HEPES buffer, pH 7.2, with the omission of sodium bicarbonate). Prior to the addition of 125I-DT, the Vero cells were incubated with 500 nM bafilomycin A1 for 30 min at 37 °C. 125I-DT (70 rig/ml) was added, and the cells were incubated for 10 min. Then the cells were washed three times with ice-cold PBS, dissolved in 0.1 M NaOH, and the lysate was counted in a γ-counter. In this paper, the values are shown as specific counts, determined by subtracting the counts obtained in the presence of a 100-fold excess of unlabeled toxin (nonspecific binding) from those obtained with 125I-DT alone (total binding). In this experiment, the nonspecific binding was less than 10% of the total binding.

Measurement of the Amount of DT Associated with Cell Surface—Vero cells incubated with 125I-DT were washed three times with ice-cold PBS and then treated with protease solution (0.5 mg/ml trypsin, 0.5 mg/ml chymotrypsin in PBS) for 6 min at 37 °C. The cells were washed once with ice-cold PBS. The protease solution and PBS used for washing were combined and centrifuged at 3000 rpm for 20 min at 4 °C. The supernatants were counted as the protease-releasable radioactivity.

Subcellular Fractionation—A stock of iso-osmotic Percoll (Pharmacia, Sweden) solution was made by combining 9 parts Percoll with 1 part 0.25 M sucrose (v/v). The 18% Percoll solution for cell fractionation was made by combining 2 parts of the Percoll stock solution with 8 parts of STE buffer (0.25 M sucrose in 10 mM Tris-HCl, pH 7.2, and 1 mM EDTA) (37). The following experiments were performed at 4 °C. Vero cells were washed with ice-cold PBS and removed using a rubber policeman. Cells pellets were resuspended in STE buffer and then homogenized in a tight-fitting Dounce homogenizer with 50 strokes. The homogenates were centrifuged at 600 x g for 10 min to remove intact cells and nuclei. The supernatant was mixed with the 18% Percoll solution and centrifuged in a SW70AT rotor (Hitachi) at 100,000 x g for 25 min at 4 °C. The gradients were fractionated from the top of the centrifuging tube. The density distribution was measured using density marker beads (Pharmacia-LKB Biotechnology).

Identification of the Endosome Fractions—Vero cells were incubated with 125I-labeled transferrin for 60 min at 37 °C, and washed to remove the unbound transferrin. Only 20% of the radioactivity associated with the cells under these conditions were removed by acid treatment (51), indicating that most of the radioactivity was in the interior of the cells. The cells were subjected by the subcellular fractionation, and the peak fractions of the radioactivity were identified as the endosome fractions.

Enzyme Assay—The lysosomal enzyme β-hexosaminidase was measured as described previously (38). p-Nitrophenyl-N-acetyl-D-glucosaminide (3.15 mM) was incubated with 367 μl of a sample containing 0.1% Triton X-100. The final reaction volume was 1.0 ml. After 30 min at 37 °C, the reaction was stopped with 1.0 ml of 500 mM glycine buffer, pH 10.5. The liberated p-nitrophenolate was measured with a spectrophotometer at 420 nm.

The plasma membrane marker, 5'-nucleotidase, was measured as described previously (52).

RESULTS

Inhibition of Acidification of Intracellular Compartments with Bafilomycin A1—As it was not clear whether bafilomycin A1 can permeate the plasma membrane, we examined whether bafilomycin effectively inhibited V-ATPase in intact cells when this drug was added to the medium. To monitor the activity of this enzyme, we measured the acidity of intracellular acidic compartments by staining with a fluorescent dye, acridine orange. This dye is membrane-permeant when uncharged at neutral pH but relatively membrane-impermeable once it becomes protonated; thus, the dye is accumulated in acidic vesicles. Vero cells were preincubated with bafilomycin A1 at 37 °C for 60 min and incubated further with acridine orange for 20 min. The cells were washed, mounted on a slide glass, and observed under a fluorescence microscope. As shown in Fig. 1, strong granular fluorescence with an orange color was observed in the control cells that were not treated with bafilomycin A1, whereas such fluorescence was not observed in the cells preincubated with bafilomycin A1. Although a faint granular fluorescence was seen in some bafilomycin-treated cells, the number of fluorescent granules was few. These results suggest that bafilomycin inhibited V-ATPase in intact Vero cells.

Effect of Bafilomycin A1 on the Cytotoxic Action of DT—To determine if bafilomycin A1 inhibits the cytotoxic action of DT, Vero cells were incubated with various concentrations of bafilomycin A1 at 37 °C for 30 min, and DT was added to the medium. The toxicity of DT was assayed by measuring the protein synthesis rate of Vero cells. Fig. 2 shows that bafilomycin inhibits the toxicity of DT in a dose-dependent manner; at 10 nM bafilomycin, the toxicity of DT was inhibited partially, and at 500 nM, it was inhibited completely. At a concentration less than 1 μM, bafilomycin per se did not affect cellular protein synthesis (data not shown).

The concentration of bafilomycin A1 required to inhibit the cytotoxic action of DT in intact Vero cells completely was similar to that required to inhibit V-ATPase in cell-free experiments (32). This concentration is at least three orders of magnitude lower than the minimum concentration showing an effect on other ATPases. Therefore, we concluded that V-ATPase is involved in the expression of the cytotoxic action of DT in Vero cells. This is the first evidence indicating that the entry of DT requires V-ATPase.

The translocation of DT to the cytoplasm requires the exposure of DT to low pH. We showed in Fig. 1 that bafilomycin A1 inhibits the acidification of intracellular compartments. Thus the effect of bafilomycin on the toxicity of DT is probably due to the inhibition at the translocation step of DT. To confirm the point at which bafilomycin A1 inhibits

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FIG. 1. Acridine orange staining of acidic compartments in the cells incubated with or without bafilomycin A1. Vero cells were incubated with (C, D) or without (A, B) bafilomycin A1 at 37 °C for 60 min and then incubated further with acridine orange at 37 °C for 20 min. After washing with ice-cold PBS, the cells were observed under a microscope. A and C, phase-contrast optics; B and D, fluorescence optics. Bar, 50 μm.
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1. The cytotoxic action of DT, we studied the association and the internalization of \(^{125}\text{I}\)-DT to Vero cells. When the binding study was performed at 4 °C with the cells pretreated with or without 500 nM bafilomycin A1, the amounts of \(^{125}\text{I}\)-DT radioactivity bound to Vero cells were similar (data not shown). However, the association profile was changed by bafilomycin A1 when the cells were incubated with \(^{125}\text{I}\)-DT at 37 °C. In the absence of bafilomycin A1, the amount of radioactivity associated with the cells increased for 1 h and then decreased as reported previously (39). In contrast, in the presence of bafilomycin A1, the amount of radioactivity continued to increase for 4 h (Fig. 3). These results indicate that at least bafilomycin A1 does not affect the binding of DT to its receptor.

We measured the amounts of toxin associated with the cell surface by determining the protease sensitivity of the bound toxin. More than 85% of the radioactivity associated with the cells at 4 °C was removed by protease treatment as reported previously (16), whereas only 20% of the total radioactivity associated with the cells incubated with the toxin at 37 °C for 3 h in the presence of bafilomycin was removed by this treatment. These results suggested that when the cells were incubated with \(^{125}\text{I}\)-DT at 37 °C in the presence of bafilomycin A1, the toxin accumulated mainly in the interior of the cells, indicating that bafilomycin did not inhibit the internalization of DT. It is, therefore, concluded that bafilomycin A1 inhibits the translocation of DT from the endocytic vesicles to the cytoplasm by inhibiting V-ATPase.

Inhibition of the Degradation of DT—The increasing association of \(^{125}\text{I}\)-DT with Vero cells in the presence of bafilomycin resulted from the accumulation of the toxin inside the cells as shown above. This increasing accumulation is attributable to both inhibition of DT degradation in the cells by the action of bafilomycin and continuous internalization of DT via newly synthesized DT receptors as shown in the case of methylene (16). In fact, when \(^{125}\text{I}\)-DT and bafilomycin were removed, the degradation occurred rapidly, and no radioactive materials were accumulated (shown in Fig. 3). Furthermore, when \(^{125}\text{I}\)-DT was removed but bafilomycin was added again, neither the increasing association nor the degradation were observed. Thus these results indicate that V-ATPase is involved in the degradation step of DT.

Reversibility of the Effect of Bafilomycin A1—When bafilomycin A1 is used in living cells, the effects are reversible. The reversibility of the effect of bafilomycin A1 was demonstrated by the toxicity assay of DT. Vero cells were preincubated with 500 nM bafilomycin A1 at 37 °C for 30 min and then incubated with 3 ng/ml of DT for 60 min. The cells were washed with ice-cold PBS and then incubated further at 37 °C with fresh medium containing (△) or not containing bafilomycin A1 (○). After incubation for the time indicated, the cells were labeled with \(^{3}\text{H}\)lucine for 60 min, and the rate of protein synthesis was measured.

Intracellular Localization of \(^{125}\text{I}\)-DT—It is of interest to
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FIG. 5. Phase-contrast micrograph of Vero cells exposed to 500 nM bafilomycin A1 (A) or 20 mM methylamine (B) at 37 °C for 100 min. C, control cells without drug. Bar, 50 μm.

FIG. 6. Subcellular distribution of 125I-DT in the cells treated with bafilomycin A1. Vero cells were preincubated with 500 nM bafilomycin A1 at 37 °C for 30 min. The cells were then incubated with 125I-DT at 4 °C for 2 h. The medium was replaced with fresh medium containing the same amount of bafilomycin A1. The cells were incubated at 37 °C for the time indicated and then subjected to fractionation in a Percoll gradient. Ly., lysosomes.

define which cellular compartments accumulated DT during incubation with bafilomycin. Earlier studies showed that DT accumulated in the cells when the acidification was blocked by acidotropic amines (16, 40). However, it was difficult to define the location of DT, because those amines caused severe vacuolation due to the increase of osmotic pressure in the vesicle by accumulation of the protonated amines (41). Because bafilomycin A1 inhibits the acidification of the vesicles through inactivation of V-ATPase, we anticipated that bafilomycin does not induce vacuolation. To examine this possibility, we incubated Vero cells with bafilomycin A1 or methylamine at 37 °C for 100 min: as expected, methylamine caused extensive vacuolation, whereas bafilomycin A1 had no effect (Fig. 5).

We examined here whether DT accumulated in lysosomes or other compartments during incubation with bafilomycin by subcellular fractionation in a Percoll density gradient (37). After preincubation with or without 500 nM bafilomycin A1 at 37 °C for 30 min, Vero cells were incubated with 125I-DT at 4 °C for 2 h and at 37 °C in fresh medium not containing 125I-DT with or without bafilomycin. The cells were collected, homogenized, and fractionated in a Percoll gradient. The plasma membrane fraction and the lysosome fraction were identified using as enzyme markers 5'-nucleotidase and β-hexosaminidase, respectively. The endosome fraction was identified by the radioactivity of 125I-labeled transferrin as described under “Experimental Procedures,” because transferrin does not enter lysosomes (53). When control Vero cells were fractionated under these conditions, the plasma membrane and the endosome were recovered in fractions 3–7 with a mean density of 1.04 g/ml, and the lysosome was in fractions 16–22 with a mean density of 1.08 g/ml.

In the absence of bafilomycin A1, at 0 min of the chasing period, the radioactivity was distributed in fractions 3–7, i.e. in the endosome-plasma membrane fraction (Fig. 6, left panel). At 40 min, or 60 min, the peak of radioactivity in
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fractions 3–7 decreased, consistent with the results shown in Fig. 3. No radioactive peaks were seen in the lysosome fraction.

In the presence of bafilomycin A1, the radioactive peak was observed in fractions 3–7 at 0 min (Fig. 6, right panel). At 40 min, the degradation was not observed. Although the lysosome fraction shifted two fractions to lower density positions by the incubation with bafilomycin, the pattern was almost the same as that at 0 time. At 60 min, although the radioactivity of the peak in fractions 3–7 was decreased and a small peak was observed in the lysosome fraction (fractions 13–19), the radioactivity was still found mainly in fractions 3–7.

To eliminate the possibility that the little accumulation of DT in the lysosome fraction is due to inadequate experimental conditions to demonstrate it or by an unexpected side effect of bafilomycin, we performed similar experiments using EGF. As carried out for DT, Vero cells were incubated with 125I-EGF at 4 °C, chased at 37 °C in the presence of bafilomycin A1 and subjected to cell fractionation. At 0 min, the radioactivity was found in the endosome-plasma membrane fraction (Fig. 7). During the chase at 37 °C, a significant amount of the radioactivity was shifted to the lysosome fraction; at 40 min, 28% of the total radioactivity was distributed to fractions 14–20; and at 60 min, 37% was distributed to fractions 13–19. These results indicate that the experimental conditions used were adequate for demonstrating the transfer of EGF to lysosomes in the cells treated with bafilomycin. Therefore, we conclude that DT accumulates mainly in the endosomes, or at least in compartments with a density similar to that of endosomes, when cells are incubated with DT in the presence of bafilomycin.

**DISCUSSION**

Although the specific inhibition of V-ATPase by bafilomycin A1 has been studied using purified enzymes (31) and vesicles containing such enzymes (32, 42), little has been reported about the effects in intact cells. Using the monkey kidney cell line Vero, we studied the efficacy of bafilomycin A1 and subjected to cell fractionation. At 0 min, the activity was found in the endosome-plasma membrane fraction (Fig. 7). During the chase at 37 °C, a significant amount of the radioactivity was shifted to the lysosome fraction; at 40 min, 28% of the total radioactivity was distributed to fractions 14–20; and at 60 min, 37% was distributed to fractions 13–19. These results indicate that the experimental conditions used here were adequate for demonstrating the transfer of EGF to lysosomes in the cells treated with bafilomycin. Therefore, we conclude that DT accumulates mainly in the endosomes, or at least in compartments with a density similar to that of endosomes, when cells are incubated with DT in the presence of bafilomycin.

Although the specific inhibition of V-ATPase by bafilomycin A1 has been studied using purified enzymes (31) and vesicles containing such enzymes (32, 42), little has been reported about the effects in intact cells. Using the monkey kidney cell line Vero, we studied the efficacy of bafilomycin A1 to inhibit the acidification of intracellular compartments. The staining with acridine orange showed that the proton gradient of intracellular acidic compartments was inhibited when the cells were incubated with 500 nM bafilomycin A1 at 37 °C for 60 min. The effectiveness of bafilomycin A1 in intact cells was also demonstrated by its ability to inhibit the cytotoxic action of DT completely. The concentration of bafilomycin A1 required for the complete inhibition of DT toxicity was sufficiently lower than the concentration at which bafilomycin A1 affects other classes of H+-ATPases (32). Thus, bafilomycin A1 seemed to be a useful agent in intact cells for investigating the role of acidification in various cellular events.

We demonstrated here that the cytotoxic action of DT was inhibited completely by bafilomycin A1. This is the first evidence that V-ATPase plays a role in the expression of DT toxicity. We also showed by the association study with 125I-DT that bafilomycin A1 inhibited the action of DT after its internalization. The requirement of low pH during the translocation of DT to the cytosol has been strongly indicated by experiments using ammonium chloride (13–15) and other amines (16, 17), by acidification-defective mutant cells (12, 18), and by cell-free experiments on the interaction of DT with model membranes (19–21, 43–45) and with the plasma membrane (22, 23). We, therefore, argue that V-ATPase is responsible for the translocation of DT to the cytoplasm by lowering the pH value in DT-containing vesicles.

Although some of the DT taken up by endocytosis is transferred to the cytoplasm where it exerts its toxicity, the remaining DT is degraded rapidly in the cells (46). Here we showed that bafilomycin inhibits the degradation of DT, indicating that V-ATPase is involved in the process. Moreover, studies by the subcellular fractionation show that DT accumulates mainly in the endosome fraction, not in the lysosome fraction, when Vero cells are incubated in the presence of bafilomycin. Why is DT accumulated in the endosome fraction? One possible explanation is that bafilomycin affects the transport mechanism of DT. In this explanation, it is assumed that the degradation of DT occurs in lysosomes. The retardation of the transport must cause the accumulation in prelysosomal compartments. Another possible explanation is that bafilomycin inhibits the degradation of DT in the endosome fraction. If DT is degraded in endosomes, or in compartments with a density similar to that of endosomes, the inhibition of the DT degradation by bafilomycin treatment causes the accumulation in the endosome fraction. However, since it is not clear in which compartments DT is degraded naturally, present study does not define which possibilities are more likely.

Intracellular processing and the sorting of EGF have been studied extensively using cell lines (47–49). 125I-EGF is processed initially in prelysosomal compartments, resulting in the removal of 6 amino acid residues from the carboxyl terminal. The processed ligands are then delivered to the lysosomes, where they are degraded into low molecular weight products. We observed here that the transfer of EGF to lysosomes proceeds even in the presence of bafilomycin A1. Thus, it is suggested that acidification is not necessarily requisite for the transfer of EGF from the endosomes to the lysosomes.

We reported previously that the treatment of cells with methylamine causes the accumulation of 125I-DT in cells (16), and facilitates the observation of the specific uptake of DT by cells with relatively fewer receptors (50). We showed here that the amount of cell-associated radioactivity continued to increase for 10 h in the cells treated with bafilomycin A1, whereas the increasing association caused by methylamine stopped by about 4 h as reported previously (16). Thus, bafilomycin A1 will be more suitable for studying the specific uptake of DT.

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