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Acylspermidine Derivatives Isolated from a Soft Coral, *Sinularia* sp., Inhibit Plant Vacuolar H^+-Pyrophosphatase

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H^+-pyrophosphatase (H^+-PPase), which pumps H^+ across membranes coupled with PP_i hydrolysis, is found in most plants, and some parasitic protists, eubacteria and archaebacteria. We assayed a number of extracts derived from 145 marine invertebrates as to their inhibitory effect on plant vacuolar H^+-PPase. Acylspermidine derivatives [$RCONH(CH_2)_3N(CH_3)(CH_2)_4N(CH_3)_2$] from a soft coral (*Sinularia* sp.) inhibited the PPi-hydrolysis activity of purified H^+-PPase and the PP_i-dependent H^+ pump activity (half inhibition concentration, 1 μM) of vacuolar membranes of mung bean. The apparent K_i was determined to be 0.9 μM. Acylspermidines did not affect the activity of vacuolar H^+-ATPase, plasma membrane H^+-ATPase, mitochondrial ATPase or cytosolic PPase. Acylspermidines inhibited the acidification of vacuoles in protoplasts, as found on monitoring by the acridine orange fluorescent method. These results indicate that acylspermidine derivatives represent new inhibitors of H^+-PPase with relatively high specificity.

Key words: acylspermidine, H^+-pyrophosphatase, inhibitor, soft coral, vacuole.

Abbreviations: DCCD, *N,N′*-dicyclohexylcarbodiimide; DMSO, dimethyl sulfoxide; H^+-PPase, H^+-translocating inorganic pyrophosphatase; PP_i, inorganic pyrophosphatase; V-ATPase, vacuolar H^+-ATPase.

The vacuolar H^+-pyrophosphatase (H^+-PPase) is an electrogenic proton pump that acidifies vacuoles in plant cells and some acidic compartments in other organisms. H^+-PPase is found not only in most plants, but also in some parasitic protists, eubacteria and archaebacteria. H^+-PPase has characteristics distinguishable from those of other types of proton pumps, F-, P- and V-type ATPases (*1*, *2*). (i) H^+-PPase consists of a single polypeptide of about 80 kDa. (ii) The enzyme utilizes a simple, low-cost substrate, pyrophosphate (PP_i), which has a high-energy phosphoanhydride bond. iii) H^+-PPase coexists with H^+-ATPase (V-ATPase) in vacuolar membranes of plants and in acidocalcisome membranes of some parasitic protists, such as *Trypanosoma cruzi* and *Plasmodium falciparum* (*1*, *3*). Thus, H^+-PPase is regarded as the fourth type of ion pump. It is a new proton pump model useful for studying the structure-function relationship, and the mechanism of coupling between substrate hydrolysis and proton translocation across the membrane. Its physiological role in relation with the V-ATPase that is located in the same membrane in several organisms also remains unknown.

Specific inhibitors of the enzyme of interest would be useful for investigating the physiological role of the enzyme in organisms and its biochemical characteristics. At present, no H^+-PPase-specific inhibitors are known. Several substrate analogues, such as imidodiphosphate, aminomethylenediphosphonate (*4*), and *N,N′*-dicyclohexylcarbodiimide (DCCD) (*5*), have been reported to inhibit H^+-PPase. However, DCCD inhibits several H^+-translocating enzymes and PP_i analogues inhibit soluble-type PPases. From the pharmacological aspect, the enzyme is a potential target for chemotherapy for diseases caused by parasites, such as *T. cruzi*, *T. brucei*, *P. falciparum* and *Toxoplasma gondii*, which contain H^+-PPase in the membranes of acidic Ca^{2+}-storage organelles (acidocalcisomes) and plasma membranes (*1*, *3*, *6*, *7*). Thus, specific inhibitors could be useful for preventing and treating several parasitic diseases such as malaria.

In order to search for new inhibitors of the enzyme, we prepared water-soluble and organic-solvent soluble fractions from 145 marine organisms, and examined their effects on PP_i-hydrolysis and PP_i-dependent H^+ pump activities. During this study, we found that a few compounds in the water-soluble fraction prepared from a soft coral inhibited H^+-PPase. Here we describe their inhibitory effects, specificities, and chemical structures.

EXPERIMENTAL PROCEDURES

Materials—The marine invertebrates used in this study were collected from seas adjoining Japan; *i.e.*, 41 organisms from the sea near Aka-jima in Okinawa and 104 from the sea near Honshu Island. A soft coral, *Sinularia* sp., was collected from Chatan Island in Okinawa. For enzyme preparation, mung bean (*Vigna radiata* cv. Wilczek) seeds were imbibed in 1 mM CaSO4 and then germinated at 26°C in the dark for 3.5 days. A vacuolar membrane fraction was prepared from seedling hypocotyls and used for H^+-PPase purification. Spermidine and yeast cytosolic PPase were purchased from Sigma-Aldrich Fine Chemicals.

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Vacuolar Membrane Preparation and Enzyme Purification—Vacuolar membranes were prepared from mung bean hypocotyls of 3.5-day-old seedlings as described previously (*5*, *8*). The vacuolar membrane preparation was treated with 0.6% (w/v) sodium cholate and 50 mM KCl to remove peripheral proteins. After centrifugation at 100,000 ×*g* for 40 min, the resulting precipitate fraction was suspended in 0.4% lysophosphatidylcholine and 0.1%-*n*-dodecyl-β-maltoside, and then centrifuged at 100,000 ×*g* for 40 min at 8°C. The supernatant obtained was used as the solubilized enzyme. The solubilized fraction was applied to a column of HiLoad Q Sepharose HP (Amersham Bioscience) equilibrated with 20 mM Bis-Tris-HCl, pH 7.0, 20% glycerol, 1 mM DTT, 1 mM EGTA, 2 mM $MgCl_2$, and 0.1%–*n*-dodecyl-β-maltoside. The column was eluted with a linear gradient of 0 to 300 mM NaCl in the same medium. H^+-PPase was eluted from the column with about 100 mM NaCl. The peak activity fractions were used as the purified H^+-PPase. The protein content was determined by the method of Bradford (*9*).

Enzyme Assay—The PP_i-hydrolysis activity of H^+-PPase was measured as described previously (*5*). The basic assay medium (30 μl) for PP_i-hydrolysis activity comprised 1 mM sodium PP_i, 1 mM $MgSO_4$, 50 mM KCl, 1 mM sodium molybdate, 30 mM Tris-Mes, pH 7.2, phospholipid (soybean phosphatidylcholine, Sigma) micelle suspension (2 μg), and purified H^+-PPase (38 ng). The assay medium (2.0 ml) for proton pump activity comprised 1 mM sodium PP_i, 5 mM Tris-Mes, pH 7.2, 0.25 M sorbitol, 5 mM DTT, 50 mM KCl, 0.25 μM acridine orange, and 50 μg of vacuolar membranes (*5*). After the addition of $MgSO_4$ to a final concentration of 3 mM, fluorescence quenching of acridine orange (excitation, 493 nm; emission, 540 nm) was measured at 25°C with a Shimadzu RF5000 fluorescence spectrophotometer.

Plasma membranes (*10*, *11*) and mitochondria (*12*) were prepared from mung bean hypocotyls, and then assayed for ATPase activity. The activities of V-ATPase (assay pH 7.0), plasma membrane H^+-ATPase (assay pH 6.5), and mitochondrial ATPase (ATP synthase) (assay pH 9.0) in the corresponding fractions were determined in the presence of 0.02% Triton X-100, as reported (*13*). SDS-PAGE was carried out in a 12% gel by the standard method.

Isolation of Acylspermidines from Soft Corals—Marine invertebrates were collected and freeze-dried. Each freeze-dried sample was homogenized in an ethanol-methanol mixture (4:1, 20 ml/g of dry weight) and then stood at room temperature for 3 days. The mixture was filtered and the solvent was evaporated off. The resulting solid material was dissolved in 50 ml of 90% aqueous methanol and then washed twice with 50 ml hexane. The aqueous methanol layer was concentrated, and the residue was partitioned between ethyl acetate (3 × 30 ml) and water (30 ml). The ethyl acetate and water fractions were used separately for the H^+-PPase assay to detect inhibitors of the enzyme.

The chemical compounds that inhibited H^+-PPase were completely purified and characterized. Soft coral specimens (*Sinularia* sp., 93 g of fresh weight) were collected near Aka-jima in Okinawa. The ethanol-methanol extract of freeze-dried soft coral was used for further fractionation, as described previously (*14*). Finally, purified acylspermidines **A** (15.9 mg), **B** (3.1 mg), **C** (4.5 mg), **D** (4.8 mg), and **E** (2.7 mg) were obtained by HPLC.

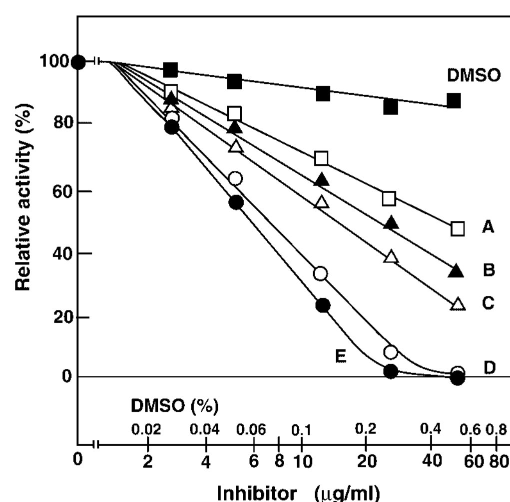

Fig. 1. **Inhibition of H^+-PPase by water-soluble compounds from a soft coral.** The PPi-hydrolysis activity of the purified H^+-PPase was assayed in the presence of the indicated amounts (μg/ml of assay medium) of the compounds (**A**, **B**, **C**, **D**, and **E**) isolated from the soft coral. Activity was expressed as a percentage of the control value without an inhibitor. DMSO, a solvent for the inhibitor, was also tested.

Protoplast Preparation and Monitoring of Vacuole Acidification—Hypocotyls were excised from 3-day-old mung bean seedlings. Tissue slices (1 g) were soaked in 10 ml of maceration medium composed of 1% cellulase Onozuka RS, 0.22% macerozyme R-10, 0.01% pectolyase Y-23, 50 mM $CaCl_2$, 0.35 M sucrose, 1 mM DTT, and 50 mM MES-KOH, pH 5.5. Then the mixture was gently agitated for 3 h at 25°C. After centrifugation at 400 ×*g* for 5 min, the top layer of protoplasts was collected, and suspended in 10 mM $CaCl_2$, 0.35 M sucrose, 1 mM DTT, and 50 mM Tris-Mes, pH 7.2. Acidification of the protoplasts was observed under a fluorescent microscope (BX-60, Olympus).

RESULTS

Screening for Inhibitors in Marine Invertebrates—We prepared both ethyl-acetate extracts and water-soluble fractions from 145 specimens of marine invertebrates, which included soft corals, sponges, starfishes, sea squirts, sea anemones, and sea cucumbers. Only the water fraction of a soft coral, *Sinularia* sp., collected in Okinawa markedly inhibited H^+-PPase. The compounds in the water fraction were separated into five distinct ones (**A** to **E**). Figure 1 shows their effects on the PP_i-hydrolysis activity of H^+-PPase. Compounds **D** and **E** completely inhibited the enzyme at a concentration of 27 μg/ml. At the same concentration, compounds **A**, **B** and **C** inhibited the enzyme by about 50%. The solvent, DMSO, did not show any inhibitory effect. Furthermore, these compounds did not inhibit yeast cytosolic PPase, even at 40 μg/ml (data not shown).

The inhibitory effect of **E** was examined with various concentrations of PP_i (2 to 40 μg/ml) (Fig. 2). The double-reciprocal plots shown in Fig. 2B suggest non-competitive

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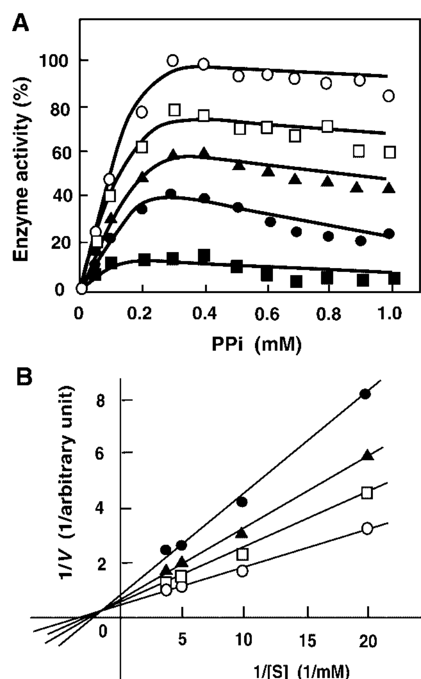

Fig. 2. **Noncompetitive inhibition of H⁺-PPase by the inhibitor.** (A) H⁺-PPase activity was measured with various concentrations of PPi in the presence of an inhibitor (compound **E**). The concentration of the inhibitor was 0 (open circles), 2 (open squares), 4 (solid triangles), 10 (solid circles), or 20 μg/ml (solid squares). Activity was expressed as a percentage of the control activity without the inhibitor at 0.3 mM PP_i. (B) Double reciprocal plots of the results show noncompetitive (or mixed) inhibition by compound **E**.

inhibition (mixed inhibition) of the inhibitor. The apparent K_i value of **E** was calculated to be 0.91 μM.

Mung bean H⁺-PPase consists of a single polypeptide of 80 kDa (73 kDa in SDS-PAGE) (*1*, *5*). Figure 3 shows an

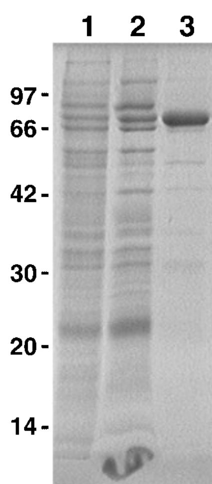

Fig. 3. **Purified preparation of H⁺-PPase used for the inhibitor assay.** The enzyme was purified from vacuolar membranes of mung bean hypocotyls. Lane 1, vacuolar membranes; lane 2, the solubilized fraction with lysophosphatidylcholine and *n*-dodecyl-β-maltoside; lane 3, purified preparation after ion exchange column chromatography. The molecular masses (kDa) of the standard proteins are shown on the left.

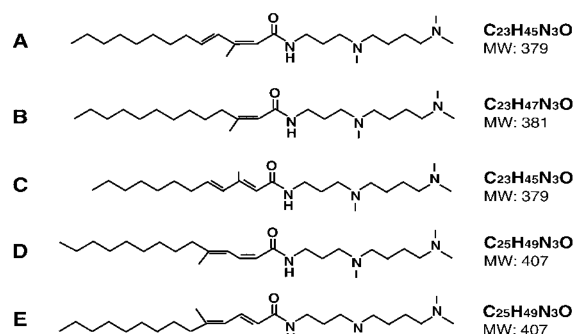

Fig. 4. **Chemical structures of acylspermidine derivatives from the soft coral.** Five inhibitors of V-PPase were identified as acylspermidine derivatives (acylspermidines **A**, **B**, **C**, **D**, and **E**). Their chemical formulas and molecular masses are shown.

SDS-PAGE profile of the purified mung bean H⁺-PPase. In the present study, we highly purified H⁺-PPase in order to decrease the secondary effect of the other protein components in the membrane. The purified enzyme was assayed in the presence of phospholipid micelles.

Chemical Identification of Inhibitors—In the present study, the purified inhibitors were subjected to the enzyme assay (Fig. 4). The five compounds isolated from the water fraction of a soft coral, *Sinularia* sp., were determined to be acylspermidine derivatives (*14*). In the present study we named them acylspermidines **A**, **B**, **C**, **D**, and **E** (Fig. 4). All the derivatives are *N′,N″,N″*-trimethylspermidines that are acylated by a methyl-branched unsaturated fatty acid. Chemical identification of acylspermidines **A** and **B** was reported previously (*15*, *16*). Acylspermidines **C**, **D** and **E** have been recently identified and reported by Ojika *et al.* (*14*).

*Effects of Acylspermidines **D** and E on **H⁺**-Pump Activity*—The inhibitory effect of acylspermidines **D** and **E** on H⁺-PPase was further substantiated by examining their influence on the PP_i-dependent H⁺ pump activity in vacu-

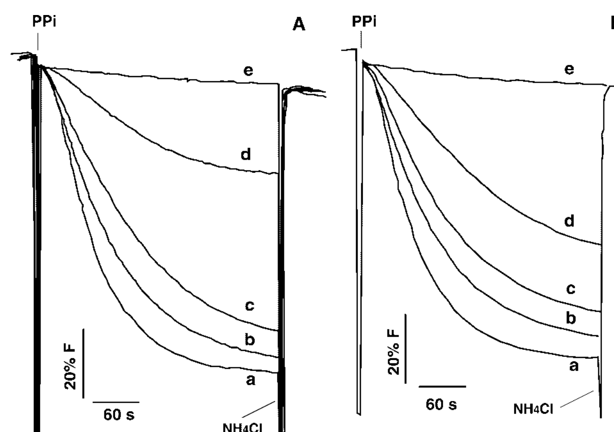

Fig. 5. **Inhibition of PP_i-dependent H⁺ pump activity by acylspermidine derivatives.** Vacuolar membrane vesicles prepared from mung bean hypocotyls were assayed in the presence of acylsperimidine **D** (A) or **E** (B). The H⁺ pump activity was measured as the rate of fluorescence quenching of acridine orange. The concentration of acylspermidine was 0 (a), 0.1 (b), 0.4 (c), 1 (d), or 2 μg/ml (e) in both (A) and (B).

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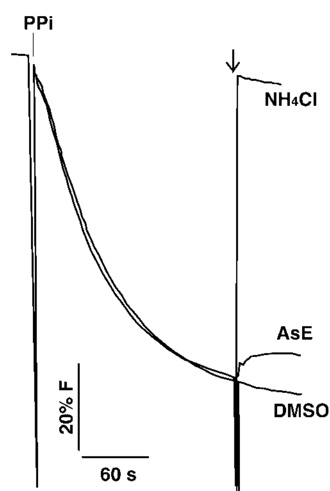

Fig. 6. **Effect of acylspermidine E on membrane integrity.** The pH gradient across the membrane of vesicles was formed by adding PP_i, and then acylspermidine **E** (AsE), DMSO (0.02%), or NH_4Cl (1 mM) was added at the indicated time.

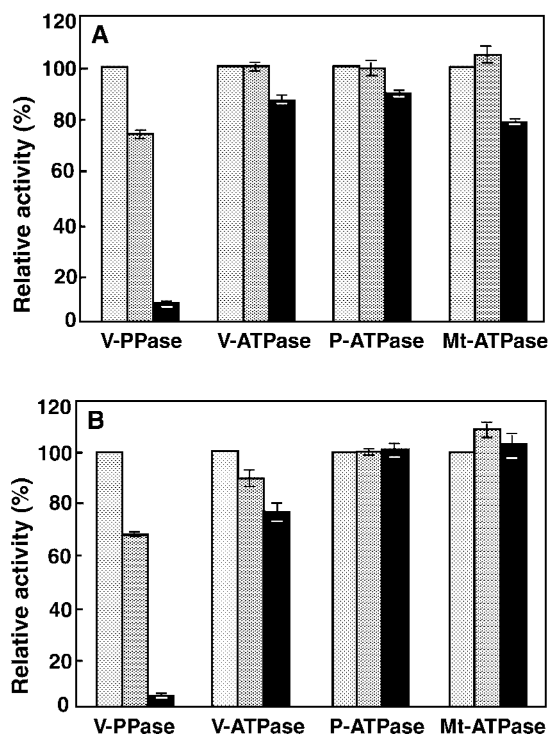

Fig. 7. **Specificity of acylspermidine derivatives.** Vacuolar membranes, plasma membranes, and mitochondria were prepared from mung bean hypocotyls. The effects of acylspermidine derivatives **D** (A) and **E** (B) on the activities of H^+-PPase, V-ATPase, plasma membrane H^+-ATPase (P-ATPase), and mitochondrial ATP synthase (Mt-ATPase) in the corresponding membrane preparations from mung bean hypocotyls were examined. The effects of acylspermidines **D** and **E** at 2 μg/ml (shaded bars) and 10 μg/ml (closed bars) are expressed as means ± SD of the control (open bars). The activity is expressed as a percentage of the control activity without an inhibitor.

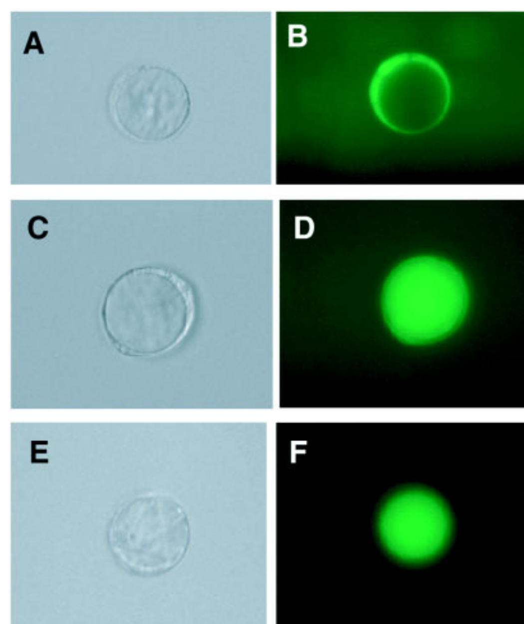

Fig. 8. **Effect of acylspermidine D on the acidification of vacuoles in protoplasts.** Protoplasts were isolated from the hypocotyls of 3-day-old mung bean seedlings. A large central vacuole was observed in each cell. Protoplasts were incubated in the standard medium (A and B), acylspermidine **D** (10 μg/ml) (C and D), or bafilomycin A_1 (100 nM) (E and F) for at least 20 min. Then vacuolar acidification was monitored using fluorescent acridine orange under a fluorescent microscope.

olar membrane vesicles. H^+ pump activity was measured as the rate of fluorescence quenching of acridine orange. As shown in Fig. 5, the membrane preparation exhibited electrogenic H^+-pump activity, since the quenched fluorescence was recovered on the addition of ammonium chloride. Both compounds inhibited the H^+ pump activity completely at 2 μg/ml (Fig. 5, line e), and by 50% at 0.4 μg/ml (line c).

Figure 6 shows the effect of acylspermidine **D** on the membrane integrity along a pH gradient across the membrane pre-formed by adding PPi to the medium. Acylspermidine **D** slightly stimulated the proton leakage from the vesicles, but its effect was weak compared with that of ammonium chloride, a potent H^+-gradient breaker.

Specificity of the Inhibitory Effect of Acylspermidine Derivatives—The ATP-hydrolysis activities of V-ATPase in the vacuolar membranes, H^+-ATPase in the plasma membranes, and ATP synthase in mitochondria were individually assayed under suitable assay conditions. As shown in Fig. 7, the inhibitory effect of acylspermidine **D** and **E** was limited on the activities of V-ATPase, plasma membrane H^+-ATPase, and mitochondrial ATP synthase, even at a high concentration (10 μg/ml), although they markedly inhibited V-PPase at this concentration. These results strongly suggest the specificity of the inhibition of H^+-PPase by acylspermidine derivatives.

Effects of Acylspermidine Derivatives on the Acidification of Vacuoles in Protoplasts—In mature vegetative cells, the central vacuoles occupy more than 90% of the cell volume (Fig. 8, A, C, and E). The acidic conditions in the vacuolar lumen can be monitored by means of fluorescent monoamine acridine orange. The fluorescence quenching of acridine orange occurs under acidic conditions. Thus, the fluorescence was weak in the vacuoles in normal pro-

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toplasts (Fig. 8B). By contrast, the vacuoles in protoplasts that had been treated with acylspermidine **D** (panel D) or bafilomycin A_1 (panel F), a potent inhibitor of V-ATPase, showed relatively strong green-yellow fluorescence. These results indicate that these vacuoles had a neutral lumenal pH. Namely, acylspermidine **D** inhibited the vacuolar acidification to a similar level as bafilomycin A_1.

DISCUSSION

Soft corals are known as rich sources of sesquiterpenoids and diterpenoids, especially cembranoid diterpens (*17–21*). The identified compounds in this study were not terpenoids or derivatives of them. The water-soluble fraction of a soft coral, *Sinularia* sp., strongly inhibited H^+-PPase. Here, we report that these acylspermidine derivatives are inhibitors of H^+-PPase. Acylspermidines **D** and **E** exhibited the strongest inhibitory activity of the five derivatives. The half inhibitory concentration (IC_{50}) of acylspermidines **D** and **E** was 15 μM (Fig. 1). Under these conditions (H^+-PPase, 10 nM), the molar ratio of acylspermidine to H^+-PPase was approximately 1,500:1. For the H^+ pump activity, acylspermidines **D** and **E** inhibited the activity by half at 1.2 μM (Fig. 5). The molar ratio of acylspermidine to H^+-PPase in the membrane was calculated to be approximately 40:1. In conclusion, acylspermidines **D** and **E** are effective inhibitors of H^+-PPase, with a K_i of 0.9 μM.

We demonstrated the inhibitory effect of acylspermidines in a protoplast system. Quantitative analysis could not be performed, but the inhibition of vacuole acidification by acylspermidine **D** was comparable to that by bafilomycin A_1, an inhibitor of V-ATPase. The activity of vacuolar H^+-PPase is equal to or more extensive than the V-ATPase activity in the elongating region of mung bean hypocotyls, which we used for protoplast preparation in the present study (*22*, *23*). Therefore, it is reasonable that the effect of inhibition of H^+-PPase by acylspermidines on the vacuole acidity was similar to that of V-ATPase by bafilomycin A_1.

One should note the difference in the IC_{50} values between the activities of PP_i hydrolysis (15 μM) and the H^+ pump (0.98 μM). The difference may be due to the assay conditions. To assay PP_i hydrolysis activity, the purified enzyme was used in the presence of 0.02% Triton X-100. On the other hand, the H^+ pump activity was assayed using membrane vesicles without a detergent. There is a possibility that the detergent suppresses the inhibitory effect by partially absorbing acylspermidines in the assay medium. Furthermore, the enzyme reaction may be partially altered by a detergent. The apparent affinity for a substrate of mung bean H^+-PPase was also different, that is, between the PP_i hydrolysis (K_m, 100 to 200 μM) and H^+ pump (K_m, < 20 μM) activities, as reported (*24*, *25*).

Neither acylspermidine **D** nor **E** inhibited V-ATPase, plasma membrane H^+-ATPase, mitochondrial ATP synthase, or soluble, cytosolic PPase. We also confirmed that spermidine, a polyamine, did not inhibit H^+-PPase, even at 50 μM. Spermidine did not protect the enzyme from the inhibition by acylspermidines. The results indicate no interaction between the enzyme and spermidine. From their chemical structures and hydrophobicity, the fatty acid moiety of acylspermidines may be inserted in the portion between the membrane-spanning α-helices, which is located at the catalytic site, of H^+-PPase or the interface between the helices and membrane lipids. The methylated spermidine moiety of the acylspermidines may prevent the interaction of the negatively charged PP_i and the catalytic amino acid residues of the enzyme, such as the Asp-253, Glu-263, and Asp-723 ones (*26*). Acylspermidines **D** and **E** are different from the other three derivatives in the carbon number and the methyl-branched site of fatty acids. These structural characteristics may be closely related to the interaction with H^+-PPase and reflected in their inhibitory effects.

In conclusion, we found five natural inhibitors of vacuolar H^+-PPase in natural sources. These new inhibitors of H^+-PPase are different from known ones, such as aminomethylenediphosphonate (*4*), imidodiphosphate (*4*), Ca^{2+} (*24*, *27*), and DCCD (*5*). From the specificity of their activity, these acylspermidines can be used for *in vitro* experiments to distinguish H^+-PPase from F-, P-, and V-type H^+-ATPases, and cytosolic PPase, and to evaluate the physiological contribution of H^+-PPase to the vacuole acidification. Before such *in vivo* experiments, the effect of the acylspermidines on other enzymes needs to be examined. We can also modify the natural acylspermidines and synthesize new compounds to obtain more effective inhibitors for medicinal uses. In plants (*1*, *2*, *28*) and parasitic protists (*3*, *6*, *7*), vacuolar H^+-PPase functions as a primary proton pump in vacuoles or acidic organelles together with V-ATPase, which are essential for the acidification and secondary transport systems of vacuoles. Thus, the acylspermidine derivatives found in this study and modified forms of them may be useful for evaluating the physiological function of H^+-PPase in plants and parasitic protists.

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