Concanamycin B, a Vacuolar H⁺-ATPase Specific Inhibitor Suppresses Bone Resorption in Vitro

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Effects of concanamycin B, a specific inhibitor of the vacuolar type H⁺-ATPase (V-ATPase), on the stimulation of bone resorption induced by parathyroid hormone (PTH) were examined in vitro. Concanamycin B was found to inhibit PTH-stimulated osteoclastic pit formation and to suppress the acidification of vacuolar organelles by V-ATPase in the osteoclasts. PTH-stimulated ⁴⁵Ca release from prelabelled chick embryonic calvariae was also inhibited by concanamycin B in a dose-dependent manner. These results suggest that osteoclastic acidification of lacunae by V-ATPase plays an essential role in mineral dissolution and degradation of the organic matrix during bone resorption.

Key words concanamycin B; vacuolar H⁺-ATPase inhibitor; bone resorption

Osteoclasts are multinucleated cells that play a key role in bone resorption. Bone resorption occurs in an acidic extracellular compartment. This space between osteoclasts and the mineralized bone matrix is covered with the ruffled border of osteoclasts during bone resorption.¹⁻³ This compartment functions as an extracellular lysosome into which acid and enzymes are secreted by the osteoclasts.⁴ The acid solubilizes the mineral and allows the proteolytic enzymes to digest the matrix proteins.⁴ An acidic environment is obtained by the proton secreted through the ruffled border membrane by osteoclastic V-ATPase.⁵⁻⁶ Mineral dissolution and enzymatic degradation of the organic matrix of mineralized bone may be modulated by the acidification of bone resorption site.⁵⁻⁶

Concanamycins, specific inhibitors of V-ATPase found in the culture of Streptomyces species, are known to inhibit ATP-dependent acidification of vacuolar organelles such as the endosomes and lysosomes of the macrophage J774 cells⁷⁻⁸ and to inhibit the activity of vacuolar ATPase from Neurospora crassa.⁹ Sasaki et al. demonstrated the expression of vacuolar H⁺-ATPase in mouse osteoclasts by immunolabeling and showed a suppressive effect of concanamycin B on pit formation using backscattered electron microscopy.¹⁰ In this paper, we have shown a reduction of pit numbers caused by concanamycin B and an inhibitory effect on acidification of osteoclastic vacuolar organelles by using acridine orange staining as a pH indicator. Furthermore, a suppressive effect on PTH-stimulated ⁴⁵Ca release from chick calvaria was also shown.

MATERIALS AND METHODS

Materials Human parathyroid hormone (PTH, 1—34) was purchased from Sigma Chemical Co. Concanamycin B was kindly given by Prof. A. Endo (Tokyo Noko University). Acridine orange was obtained from Wako Chemical Co., and [¹⁰⁸Ca]calcium chloride was from NEN Research Product. All the other chemicals were of analytical grade. Sprague-Dawley rats (1—2 d old) were used to prepare unfractionated bone cells.

Pit Formation Assay The pit formation assay was carried out according to the method of McSheehy and Chambers¹¹ with some modifications. Unfractionated bone cells were prepared from tibiae, femora and humeri of 1—2 d old Sprague-Dawley rats. The bones were dissected free from soft tissues in the iced tissue culture medium (α-MEM (minimum essential medium), pH 7.2, supplemented with 100 IU per milliliter benzylpenicillin containing 10 mm N-2-hydroxyethyl-piperazine-N-ethanesulfonic acid (HEPES). To prepare bone cells, the long bones were cut up with scissors in the same medium and the suspension was then titrated with a wide-bored plastic pipette. Two hundred and fifty microliters of this suspension (1 × 10⁶ cells per milliliter) were added to each well of a 96-well plate containing a bone slice, and the slices were incubated at 37 °C in a CO₂ incubator (5% CO₂—95% air) for 1 h. The slices were then rinsed with α-MEM and transferred to fresh medium containing 10% fetal bovine serum (FBS), concanamycin B (0, 1.25 ng/ml) and 100 nm of PTH, and then incubated for 24 h. The cells were removed from the slices by vigorous washing with distilled water, and then the slices were stained with acid hematoxylin for 5 min. The total number of pits formed by osteoclastic bone degradation was counted under an optical microscope. The osteoclastic cell number was determined by counting the adhesive TRAP (tartrate-resistant acid phosphatase)-positive cells on a femur slice cultured by the same method as the pit formation assay.

Vital Fluorescence Microscopy Unfractionated bone cells cultured on the bone slice were stained with acridine orange according to the method by Geisow et al. to investigate the pH distribution in the cells.¹² The cells grown on the bone slice were incubated at 37 °C for 30 min with α-MEM containing 1/100 volume of concanamycin B (final concentration: 1.25 ng/ml) dissolved in dimethyl sulfoxide (DMSO). For control experiments, DMSO alone was added to the medium instead of the concanamycin B.

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solution. Then the cells were incubated at 37°C for 10 min with 0.5 μg/ml acridine orange. After washing four times with PBS (−), the cells on the slice were examined by a fluorescence light microscopy.

**Bone Culture** Calvaria were isolated from 17 d-old chick embryos. Chick calvarium is composed of paired identical pieces of bones. One-half of each calvarium served as a control for the corresponding half treated with test sample. Each half-calvarium (40—50 mg wet weight) was cultured in a 24-well plate in 1 ml of serum-free medium consisting of BGJb medium supplemented with 0.5% bovine serum albumin plus kanamycin (60 μg/ml). Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO₂ and 95% air.

**45Ca Release Assay** Bone resorption assay was performed by the modified method of Saito et al. Calvaria were preincubated in the medium. After 24 h, the bones were labelled with medium containing 45Ca (1 μCi/ml) for 2 h. The labelled bones were washed twice in calcium- and magnesium-free phosphate buffered saline [PBS (−)] and then transferred to fresh medium without 45Ca and cultured for 24 h. This step was necessary to determine nonspecific 45Ca release from labelled bone in this assay system. After 24 h incubation, the bones were washed with PBS (−) again and transferred to fresh medium supplemented with PTH, with or without concanamycin B, and then cultured for 96 h. At the end of this culture period, the medium (100 μl) was withdrawn for liquid scintillation counting to measure the radioactivity released into the medium from labelled bone during cultivation. To measure the 45Ca remaining in bone, the bones were immersed in 1 N HCl (1 ml) overnight and the extracted radioactivity (100 μl) was counted. The amount of bone resorption was calculated as the release of 45Ca, expressed as follows: 45Ca release (%) = 45Ca in medium/(45Ca in medium + 45Ca in bone) × 100. Each value indicated is the mean of 5 cultures ± S.E.

**RESULTS**

The effect of concanamycin B on pit formation induced by PTH was investigated. More than three hundred pits were observed in the PTH-treated group, whereas the number in the control (− PTH) group was only 10 (Fig. 1). This means that demineralization and degradation of calcified collagen was enhanced by PTH. Addition of concanamycin B (1.25 ng/ml) reduced the pit number to about 50% of the PTH-treated group. This result agrees with the data of Sasaki et al. The number of TRAP-positive adherent cells was not affected by this concentration of concanamycin B. This means that concanamycin B suppresses pit formation without affecting the differentiation or viability of the osteoclast.

To examine inhibition of vacuolar acidification by concanamycin B in intact osteoclasts, acridine orange staining was performed. Acridine orange exhibits an orange fluorescence at low pH, whereas at higher pH this changes to green. When osteoclasts were stained with acridine orange, nuclei and cytoplasm exhibited a green fluorescence, whereas an orange fluorescence was observed in the granular pattern in the cytoplasm (Fig. 2A). This distribution pattern suggests that normal osteoclasts have

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**Fig. 1. Effects of Concanamycin B(C.B) on Pit Formation (A) and Osteoclastic Cell Numbers (B)**

Concentration of PTH and concanamycin B was 100 nM and 1.25 ng/ml, respectively. Each value represents the mean ± S.E.M. (n = 5). * p < 0.01 vs. + PTH. ** p < 0.05 vs. none.

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**Fig. 2. Acridine Orange Staining of Osteoclasts**

A: no treatment, B: treated with concanamycin B (1.25 ng/ml).
acidified vacuolar organelles. Treatment with 1.25 ng/ml concanamycin B for 30 min before acridine orange-staining markedly reduced the orange fluorescence in osteoclasts (Fig. 2B). This suggests that concanamycin B inhibits the acidification of vacuolar organelles by interacting with V-ATPase in the vacuolar organelle membranes.

The effect of concanamycin B on \(^{45}\)Ca release induced by PTH was examined in organ cultures of 17-d-old embryonic chick calvaria. In the \(^{45}\)Ca release assay system, PTH stimulated the \(^{45}\)Ca release from bone into medium. The \(^{45}\)Ca release of the control (−PTH) groups was 8%, whereas in the PTH-treated group the figure was 12%. These results show that PTH stimulates \(^{45}\)Ca release from bone into medium. On the other hand, addition of concanamycin B to PTH-stimulated chick calvaria reduced the increase in \(^{45}\)Ca release in a dose-dependent manner. When the increase in PTH-induced \(^{45}\)Ca release (%) is taken as 100 (%), 20 ng/ml of concanamycin B reduced it to 20 (%) (Fig. 3). Concanamycin B did not have any cytotoxic effects on cultured calvaria at the concentrations used in this experiment.

**DISCUSSION**

Sundquist et al. have reported that bone resorption and \(H^+\)-transport in chicken osteoclastic cultures are inhibited by bafilomycin A1, a 16-membered macrofide V-ATPase inhibitor.\(^{14}\) Using two types of typical bone resorption assay, pit formation and \(^{45}\)Ca release, we showed that the 18-membered macrofide V-ATPase-specific inhibitor, concanamycin B also suppresses osteoclastic bone resorption, although the effective dose for \(^{45}\)Ca release is much higher than that in pit formation. This might be due to a difference in sensitivity for concanamycin B between chick calvarial cells and rat long bone cells. Another possibility is that the ability of the drug to permeate into cells in the organ culture is different from that in the suspension cell culture.

Our data obtained in these two experiments suggest that osteoclastic acidification by V-ATPase plays an essential role in mineral dissolution during bone resorption. This agrees with the hypothesis that osteoclastic acidification is a necessary precursor step to mineral dissolution and collagen degradation and that osteoclastic acidity is increased by PTH.\(^{15}\) Our data in Fig. 2B show that the acidic vacuolar organelles in osteoclast is clearly eliminated by the presence of concanamycin B. The acidic extracellular compartment of osteoclast functions as an extracellular lysosome into which acid and enzymes are secreted to degrade the mineralized bone matrix. Therefore, inhibition of the acidification of vacuolar organelles means the destruction of the acidic microenvironment of osteoclasts and results in significant damage to the osteoclastic function in bone resorption.

\(H^+\)-ATPases can be distinguished from each other on the basis of their structure and sensitivity to specific inhibitors. V-ATPases are sensitive to \(N\)-ethylmaleimide and bafilomycin A1, but not to vanadate. Unlike vesicles derived from any other cell type or organelles, chicken osteoclastic V-ATPase is inhibited by vanadate.\(^{16}\) It is suggested that the ruffled-border osteoclastic V-ATPase possibly differs from that of other cells or organelles. Taken together, we believe that a specific inhibitor of osteoclastic V-ATPase would be a good candidate agent for inhibiting bone resorption.

**REFERENCES**