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INHIBITION OF OSTEOCLAST PROTON TRANSPORT BY BAFILOMYCIN A_1 ABOLISHES BONE RESORPTION

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Osteoclasts are the main bone resorbing cells with capacity to acidify their intimate contact area with bone. Recent studies have suggested that osteoclast acid secretion is carried out by an H^+-ATPase. We demonstrate here, that specific inhibitor of vacuolar type H^+-ATPases, bafilomycin A_1, inhibits bone resorption in osteoclast cultures as well as blocks proton transport in isolated medullary bone derived microsomes containing a vacuolar type H^+-ATPase. These results demonstrate an important role of vacuolar H^+-ATPase in bone resorption.

Osteoclasts are known to acidify the underneath lacunae during bone resorption (1,2 for rev. 3). Recent data support the view that this is carried out by proton secretion through the ruffled border membrane by a vacuolar type of proton transport ATPase (4,5). Recently Bowman et al. (6) described the highly specific inhibition of the vacuolar type ATPases by bafilomycin. Bafilomycins can thus be used to specifically differentiate the vacuolar class of ATPases from other type of proton transporting ATPases.

We demonstrate here that bone resorption by isolated and cultured osteoclasts as well as H^+-transport in chicken medullary bone derived microsomes, is inhibited by bafilomycin A_1.

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Materials and Methods

Bone resorption assay

The procedure to culture isolated rat osteoclasts on bovine bone slices was modified from original methods of Chambers et al. (7) and Boyde et al. (8) and has been described earlier in detail (9). In brief, osteoclasts were mechanically dis-aggregated from long bones of 3-4-day old rat pups in Hepes-buffered DMEM containing 0.84 gr/l sodium bicarbonate, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated fetal calf serum, pH 6.9 and allowed to attach to transverse slices (150 µm thick) of dense cortical bovine bone (0.5 cm^2 surface area). After 30 min of incubation at 37°C in 5% CO_2/95% air non-attached cells were washed away and bone slices with attached cells were removed either to culture medium with approciate vehicles or with inhibitors. Synthetic salmon calcitonin was purchased from Sigma Chemical Co (St. Louis, MO) and bafilomycin A_1 was a generous gift from Dr. Altendorf (University of Osnabrück, BRD).

After incubation for indicated time period, the bone slices were removed, gently washed in PBS and fixed in 3% paraformaldehyde containing 2% sucrose in PBS. After fixation bone slices were washed again in PBS and stained with 1% Toluidine Blue in 1% sodiumborate (10). Each bone slice was screened systematically using X25 objective to count the number of osteoclasts. Cells were removed by ultrasonication for 30 s in 0.25 mmol ammonium hydroxide and resoption pits were counted using phase contrast optics. Resorption index for each slice was counted as a number of resorption pits per number of osteoclasts. Some slices were also examined in a JEOL JSM-35 scanning electron microscope after critical point drying and gold sputter-coating.

Proton transport measurements

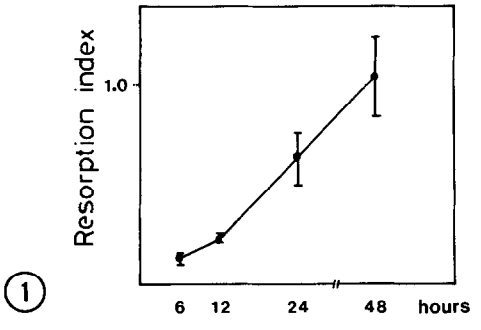
Bone microsomes were prepared from medullary bone of regularly laying hens. Medullary bone from the tibia and femur was dissected and the resulting tissue was immediately homogenized in a medium containing 5 mM Tris pH 7.4, 250 mM sucrose, 1 mM K_2CO_3, 1 mM dithiotreitol and 1 mM EGTA in a glass-teflon homogenizer. The homogenate was centrifuged at 1000 x g for 10 min and the pellet discarded. The supernatant was initially centrifuged at 10 000 x g for 30 min and the resulting supernatant centrifuged again at 100 000 x g for 60 min. The final pellet was suspended in the homogenization buffer.

Proton transport by isolated membrane vesicles was assayed in a dual beam, dual wavelength spectrophotometer (Shimadzu UV-3000) by measuring the uptake of acridine orange at room temperature. The membrane vesicles (30 µg protein) were suspended in 2 mM Hepes buffer, pH 7.5, containing 10 µM acridine orange, 2 mM $MgCl_2$, 1 µM valinomycin and 175 mM KCl in a final volume of 1 ml. The reaction was initiated by adding 100 µl 5 mM Na_2 ATP (pH was adjusted to 7.5 with Tris base) and the pH gradient was dispatced by 1 µg of nigericin. Different concentrations of bafilomycin A_1 were added 5 min before ATP when used. Acridine orange fluorescence was measured with excitation and emission wavelengths of 492 nm and 547 nm, respectively. Protein concentration was measured as described by Bradford (11).

Results and Discussion

In our cultures, osteoclasts were isolated from neonatal rat bones and resorbed approximately one visible cavity per cell during 48 hours (Fig. 1). This is about the rate of resorption described earlier by others (10). Although resorption pits were observed already after 6 hours, 2 days was selected for further experiments in order to determine the possible cytotoxicity of used drugs. In this culture model osteoclasts continue resorption up to three weeks. We observed that light microscopical examination and counting of resorption pits after Toluidine Blue staining is as sensitive as counting pits using SEM. This has been also shown earlier by Arnett and Dempster (10). In this experimental model, correlation between the number of resorption pits and the total area of resorption cavities has been shown to be excellent (10). Thus, this assay method is a specific, reliable and sensitive way to measure osteoclast-mediated bone resorption. Calcitonin which is known to be a potent and specific inhibitor of osteoclast mediated bone resorption is effective also in this assay system, showing an IC_{50} of about 6×10^{-12}M at two days assay (Fig. 2). About 20% of the bone resorption was resistant to calcitonin even at concentrations of 10^{-7}M (Fig. 2). Calcitonin might be more potent at shorter culture times, since the escape from inhibition takes place also in this assay system.

Several different concentrations (10^{-10}M to 10^{-7}M) of bafilomycin A_1 were tested in the isolated osteoclast and an



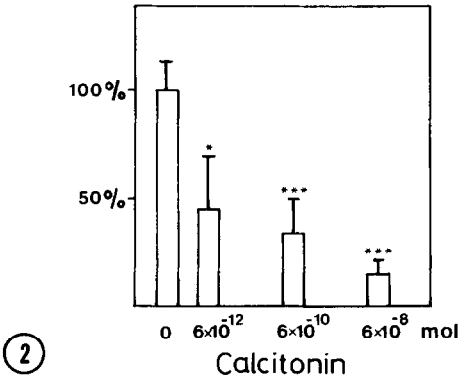
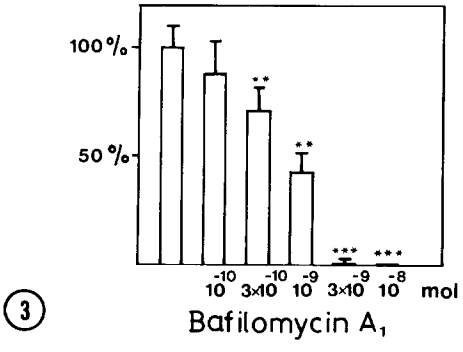


Fig. 1. Resorption activity of isolated rat osteoclast in culture. (bar = mean ± SEM, n = 4)

Fig. 2. Inhibition of bone resorption by calcitonin. (bar = mean ± SEM, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

IC_{50} of about 1 nmol was obtained (Fig. 3). With concentration of 3 x 10^{-9}M almost total inhibition of bone resorption was reached and in several different experiments with 10^{-8}M no resorption pits were found. The number of osteoclasts were not affected at bafilomycin concentrations of 10^{-8}M or less (152 ± 23 and 133 ± 22 in controls and with 10^{-8}M bafilomycin A_1, respectively, n.s.). However, at bafilomycin A_1 concentration of 10^{-7}M statistically significant reduction in the osteoclasts number was observed (34 ± 4, $p<0.001$).

The effect of bafilomycin A_1 on ATP driven H^+-transport in isolated medullary bone microsomes was investigated. These microsomes have previously been shown to exhibit similar characteristics of H^+-transport as membrane vesicles isolated from kidney or from isolated osteoclasts (4,5) Among bone cells, only osteoclasts have been shown by immunohistochemistry to exhibit staining with antibodies rised against vacuolar type H^+-ATPase (4,5). The effect of bafilomycin A_1 on H^+-transport by H^+-ATPase of bone microsomes is shown is Fig. 4. Acidification of isolated bone microsomes was ATP-dependent and bafilomycin A_1 inhibited H^+-transport with the IC_{50} of about 3 nmol/l. Almost complete inhibition was obtained at 2 x 10^{-8} mol/l. This is close to what was observed in the bone resorption. The present observation that bafilomycin A_1, a selective vacuolar H^+-ATPase inhibitor, prevents bone resorption strengthen the view that this type of



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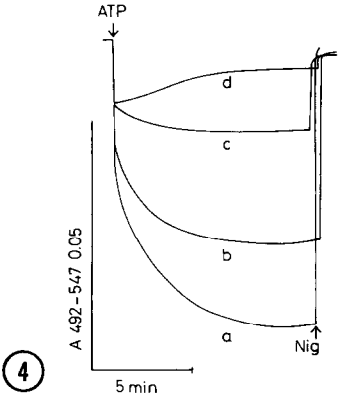

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Fig. 3. Inhibition of bone resorption by bafilomycin A_1. (bar = mean ± SEM, ** $p \leq 0.01$, *** $p \leq 0.001$)

Fig. 4. The effect of bafilomycin A_1 to H^+-transport of isolated bone microsomes. (a = control, b,c and d = 1,10 and 100 nmol of bafilomycin A_1, respectively)

proton pump is essential for bone resorption and suggests that acidification of the resorption lacunae is due to this enzyme.

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