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Inhibition of Bone Resorption by Bisphosphonates: Interactions Between Bisphosphonates, Osteoclasts, and Bone

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Summary. Bisphosphonates are nonbiodegradable pyrophosphate analogues that are being used increasingly to inhibit bone resorption in disorders characterized by excessive bone loss. We have previously found that dichloromethylene bisphosphonate (Cl_2MBP) inhibits bone resorption through injury to the cells that resorb Cl_2MBP-contaminated surfaces. 3-amino-1-hydroxypropylidene-1,1-bisphosphonate (AHPrBP) is a more potent inhibitor of bone resorption *in vivo*, and we have attempted to identify a step in the resorptive pathway that accounts for this increased potency. We found that when osteoclasts, isolated from neonatal rat long bones, were incubated on bone slices in the presence of bisphosphonates, AHPrBP was less, rather than more potent as a resorption-inhibitor than Cl_2MBP. The greater sensitivity of resorption to AHPrBP *in vivo* could neither be attributed to an effect of AHPrBP on the ability of osteoblastic cells to stimulate resorption in response to calcium-regulating hormones *in vitro* nor to an effect on osteoclast generation: osteoclast formation was unaffected by concentrations of AHPrBP 10-fold higher than those of Cl_2MBP which inhibit bone resorption in the bone slice assay. We also found no evidence for impaired osteoclast generation *in vivo* in AHPrBP-treated rats. These results suggest that the comparisons of potency *in vitro* do not include all the factors responsible for determining bisphosphonate potency *in vivo*. Because bisphosphonates owe the specificity of their actions to their ability to bind to bone surfaces, we performed experiments using bone slices that had been immersed in bisphosphonates before use. Bone resorption was virtually abolished on bone slices preincubated in 10^{-3} M AHPrBP. Inhibition was associated with degenerative changes in osteoclasts and a more rapid decrease in the number remaining on the bone surface than occurred with Cl_2MBP. The effect was specific for osteoclasts, could be prevented if bone resorption was suppressed by calcitonin, and was not seen in osteoclasts incubated in AHPrBP on plastic coverslips. These observations suggest that AHPrBP inhibits bone resorption through injury to osteoclasts when they solubilize bisphosphonate-contaminated bone. We found that the concentration of AHPrBP used in the preincubation phase could be reduced by an order of magnitude if the volume of the AHPrBP solution was correspondingly increased. This implies that the concentration of bisphosphonate is less relevant to potency comparisons than the density of bisphosphonate on the bone surface. The latter will be strongly influenced *in vivo* not only by affinity for bone but by the pharmacokinetic and other properties of the compound.

Key words: Osteoclast – Osteoblast – Bisphosphonates – Bone resorption.

Bisphosphonates (PCPs) are nonbiodegradable analogues of pyrophosphate with a high binding affinity for hydroxyapatite crystals [1]. Administration of PCPs to experimental animals rapidly curtails bone resorption [2–4] and it is for this reason that they have become established as therapeutic agents in clinical disorders characterized by increased bone degradation, including Paget's disease of bone [5–8], and hypercalcemia of malignancy [6, 9, 10], and may prevent osteoporosis [11–13]

The mechanism by which these compounds inhibit bone resorption is unknown. It was originally thought that they might act through physicochemical inhibition of hydroxyapatite crystal dissolution [14, 15], but no correlation has been found between the ability of PCPs to inhibit bone resorption *in vitro* or *in vivo* and their ability to inhibit hydroxyapatite dissolution [16]. This suggests that their action on bone resorption is mediated through mechanisms other than physicochemical effects on hydroxyapatite. For dichloromethylene bisphosphonate (Cl_2MBP, formerly Cl_2MDP), inhibition of bone resorption is associated with morphological evidence of degenerative changes in osteoclasts, the cells that resorb bone, without morphological effect on cells other than osteoclasts [17, 18]. Toxicity to osteoclasts is considerably increased if bone is used as a substrate; and calcitonin, which prevents the osteoclasts from resorbing bone, protects against the toxic effects of Cl_2MBP. PCPs interfere with many aspects of the intermediary metabolism of cells [19–23], and the above observations suggest that Cl_2MBP acts as a toxic compound with a high affinity for bone, which inhibits bone resorption through injury to the cells that solubilize Cl_2MBP-contaminated surfaces.

It may well be that other PCPs have a different mechanism of action. The relative potency of 3-amino-1-hydroxypropylidene-1,1-bisphosphonate (AHPrBP, formerly APD), as judged by inhibition of bone resorption in organ culture, does not correlate with the increased relative potency of AHPrBP *in vivo* [16]. This has led to the suggestion that AHPrBP may have an additional effect on osteoclast precursors [24–26]. In this report we examine the effects of AHPrBP on osteoclast generation *in vivo* and *in vitro*, and assess the effects of AHPrBP on osteoclast isolated from neonatal rat long bones.

Materials and Methods

Hepes-buffered medium 199 (Flow Laboratories, Irvine, Scotland) (199) was used for isolation and sedimentation of osteoclasts from rat long bones and in the preparation of mouse bone marrow cul-

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tures. Phosphate-free Eagle's Minimal Essential Medium with Hanks' salts (Flow) (MEM/Hanks') was used for incubation of isolated osteoclasts, and mouse bone marrow was cultured in MEM with Earle's salts (Flow) (MEM/Earle's). All medium used for incubation of cells contained 10% heat-inactivated fetal calf serum (FCS) (Gibco, Paisley, Scotland). L-Glutamine (2 mM) (Glaxo, Middlesex, England) and benzyl penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Glaxo) supplemented all media. Hydrocortisone (Sigma, Poole, Dorset, UK) and 1,25 dihydroxyvitamin D_3 (Leo Laboratories, Bucks, UK) were dissolved in ethanol and prepared as stock solution of 10^{-2} M and 10^{-5} M, respectively. AHPrBP (donated by Ciba-Geigy, Basle, Switzerland) and Cl_2MBP were dissolved in 0.9% sodium chloride (BDH Chemicals Ltd, Poole, England), corrected to pH 7.4 and filter sterilized (Millipore, Molsheim, France). Salmon calcitonin (CT) was donated by Sandoz Pharmaceuticals (Basle, Switzerland) and parathyroid hormone (PTH) was a gift from Dr. J Zanelli (National Institute for Biological Standards, Hampstead, London, UK).

Preparation of Bone Slices

Devitalized bone slices were prepared from bovine femoral cortical bone, as previously described [27]. Cortical bone slices (0.4 × 0.4 × 0.01 cm) were prepared from wafers of bone cut with a low speed saw (Isomet, Buehler, Illinois, USA). The slices were cleaned by ultrasonication for 20 minutes in distilled water, immersed in acetone for 10 minutes, followed by alcohol (10 minutes), and stored dry at room temperature.

Preincubation of Bone Slices in Bisphosphonates

Each bone slice was placed in separate 200 μl or 5 ml volumes of either Cl_2MBP or AHPrBP and incubated for 24 hours at room temperature. The bone slices were washed three times in 0.9% sodium chloride and replaced in individual 100 μl of MEM/Hanks' in a 96-well plate (Falcon, Becton Dickinson Laboratories, Oxford, UK). They were then ready for sedimentation of disaggregated osteoclasts.

Isolation and Sedimentation of Osteoclasts

Osteoclasts were isolated from neonatal rat long bones as previously described [28]. Neonatal Wistar rats were killed by cervical dislocation; the long bones were removed and cleaned of adherent soft tissue. The epiphyses were cut across and the bones were curetted with a scalpel blade in 2 ml of 199. The fragments were vigorously agitated using a Pasteur pipette. The larger fragments were allowed to settle for 10 seconds and the suspension was added to bone slices or 6 mm plastic coverslips (Lux, Flow).

Bone slices for sedimentation were either placed in 2 wells of a Sterilin 100 × 18 mm multiwell dish (Teddington, England), if the osteoclasts were to be subsequently incubated in medium containing bisphosphonate, or in individual wells of a 96-well plate if the bone slices had been preincubated in either Cl_2MBP or AHPrBP. Plastic coverslips were placed in a 96-well plate (Falcon) for sedimentation purposes. The cells were sedimented onto bone slices or plastic discs for 10 minutes in 5% CO_2 at 37°C. After sedimentation, the substrates were removed, washed vigorously in 199, and placed in 100 μl of MEM/Hanks'. The same volume containing the appropriate concentration of agent or vehicle was then added. The cultures were incubated for 5 or 24 hours. The experiments were terminated by fixing the cells in 10% buffered formalin (BDH Chemicals) or glutaraldehyde (Emscope Laboratories, Ashford, Kent, UK). Experiments were designed in such a way that all variables received osteoclasts from the same suspension.

Isolation of Osteoclasts from Neonatal Rats Treated with AHPrBP

Newborn Wistar rats were injected subcutaneously with 1.0 mg P/kg body weight of AHPrBP. The first injection was administered within 12 hours of birth, and was followed by a similar injection on 4 subsequent days. On day 6, the animals were killed by cervical dislocation. Control animals from the same litter were injected with normal saline. Osteoclasts were then isolated from these animals and sedimented onto bone slices as described above. In experiments where hormone-responsiveness was required, sedimentation time was increased to 30 minutes [29].

Table 1. Effect of AHPrBP (10^{-7} M) on bone resorption by disaggregated rat osteoclasts, and co-cultures of UMR 106 cells and osteoclasts

	Mean plan area of bone resorbed per bone slice $\mu m^2 \times 10^{-3}$ (±SEM)		
	Control	AHPrBP (10^{-7} M)	n
OC	13.7 (±6.4)	13.6 (±3.5)	12
Co-culture experiments			
UMR + OC	23.2 (±4.0)		
UMR + OC + PTH	44.8[a] (±4.2)	40.5[a] (±8.8)	12

Osteoclasts were disagregated from neonatal rats and incubated in the presence or absence of AHPrBP for 24 hours. Bone resorption was quantified by scanning electron microscopy after incubation. OC = osteoclasts; n = number of bone slices
[a] $P < 0.05$ vs UMR + OC

Co-culture Experiments

UMR 106 cells (a clonal osteoblastic cell line) (Dr. T. J. Martin, Melbourne, Australia) were grown to confluency in RPMI (Flow). The cells were suspended by incubating the cells in Trypsin-EDTA (Gibco) for 5 minutes. The cells were then centrifuged and resuspended in MEM/Hanks' and were added (5×10^{-4} cells/well) to bone slices in microtiter wells, onto which osteoclasts had already been sedimented. The co-cultures were incubated for 24 hours in a final volume of 200 μl of AHPrBP (10^{-6} M) or vehicle. Hormone responsiveness of the osteoclastic population was assessed by adding PTH (0.1 IU/ml final concentration) to some of the wells that did not contain UMR 106 cells. The cultures were terminated by fixation with 10% buffered formalin.

Assessment of Cell Morphology

Cultures were fixed after incubation in 5% glutaraldehyde in 0.2 M cacodylate for 30 minutes. The specimens were dehydrated through a graded series of alcohols and critical-point-dried from CO_2. The specimens were sputter-coated with gold palladium and examined in a Cambridge Stereoscan S90 scanning electron microscope (Cambridge Instruments, Cambridge, UK)

Measurement of Bone Resorption

After enumeration of osteoclasts by light microscopy, cells were removed from the bone surface by immersion in 10% sodium hypochlorite (for 10 minutes). The entire surface of each bone slice was examined, blind, in a Cambridge S90 scanning electron microscope. The number of resorption pits and the plan area of bone resorption were assessed as previously described [29]. To measure the depth of excavations, bone slices were scanned with the Petran version of the Tandem Scanning Reflected Light Microscope. The depth of each pit was calculated as the distance through which the objective, focused on the surface of the bone slice, had to be moved to become focused on the base of the excavation under observation, as previously described [30].

Table 2. Bone resorption by osteoclasts disaggregated from animals injected with AHPrBP (1 mg/Kg body weight) or vehicle

	Bone resorption							
	Mean plan area/bone slice $\mu m^2 \times 10^{-3}$ (±SEM)	n	x	y	Osteoclasts/cm^2 (±SEM)	n	x	y
Control/vehicle	22.0 (±2.6)	12	2	2	54.8 (±4.4)	22	4	2
Control + PTH	39.8* (±3.8)	12	2	2				
Control/AHPrBP	17.0 (±3.7)	12	2	2	45.7 (±3.8)	22	4	2
AHPrBP + PTH	51.8* (±5.4)	12	2	2				

AHPrBP or vehicle was injected into neonatal rats 12, 36, 60, and 84 hours after birth. On day 6, osteoclasts were disaggregated from long bones and incubated on bone slices in the presence or absence of PTH. In a separate set of experiments, osteoclasts disaggregated from animals that had been administered AHPrBP or vehicle were enumerated. Bone resorption and osteoclast numbers were quantified after 24 hours. N = no. of bone slices used in-toto; x = no. of experiments; y = animals per experiment

[a] $P < 0.05$ vs relevant controls

Effect of AHPrBP on Osteoblast-like Cells

UMR 106 cells (5×10^4 cells/ml; 100 µl) were incubated for 24 hours on bone slices preincubated in AHPrBP (200 µl per bone slice: 10^{-3} – 10^{-6} M). The cells were fixed in formalin and stained with toluidine blue. The cells in 10 random high power fields were counted.

Long-term Bone Marrow Cultures

Six to eight-week-old CBA/ca mice were killed by cervical dislocation. The long bones were removed and dissected free of adherent soft tissue. The epiphyses were cut, and the marrow cavity was flushed through with 199, using a sterile 25-gauge needle. The bone marrow cells were washed and suspended (2×10^6 cells/ml) in MEM/Earle's. The suspension was placed in wells of a microtiter well plate containing bone slices in the presence or absence of AHPrBP for 2 weeks. Hydrocortisone (10^{-6} M) was present throughout the cultures and $1,25(OH)_2D_3$ (10^{-8} M) was added on day 7. Cultures were fed every 3–4 days by replacing 100 µl of culture medium with fresh medium, hormone, vehicle, or AHPrBP. After 2 weeks, bone slices were prepared for scanning electron microscopy and the plan surface area of bone resorption was calculated. The experiment was repeated using bone slices preincubated in AHPrBP, and without further addition of AHPrBP during incubation.

The values for the numbers of cells on bone and plastic, and the number of resorption pits, and the total mean plan area were calculated for each culture. Each result was expressed as the mean ± standard error of the mean (SEM) of 12–18 cultures. Significance was determined by Student's *t* test.

Results

We found that incubation in AHPrBP (10^{-7} M) had no effect on bone resorption by osteoclasts disaggregated from rat long bone (Table 1). Likewise, the compound had no effect on the stimulation of bone resorption by PTH in co-cultures of osteoblastic cells with osteoclasts (Table 1), nor on the generation of bone marrow resorptive cells from bone marrow cultures (mean % of bone slice resorbed; control versus AHPrBP (10^{-6} M) ± SEM; 10.5 ± 4.6 versus 11.5 ± 5.1; $P > 0.05$: n = 12). Osteoclastic and osteoblastic cells visualized by light and scanning electron microscopy appeared morphologically unchanged. The resorptive capacity of osteoclasts isolated from rats administered AHPrBP was unimpaired compared with control animals, and the disaggregated bone cells, obtained after prolonged sedimentation, demonstrated a normal response to PTH stimulation (Table 2) [29]. The dose of the bisphosphonate used (1 mg P/kg body weight for 4 days) compares with the range that reduces bone resorption *in vivo* within 1 week [9, 31, 32].

Experiments similar to those above were performed using bone slices preincubated in bisphosphonates. We found inhibition of bone resorption by osteoclasts incubated for 24 hours on bone slices previously immersed in Cl_2MBP (10^{-5}–10^{-3} M) (see Fig. 1). The extent of inhibition was similar to that previously observed using osteoclasts immersed in Cl_2MBP [17], supporting the notion that it is the bone-adsorbed Cl_2MBP that is responsible for a curtailment of bone resorption. Consistent with our earlier results [17] is the finding that inhibition by Cl_2MBP was more marked after prolonged incubation; bone resorption was significantly impaired after 5 hours incubation when osteoclasts were cultured on bone slices preimmersed in 10^{-3} M Cl_2MBP but not in lower concentrations (mean plan area ($\mu m^2 \times 10^{-3}$) of bone resorbed per bone slice ± SEM; control versus Cl_2MBP (10^{-3} M): 4.8 ± 1.3 versus 1.4 ± 0.2; $P < 0.0005$: n = 12). These results suggest a relatively gradual onset of inhibition. Preimmersion of bone slices in 10^{-3} M AHPrBP caused inhibition of bone resorption after 24 hours (Fig. 2). At this concentration, inhibition of resorption was considerably more complete than for Cl_2MBP. Moreover, inhibition was seen under similar conditions after only 5 hours incubation (mean plan area ($\mu m^2 \times 10^{-3}$) of bone resorbed per bone slice ± SEM; control versus AHPrBP 10^{-3} M: 7.5 ± 1.2 versus 0.4 ± 0.2; $P < 0.0005$: n = 12) whereas an effect at concentrations lower than 10^{-3} M Cl_2MBP was demonstrated only in the 24 hour cultures (Fig. 1).

The number of cells found adherent to bone slices paralleled the effects on bone resorption by isolated rat osteoclasts. Osteoclast number was unaffected after 5 hours incubation on Cl_2MBP-treated bone slices, even at 10^{-3} M, but numbers were reduced by approximately 50% of the control levels when incubated for 24 hours on Cl_2MBP (10^{-5} M) precoated bone (Fig. 3). In contrast, cell number was reduced by AHPrBP only on bone slices preimmersed at 10^{-3} M, but numbers were reduced after a relatively short incubation time (5 hours) (Fig. 4).

Osteoclasts incubated on bone slices preimmersed in AHPrBP at concentrations of 10^{-4} M and above were found to have atypical morphology by light microscopy. The appearances were similar to that noted in cell cultured on Cl_2MBP-treated slices [17]. Osteoclasts showed cytoplasmic degeneration and nuclear fragmentation, hyperchromatic nuclei, and cytoplasmic contraction (Fig. 5).

Atypical excavations were observed on bone slices preimmersed in AHPrBP at concentrations of 10^{-4} M and above. These ranged from shallow excavations (Fig. 6) with irregular margins, often with islands of unresorbed bone within the excavated area, but clearly recognizable as osteoclastic in origin, to areas of the bone surface showing no

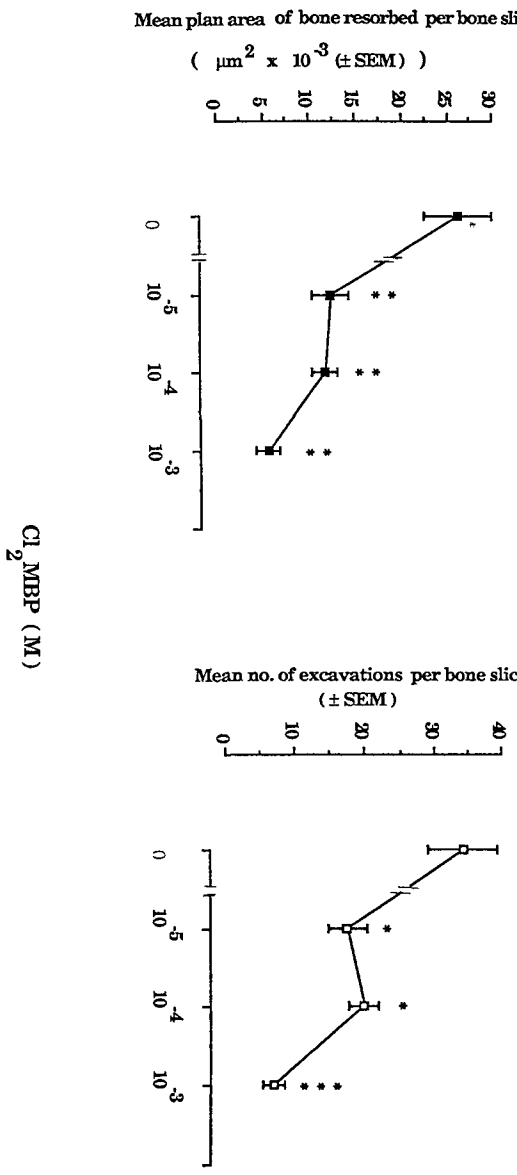

Fig. 1. Bone resorption on Cl_2MBP precoated bone slices. Bone slices were immersed in Cl_2MBP (200 μl/bone slice) and washed in medium 199 before addition of osteoclasts; bone resorption was assessed after 24 hours. (n = 12; $*P < 0.01$; $**P < 0.005$; $***P < 0.0005$).

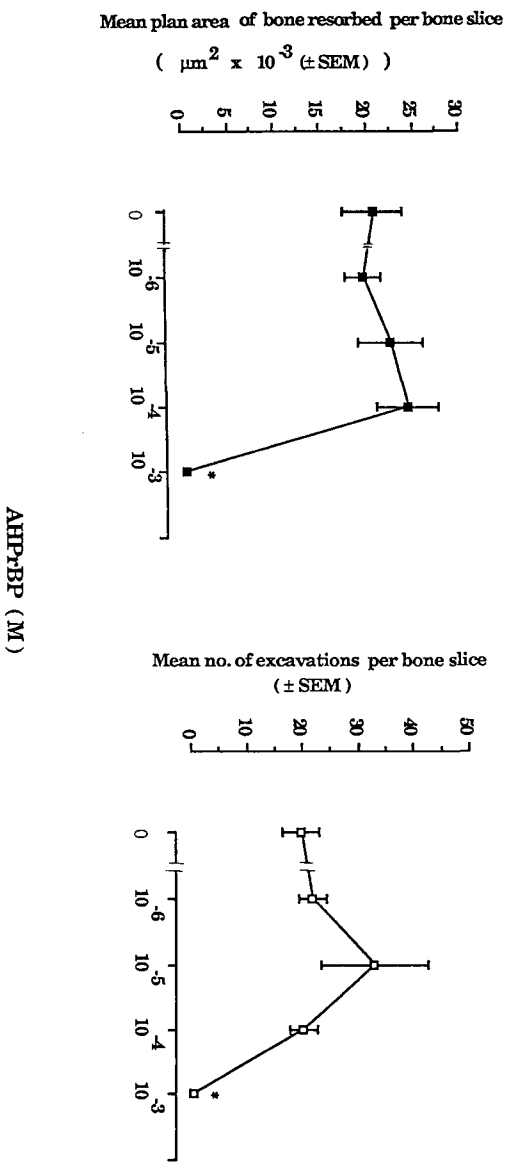

Fig. 2. Bone resorption on AHPrBP precoated bone slices. Bone slices were immersed in AHPrBP (200 μl per bone slice) and washed in medium 199 before addition of osteoclasts; bone resorption was assessed after 24 hours. (n = 12; $*P < 0.0005$).

more than a blurring of the marks of the bone saw and a change in the texture and color of the bone surface (Fig. 5). These superficial erosions were seen on bone slices preincubated in 10^{-4} M AHPrBP (Fig. 6) in similar numbers and areas to the numbers (see Fig. 4) and areas of well-formed excavations on control bone slices (mean surface area of excavations ($\mu m^2 \times 10^{-3}$) per bone slice; control versus AHPrBP (10^{-4} M) ± SEM: 0.9 ± 0.1 versus 1.0 ± 0.1; n = 12; 24 hour incubation; $P > 0.05$).

Atypical resorption pits were not identified when osteoclasts were incubated on Cl_2MBP-treated bone, nor were the excavations in these cultures more shallow than those in the control samples (control versus Cl_2MBP (10^{-4} M) ± SEM: 5.9 μm ± 0.27 versus 5.4 μm ± 0.26, n = 6). This finding may reflect a more gradual onset of effect by Cl_2MBP compared with AHPrBP, and supports the *in vivo* observations of Ralston et al. [33] who have shown that AHPrBP inhibits bone resorption more rapidly *in vivo* than does Cl_2MBP.

The number of osteoclasts that remained adherent to plastic at a concentration as high as 10^{-3} M AHPrBP was unaffected by 12 hours incubation (mean number of osteoclasts per cm^2 ± SEM; control versus AHPrBP 10^{-3} M; 38.8 ± 15.5 versus 33.4 ± 15.5; n = 19: $P > 0.05$). Thus, bone seems to be required for the effect of AHPrBP on osteoclasts to be exerted. It may be required in two ways: it may be that AHPrBP on osteoclasts to be exerted. It may be required in two ways: it may be that AHPrBP binds to bone and is toxic to cells with which it comes into contact. Alternatively, bone resorption may release cytotoxic concentrations of AHPrBP from the bone mineral. To distinguish between these two possibilities, two types of experiments were performed.

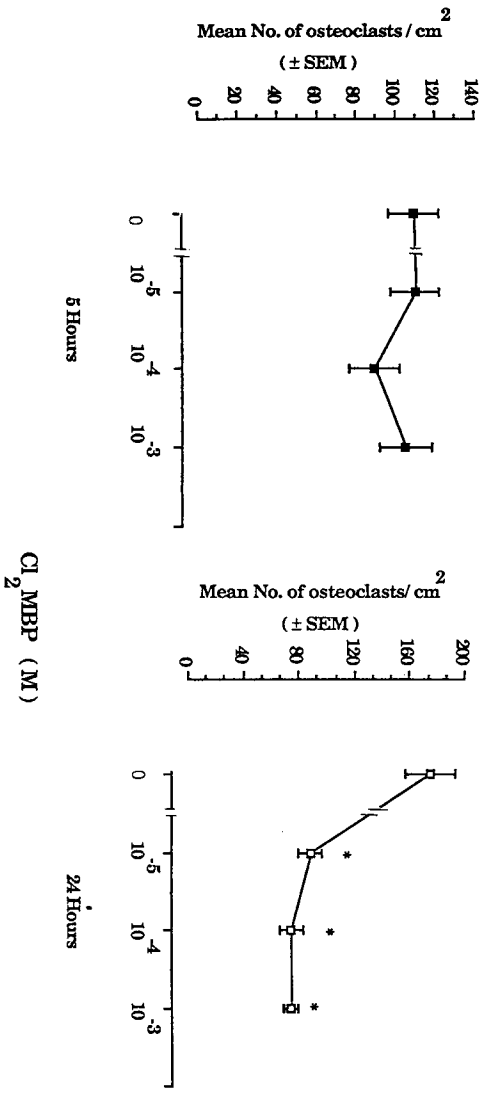

Fig. 3. The number of osteoclasts remaining adherent to Cl_2MBP precoated bone slices. Bone slices were immersed in Cl_2MBP (200 µl per bone slice) and washed in medium 199 before addition of osteoclasts; osteoclasts were enumerated after 5 and 24 hours incubation. (Two separate sets of experiments; n = 12; $*P < 0.0025$).

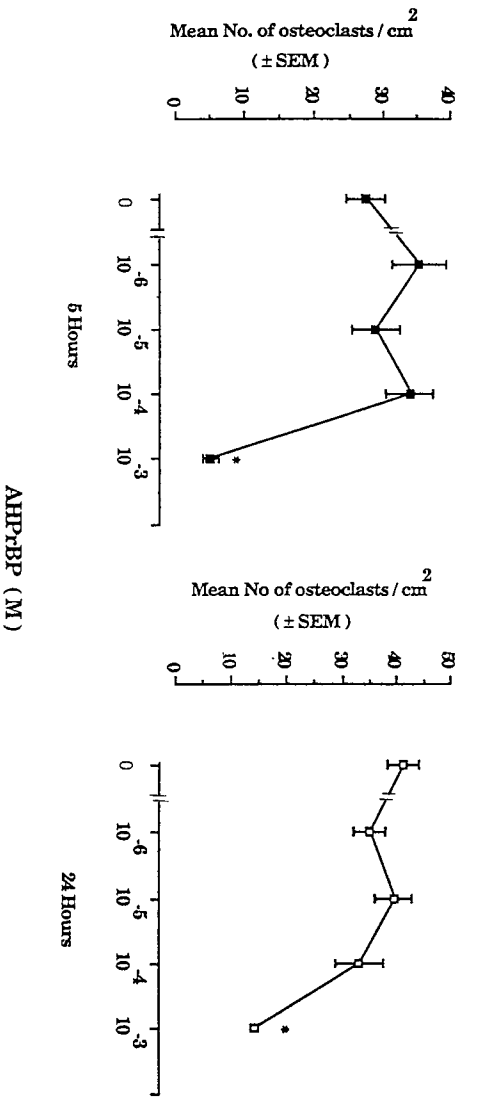

Fig. 4. The number of osteoclasts remaining adherent to AHPrBP precoated bone slices. Bone slices were immersed in AHPrBP (200 µl per bone slice) and washed in medium 199 before addition of osteoclasts; osteoclasts were enumerated after 5 and 24 hours incubation. (Two separate sets of experiments; n = 12; $*P < 0.001$).

First, UMR 106 cells were incubated on AHPrBP-coated bone slices. We found no difference in morphology or cell numbers in the presence of AHPrBP (cells/high power field ± SEM; control versus AHPrBP 10^{-3} M; 55.7 ± 30.5 versus 70.3 ± 27.3; n = 15: $P > 0.05$). Second, osteoclasts that were inhibited from resorbing bone by CT showed no change in number after incubation with AHPrBP (osteoclasts/cm^2 ± SEM; control versus AHPrBP 10^{-3} M; 59.8 ± 14.7 versus 52.6 ± 13.1; n = 17: $P > 0.05$).

To test the sensitivity of osteoclast generation to the presence of AHPrBP, bone marrow cells were incubated on bone slices that had previously been incubated in 10^{-4} M AHPrBP. We found no detectable effect of such bone slices on osteoclast generation (mean plan area ($\mu m^2 \times 10^{-3}$) of bone resorbed per bone slice ± SEM; control versus AHPrBP 10^{-4} M; 79.3 ± 42.2 versus 63.1 ± 25.3; n = 12: $P > 0.05$).

In all the previous experiments we were careful to pre-incubate bone slices in the same volume of medium (0.2 ml) as that used for the experiments in which osteoclasts were incubated in the presence of PCPs. As PCP may exert a cytotoxic effect when adsorbed onto bone rather than in fluid phase (cf effects of plastic versus bone slices on osteoclast number), this suggests that it may be the density of PCP achieved on the bone surface rather than the original concentration of PCP used in the preimmersion phase that is important. To test this we compared the effect of bone slices preincubated in 5 ml PCP with previous results using 0.2 ml. We found considerably greater inhibition of resorption by bone slices preincubated in the larger compared with smaller volume of PCP (Fig. 7).

Discussion

AHPrBP is known to exceed Cl_2MBP in potency *in vivo* by an order of magnitude [16]. In our initial experiments we

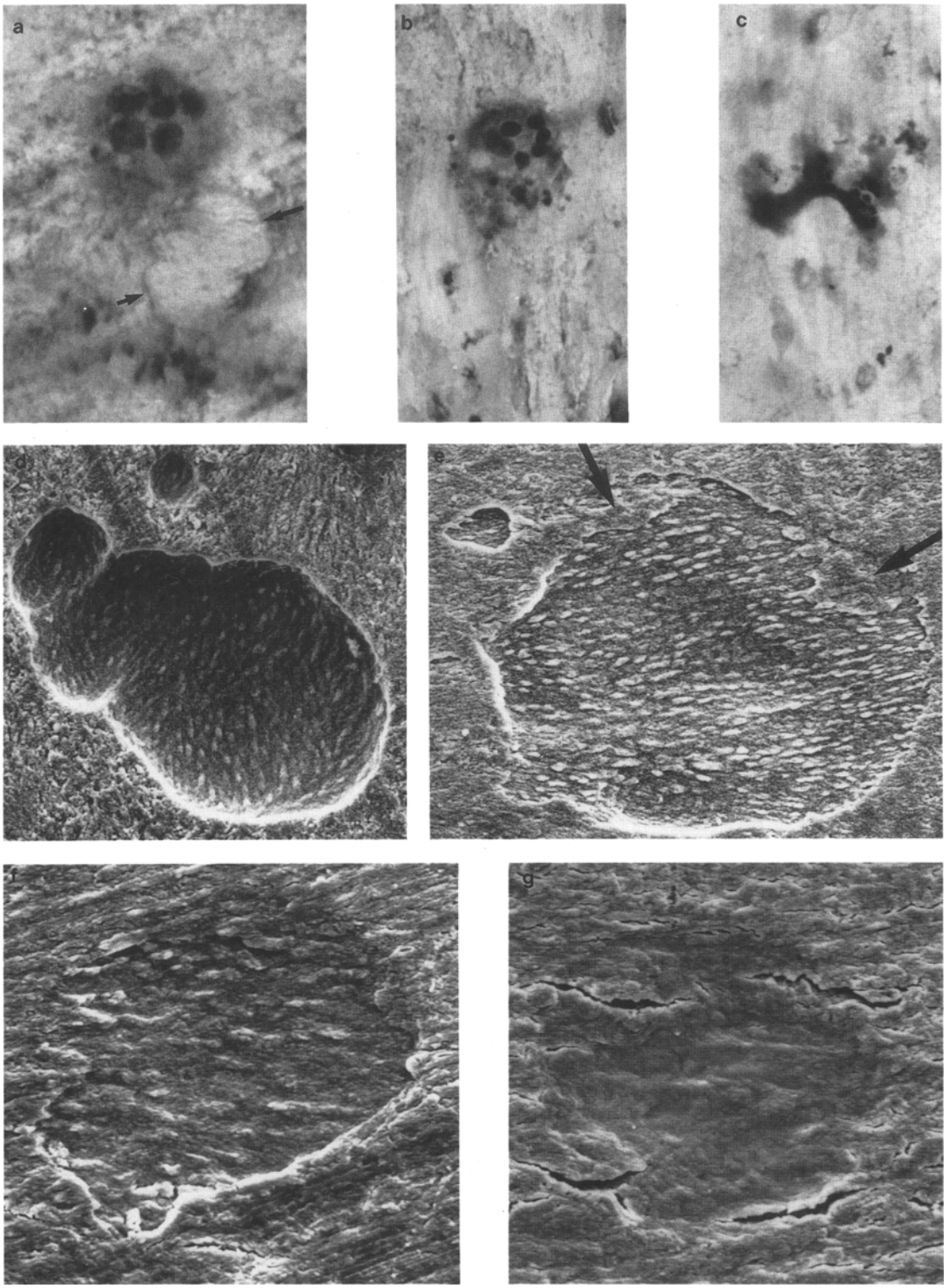

Fig. 5. Light photomicrographs of osteoclasts on bone stained with toluidine blue **(a–c)**. A well-spread osteoclast on a control bone slice lies adjacent to a resorption pit (arrows) **(a)**. Its appearance is in marked contrast to the degenerate cell that shows nuclear and cytoplasmic fragmentation **(b)**, and to the contracted osteoclast with hyperchromatic nuclei **(c)**, both of which were incubated on bone slices preimmersed in AHPrBP (10^{-4} M). Scanning electron photomicrographs of osteoclastic resorption pits **(d–g)**. Note the smooth, sharply defined margins in the excavation formed in control medium **(d)** compared with the irregular edges of the atypical excavations on the 10^{-4} M AHPrBP-treated bone **(e,f)**, and the islands of unresorbed bone at the periphery of the excavation (arrows) **(e)** (×940). The atypical pits were shallow, some showing no more than a change in texture and color of the bone **(g)** (×964).

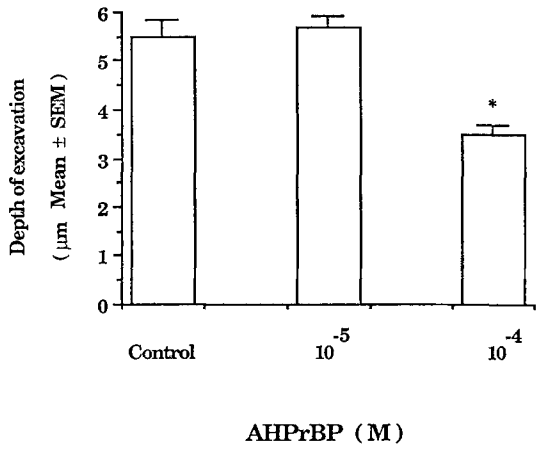

Fig. 6. Depth of excavations on AHPrBP precoated bone slices. Bone slices were immersed in AHPrBP (200 μl per bone slice) and washed in medium 199 before addition of osteoclasts. The depth of 6–10 excavations was measured on six bone slices and the mean depth/bone slice was calculated (*$P < 0.05$).

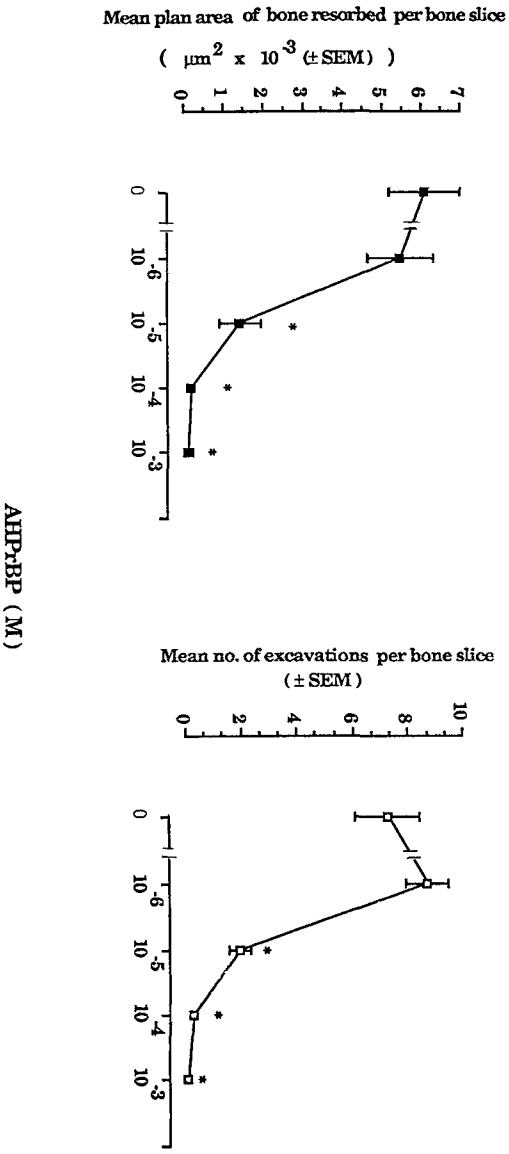

Fig. 7. Effect on osteoclastic bone resorption when bone slices were preincubated in larger volumes of AHPrBP. Bone slices were immersed in AHPrBP (5 ml/bone slice) and washed in medium 199 before addition of osteoclasts; bone resorption was assessed after 5 hours (n = 12; *$P < 0.0005$).

therefore attempted to identify an experimental system that reflected this differential potency. We have previously found [17] that concentrations of 10^{-7} M and above of Cl_2MBP strongly inhibit bone resorption by isolated osteoclasts; an experimental system that reflects *in vivo* potency differentials should thus show inhibition of bone resorption by AHPrBP at this and lower concentrations. However, we found no inhibition by 10^{-7} M AHPrBP, either on isolated osteoclasts or on the responsiveness of osteoblast-osteoclast cocultures to PTH. This suggests that there may be an effect at some point in the sequence of events that leads to bone resorption that is more sensitive to the effects of AHPrBP than are mature osteoclasts.

Other experimental models that test the function of mature osteoclasts have similarly failed to demonstrate increased potency for AHPrBP, and this has led to the suggestion that AHPrBP impairs osteoclast generation [25]. We found, however, that even 10^{-4} M AHPrBP was without significant effect on the generation of osteoclasts *in vitro*. Similarly, we found no evidence for defective generation of bone-resorptive cells in animals treated with AHPrBP. This evidence, from experiments *in vivo*, that defective formation of resorptive cells does not account for inhibition of bone resorption by AHPrBP is supported by histomorphometric data, which show increased, rather than decreased, numbers of osteoclasts in the bones of AHPrBP-treated animals [34–36]. The results are at odds with the conclusions of Hughes et al. [25]. However, in these experiments the criteria used to identify osteoclast differentiation were morphological, rather than functional; morphology is not a reliable guide to osteoclast differentiation *in vitro* [37].

Our results differ from those of Carano et al. [38] who, using chick osteoclasts, found that AHPrBP was more potent than Cl_2MBP by an order of magnitude. However, the dissolution of bone powder that occurs in this model may be a different process from that by which osteoclasts excavate bone surfaces, and it is difficult to compare the results in the two systems.

Preimmersion of bone slices in 10^{-3} M AHPrBP virtually abolished bone resorption by rat osteoclasts. We have previously found that inhibition of bone resorption by Cl_2MBP is associated with osteoclast cytotoxicity, which is caused not by fluid-phase Cl_2MBP but by bisphosphonate adsorbed onto the bone surface [17]. The same seems to hold for AHPrBP: though the number of osteoclasts that remained adherent to plastic in the presence of 10^{-3} M AHPrBP was unchanged at 12 hours, preincubation of bone slices in the same concentration caused a dramatic reduction in the number of osteoclasts adherent to bone. This reduction was accompanied by degenerative morphological changes similar to those previously observed using Cl_2MBP [17]. It seems that mere contact with AHPrBP-coated bone surfaces is not sufficient to injure cells: such surfaces did not influence the morphology or number of osteoblastic cells similarly incubated. Moreover, osteoclasts prevented from resorption by calcitonin were protected from injury. Thus, inhibition of bone resorption by AHPrBP may be due to injury to the osteoclast induced by AHPrBP released during resorption of AHPrBP-contaminated bone. The rate at which this action is

impaired would account for the shallow, irregular, poorly formed appearance of excavations.

The mechanism by which AHPrBP injures osteoclasts remains unknown. Because injury seems resorption dependent, the bisphosphonate is likely to act on the osteoclast after solubilization of AHPrBP-contaminated bone in the resorptive hemivacuole, within which it may achieve a high local concentration, and may impair membrane functions or may be absorbed into the cell and interfere with cellular metabolism.

Although Cl_2MBP and AHPrBP both appear to inhibit bone resorption through exposure of osteoclasts to bisphosphonate released during resorption, AHPrBP seemed to exert a more rapid and profound effect on osteoclast activity than did Cl_2MBP. The finding that excavations were well formed on Cl_2MBP-treated bone suggests that injury induced by release of Cl_2MBP on the bone surface was not sufficiently intense or rapid in onset to prevent completion of excavations. This is reflected in the absence of a reduction in the number osteoclasts that remained adherent to the Cl_2MBP-contaminated bone after 5 hours incubation. In contrast, on AHPrBP-treated bone there were poorly formed, shallow excavations and a striking decrease in cell number after a short incubation period, consistent with a more rapid onset of impairment of osteoclast function. These differences may be factors in the increased potency of AHPrBP *in vivo*, as the partial and relatively slow inhibition of resorption by successive osteoclasts by Cl_2MBP may be more readily compensatable through increased recruitment.

We found no evidence to suggest that osteoclast precursors were more sensitive than mature osteoclasts to the effect of AHPrBP on bone slices. If inhibition of osteoclast function is caused by bisphosphonate contamination of bone surfaces, then the concentration of bisphosphonate in the incubation medium will be less relevant to potency comparisons than the density of bisphosphonate adsorbed onto the bone surface [39]. We found that the potency of AHPrBP could be increased by at least an order of magnitude merely by preincubation of bone slices in a larger volume of the bisphosphonate. This phenomenon may underly the increased sensitivity to resorption-inhibition by AHPrBP of bone explants taken before osteoclasts are present compared with explants already containing osteoclasts (radii) [24]. Although these experiments were interpreted to show that AHPrBP acts predominantly on osteoclast precursors, and alternative explanation would be that the much smaller quantity of mineral in metatarsals compared with radii would increase the density of AHPrBP deposited on the surface of mineral in metatarsals. Cl_2MBP, with its more shallow dose-response curve, would be less potentiated.

These experiments identify two mechanisms through which the potency of AHPrBP may be greater *in vivo* than would be predicted *in vitro*. First, more rapid impairment of osteoclast function may be more difficult to compensate *in vivo*. Second, as it is not the concentration of bisphosphonate but its density on bone surfaces that determines its biological effect, relative potency of AHPrBP *in vivo* would be increased if a larger proportion of the administered dose were adsorbed onto bone surfaces. The available evidence suggests that a 6–8 times greater proportion of an intravenous dose of AHPrBP remains unexcreted after 24 hours, most of which is bound to bone, compared with Cl_2MBP [40, 41].

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