Journal of Cellular Biochemistry 56:118–130 (1994)

Inhibition of Bone Resorption by Selective Inactivators of Cysteine Proteinases

Peter A. Hill, David J. Buttle, Sheila J. Jones, Alan Boyde, Mitsuo Murata, John J. Reynolds, and Murray C. Meikle

Departments of Cell and Molecular Biology (P.A.H., J.J.R., M.C.M.) and Biochemistry (D.J.B.), Strangeways Research Laboratory, Cambridge CB1 4RN, United Kingdom; Department of Orthodontics and Paediatric Dentistry, UMDS of Guy's and St. Thomas's Hospitals, University of London, London SE1 9RT, United Kingdom (P.A.H., M.C.M.); Department of Anatomy and Embryology, University College London, London WC1E 6BT, United Kingdom (S.J.J., A.B.); and Research Centre, Taisho Pharmaceutical Co., Ltd., Saitama 330, Japan (M.M.)

Abstract Inactivators of cysteine proteinases (CPs) were tested as inhibitors of bone resorption in vitro and in vivo. The following four CP inactivators were tested: Ep475, a compound with low membrane permeability which inhibits cathepsins B, L, S, H, and calpain; Ep453, the membrane-permeant prodrug of Ep475; CA074, a compound with low membrane permeability which selectively inactivates cathepsin B; and CA074Me, the membrane-permeant prodrug of CA074. The test systems consisted of 1) monitoring the release of radioisotope from prelabelled mouse calvarial explants and 2) assessing the extent of bone resorption in an isolated osteoclast assay using confocal laser microscopy. Ep453, Ep475, and CA074Me inhibited both stimulated and basal bone resorption in vitro while CA074 was without effect; the inhibition was reversible and dose dependent. None of the inhibitors affected protein synthesis, DNA synthesis, the PTH-enhanced secretion of β-glucuronidase, and N-acetyl-β-glucosaminidase, or the spontaneous release of lactate dehydrogenase. Ep453, Ep475, and CA074Me dose-dependently inhibited the resorptive activity of isolated rat osteoclasts cultured on bone slices with a maximal effect at 50 μM. The number of resorption pits and their mean volume was reduced, whilst the mean surface area remained unaffected. Again, CA074 was without effect. Ep453, Ep475, and CA074Me, but not CA074, when administered subcutaneously at a dose of 60 μg/g body weight inhibited bone resorption in vivo as measured by an in vivo/in vitro assay, by about 20%. This study demonstrates that cathepsins B, L, and/or S are involved in bone resoprtion in vitro and in vivo. Whilst cathepsin L and/or S act extracellularly, and possibly intracellularly, cathepsin B mediates its effect intracellularly perhaps through the activation of other proteinases involved in subosteoclastic collagen degradation.

Key words: bone remodelling, osteoclasts, proteinases, collagen, degradation

Bone resorption occurs beneath the osteoclast ruffled border in a segregated extracellular microenvironment which resembles a secondary lysosome [Baron, 1989]. Solubilization of bone mineral is achieved by acidification [Baron et al., 1985], whereas the organic matrix (mainly type I collagen) is degraded by proteolytic enzymes, especially the lysosomal cysteine proteinases (CPs) such as cathepsins B, L, and S and the matrix metalloproteinases (MMPs), including collagenase [Délaisse and Vaes, 1992].

The involvement of CPs in matrix resorption was originally suggested by their ability to degrade type I collagen at acid pH [Etherington, 1972; Burleigh et al., 1974; Kirschke et al., 1982]. Direct evidence for their participation in bone resorption was subsequently provided by the finding that inhibitors of CPs prevented resorption of bone explants [Delaissé et al., 1980, 1984; Lerner and Grubb, 1992]. Furthermore,

Abbreviations used: Ep 475, *trans*-epoxysuccinyl-leucyl-amido-(3-methyl)butane; Ep453, *trans*-epoxysuccinyl-leucyl-amido-(3-methyl)butane ethyl ester; CA074, N-(L-3-*trans*-propylcarbamoyl oxirane-2-carbonyl)-L-isoleucyl-L-proline; CA074Me, N-(L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline methyl ester.

Received February 22, 1994; accepted March 25, 1994.

David J. Buttle's present address is Department of Human Metabolism and Clinical Biochemistry, University of Sheffield Medical School, Beech Hill Road, Sheffield S10 2RX, United Kingdom.

Address reprint requests to P.A. Hill, Department of Cell and Molecular Biology, Strangeways Research Laboratory, Worts Causeway, Cambridge CB1 4RN, United Kingdom.

ultrastructural studies have demonstrated that inhibitors of CPs prevent collagen degradation within the subosteoclastic resorption zone whilst allowing demineralization to proceed [Everts et al., 1988, 1992]. Although there is evidence for the involvement of cathepsin L in bone collagen degradation, recent findings have questioned the involvement of cathepsin B in this process [Rifkin et al., 1991; Kakegawa et al., 1993].

Three CPs have been isolated from mouse calvarial tissue; cathepsins B, L, and a cathepsin L-like 70 kDa proteinase [Delaissé et al., 1991] and multiple forms of cathepsin B have been isolated from human osteoclastomas [Page et al., 1992]. While cathepsins B and L have been immunolocalized in both osteoclasts and the subosteoclastic resorption zone [Sasaki and Ueno-Matsuda, 1993; Ohsawa et al., 1993; Goto et al., 1993] a recent study has suggested that cathepsin L is the main CP responsible for bone collagen degradation since the epoxy-peptide inhibitor CA074, specific for the inactivation of cathepsin B [Murata et al., 1991; Buttle et al., 1992a,b], failed to inhibit bone resorption [Kakegawa et al., 1993]. However, since CA074 is a negatively charged molecule, its ability to enter cells is limited, so the involvement of intracellular cathepsin B may not have been detected. The methyl ester of CA074 (CA074Me) is a proinhibitor of cathepsin B that is inactive but can enter cells and inhibit cathepsin B presumably following de-esterification [Buttle et al., 1992a,b]. This is analogous to a similar epoxy-peptide, Ep475, which is capable of traversing membranes only as its ethyl ester (Ep453) and following de-esterification, binds to intracellular cysteine proteinases [Tamai et al., 1986; Wilcox and Mason, 1992]. Whereas CA074 and CA074Me are selective inhibitors of cathepsin B, Ep475 and Ep453 inhibit the collagenolytic cathepsins B, L, and S [Buttle and Saklatvala, 1992; Buttle et al., 1992a,b].

The control of bone resorption by CP and/or MMP inhibitors could be valuable in the clinical management of pathological conditions associated with increased levels of bone loss such as osteoporosis, arthritis, Paget's disease, tumor osteolysis, and periodonitis. Our strategy has been therefore to use inactivators that are specific for one particular CP or MMP to determine which enzyme(s) is involved. The recent findings that the membrane permeant proinactivators, Ep453 and CA074Me are potent inhibitors of cartilage proteoglycan degradation [Buttle et al., 1993] prompted us to assess the effects of Ep475, Ep453, CA074, and CA074Me on bone resorption in vitro and in vivo to determine the roles of cathepsin B, L, and/or S in this process.

MATERIALS AND METHODS

Materials

Synthetic human parathyroid hormone [PTH-(1-84)] was a gift from the Division of Biological Standards, National Institute of Medical Research, Mill Hill, UK, and 1,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] was a generous gift from Dr. Ian Dickson, Brunel University, Uxbridge, UK. Actinomycin D, L-proline, thymidine, indomethacin, and alpha minimum essential medium (α-MEM) were purchased from Sigma Chemical Co (Poole, Dorset, UK); $^{45}CaCl_2$, methyl-[^{3}H]-thymidine, and L-[5-^{3}H]-proline were purchased from Amersham International plc (Amersham, Buckinghamshire, UK). BGJ medium was obtained from Flow Laboratories (Irvine, Scotland).

The LL-isomer of Ep453 (also known as EST, E64d, and loxistatin) and Ep475 (also known as E64c) were synthesized as described [Tamai et al., 1987]. CA074 was synthesized as described [Murata et al., 1991] and converted to CA074Me by treatment with diazomethane [Buttle et al., 1992a,b].

METHODS

Bone Resorption Assays

Bone cultures. Bone resorption was assessed either by analysing $^{45}Ca^{2+}$ or [^{3}H]-proline release from cultured neonatal mouse calvarial bones [Reynolds and Dingle, 1970]. Briefly, 1-day-old mice were injected subcutaneously with either 1–4 μCi $^{45}CaCl_2$ or 10 μCi [^{3}H]-proline. After 6 days the calvariae were excised and the posterior two thirds of the parietal bones were dissected into four pieces and pre-cultured in BGJ medium (1 ml) containing indomethacin (1 μM) for 24 h [Lerner, 1987; Ljunggren et al., 1991]. Bones that were stimulated by PTH or 1,25$(OH)_2D_3$ were also treated with the hormones during this initial preincubation period. Paired bones were subsequently cultured in pairs in fresh BGJ medium (1 ml) with and without the CP inhibitors. Mobilization of radioactivity was expressed as the percentage release of initial isotope (calculated as the sum of radioactivity in medium and bone after culture). To determine $^{45}Ca^{2+}$ release due to passive exchange of

isotope with cold Ca^{2+} in the culture medium, four parietal bone quarters from each litter were devitalized by three cycles of freeze-thawing. The percentage release from the devitalized bone was subtracted from each living bone to give the amount of cell-mediated resorption (CMR).

Isolated osteoclast-bone resorption assay. The osteoclast bone resorption assay is based on the ability of isolated osteoclasts to resorb devitalized cortical bone or ivory slices in vitro [Osdoby et al., 1982; Zambonin-Zallone et al., 1982; Chambers et al., 1985a,b]. Bone slices 200 μm thick were cut with a low-speed, water-cooled diamond saw (Isomet, Buehler UK Ltd., Coventry, Warwickshire, UK) from a 1 cm^2 square rod. Slices were cleaned by ultrasonication for 20 min in distilled water, sterilized in 70% ethanol for 1 h, and stored dessicated at room temperature until use. Osteoclasts were prepared from 2–3-day-old rats (Wistar). After killing the animals, femora and tibiae were removed and freed of adherent soft tissue adherent soft tissues, cut across their epiphyses, and sectioned longitudinal. Osteoclasts were mechanically disaggregated by curetting the bones into 4 ml phosphate-buffered saline and agitating the cell suspension with a pipette. Larger fragments were allowed to settle for 10 s before 500 μl aliquots of the supernatant cell suspension were immediately transferred to six wells of 24 well culture dishes (Costar), each containing a single bone slice. Cells were allowed to settle for 1 h at 37°C. The substrate was then washed free of nonadherent cells and the slices incubated for 20 h in a humidified atmosphere of 5% CO_2/95% air at 37°C in 500 μl α-MEM supplemented with 10% FCS, 2.0 g/L $NaHCO_3$, 2 mM L-glutamine, 100 μ/ml penicillin, and 100 μg/ml streptomycin; these culture conditions result in a culture medium pH of approximately 7.2 [Arnett and Dempster, 1986].

Owing to the variability in the number of osteoclasts isolated from each rat, a single experiment consisted of six bone slices bearing the cells from one rat with three slices for each control and test variable. Each experimental variable was repeated five times and the results were expressed as the percentage inhibition of the control which was set at 100%.

At the completion of the culture period, the bone slices were either stained unfixed with neutral red so that live osteoclasts could be counted, or fixed with warm (37°C) formaldehyde-acetone-citrate (1:6.5:2.5) solution. Following fixation the specimens were stained for 30 min at 37°C in darkness for tartrate resistant acid phosphatase (TRAP) activity; osteoclasts were identified as large multinucleated (three or more nuclei) strongly TRAP-positive cells. In those experiments where the maximum concentration of inhibitor was used, osteoclast counts were made over the entire surface of each slice. Cells were then dislodged from the bone slices by rubbing each slice with gloved fingers and the specimens restained with toluidine blue in order to count the resorption lacunae [Boyde et al., 1984]. The method used for the precise quantitation of the resorption capacity of the osteoclasts involved estimating the surface area and volume of each lacuna [Jones et al., 1992].

In vivo/in vitro bone cultures. This method has been described in detail [Reynolds, 1972]. Briefly, 1-day-old mice were injected subcutaneously (s.c.) with 1 μCi $^{45}CaCl_2$ and after 6 days the mice were weight-paired with one mouse acting as control. The control was injected s.c. with 30 μl of 0.15 M NaCl (vehicle), while the other mouse was injected s.c. with vehicle supplemented with 60 μg/g bodyweight of either CA074, CA074Me, Ep475, or Ep453. The mice were sacrificed 3 h after injection and pairs of explants of half-calvariae were prepared from each mouse; one bone of each pair was devitalized by freeze-thawing to calculate CMR. The in vitro incubation lasted for 24 h and mobilization of radioactivity was expressed as a percentage inhibition of control cultures.

Protein Synthesis

Protein synthesis in cultured calvariae was assessed by preculturing calvarial halves for 24 h in 1 ml BGJ medium with and without the different CP inhibitors. One group of bones was incubated in medium containing actinomycin D (100 μM) as a control. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. During the last 6 h, the explants were labelled with 2 μCi of [^{3}H]-proline in the presence of cold carrier proline (100 μM). The bones were washed in 3% (w/v) ice-cold trichloroacetic acid (TCA; 3 × 15 min) and water (3 × 10 min). Each bone was dissolved in 200 μl formic acid for 45 min at 60°C. A scintillation cocktail was added and the mixture was counted for radioactivity.

DNA Synthesis

DNA synthesis in calvarial bones was estimated by incubating the explants in the presence of [^{3}H]-thymidine and isolating labelled DNA by a method described by Lerner and Granstrom [1984]. Briefly, the calvarial halves were preincubated for 24 h in 1 ml of BGJ medium with and without the different CP inhibitors. The culturs were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. For the last 6 h the explants were labelled with 5 μCi of [^{3}H]-thymidine in the presence of cold carrier thymidine (10^{-4} M). The explants were then homogenized in 1 ml of cold saline and precipitated with 10% TCA and centrifuged. The precipitate was suspended in 5% TCA and heated at 60°C for 20 min, cooled, and recentrifuged. The radioactivity in the hot 5% TCA soluble pool (DNA fraction) was determined by liquid scintillation.

Enzyme Release

The release of the lysosomal enzymes, β-glucuronidase and N-acetyl-β-glucosaminidase, and the cytosolic enzyme lactate dehydrogenase from the calvarial bones was measured. In these experiments calvarial bones were cultured in 2 ml medium containing the various CP inhibitors for 24 h.

β-Glucuronidase was analysed using phenophthalein glucuronic acid as the substrate [Fishman et al., 1967]; 1 unit of β-glucuronidase activity will liberate 1 μg of phenolphthalein in 20 h at 37°C. N-acetyl-β-glucosaminidase was analysed using p-nitrophenyl-N-acetyl-β-D-glucosaminide as the substrate; 1 unit refers to the decomposition of 1 μmol substrate/min. The activity of lactate dehydrogenase (LDH) was assayed by monitoring the oxidation rate of reduced nicotinamide adenine dinucleotide at 340 nm [Cabaud and Wroblewski, 1958]; 1 unit of LDH converts 1 μmol of pyruvate per minute at 25°C.

Statistical analysis. Statistical evaluation of the data was done using the Mann-Whitney test.

RESULTS

Effect of CP Inhibitors on Calvarial Bone Resorption In Vitro

Initially we examined the effects of the CP inhibitors on both the basal and stimulated (either PTH or 1,25$(OH)_2D_3$) release of $^{45}Ca^{2+}$ and [^{3}H]-proline from prelabelled mouse calvarial bones during a 24 h culture period (Fig. 1). The inhibitory effects of Ep453 (0.1 μM), Ep475 (1 μM), and CA074Me (5 μM) on stimulated bone resorption were complete; CA074 was without effect. Although Ep453, Ep475, and Ca074Me produced a significant inhibition of basal resorption at a 50 μM concentration, their effects were incomplete, although it is noteworthy that they prevented the basal release of [^{3}H]-proline to a greater extent than that of $^{45}Ca^{2+}$; CA074 was again without effect.

We then extended the study by testing a range of doses of the CP inhibitors of the PTH-stimulated release of [^{3}H]-proline from prelabelled mouse calvarial bones during a 48 h culture period (Fig. 2). CA074Me, Ep475, and Ep453 inhibited the release of [^{3}H]-proline dose-dependently, with Ep453 producing a statistically significant inhibition at 1 nM, Ep475 at 0.1 μM, and CA074Me at 1 μM. CA074 was again without effect even at a dose of 50 μM. During the 48 h culture period we found that a 10-fold higher concentration of the respective CP inactivators was required to completely prevent PTH-stimulated resorption compared to the 24 h cultures.

A time-course experiment (Fig. 3) was then carried out in which bones were cultured for 96 h with CA074 (50 μM), CA074Me (50 μM), Ep475 (50 μM), and Ep453 (5 μM). CA074Me, Ep475, and Ep453 produced a significant inhibition of PTH (20 nM)-stimulated $^{45}Ca^{2+}$ release throughout the culture period of 96 h, although the effects of CA074Me were incomplete during the latter 48 h of culture.

To exclude the possibility that cell death was contributing to the inhibition a recovery experiment was performed. Calvarial bones were treated with PTH and one of the inhibitors for the first 48 h and then cultured with PTH alone for a further 4 days. The inhibitory effects of CA074Me, Ep475, and Ep453 seen during the initial culture period were gradually lost, the amount of PTH-stimulated $^{45}Ca^{2+}$ release returning to normal by 96–144 h (Table I). Further evidence that inhibition was not due to toxic effects included the findings that none of the CP inhibitors at a concentration of 50 μM affected either protein or DNA synthesis, as assessed by the incorporation of [^{3}H]-proline and [^{3}H]-thymidine into the explants (Table II).

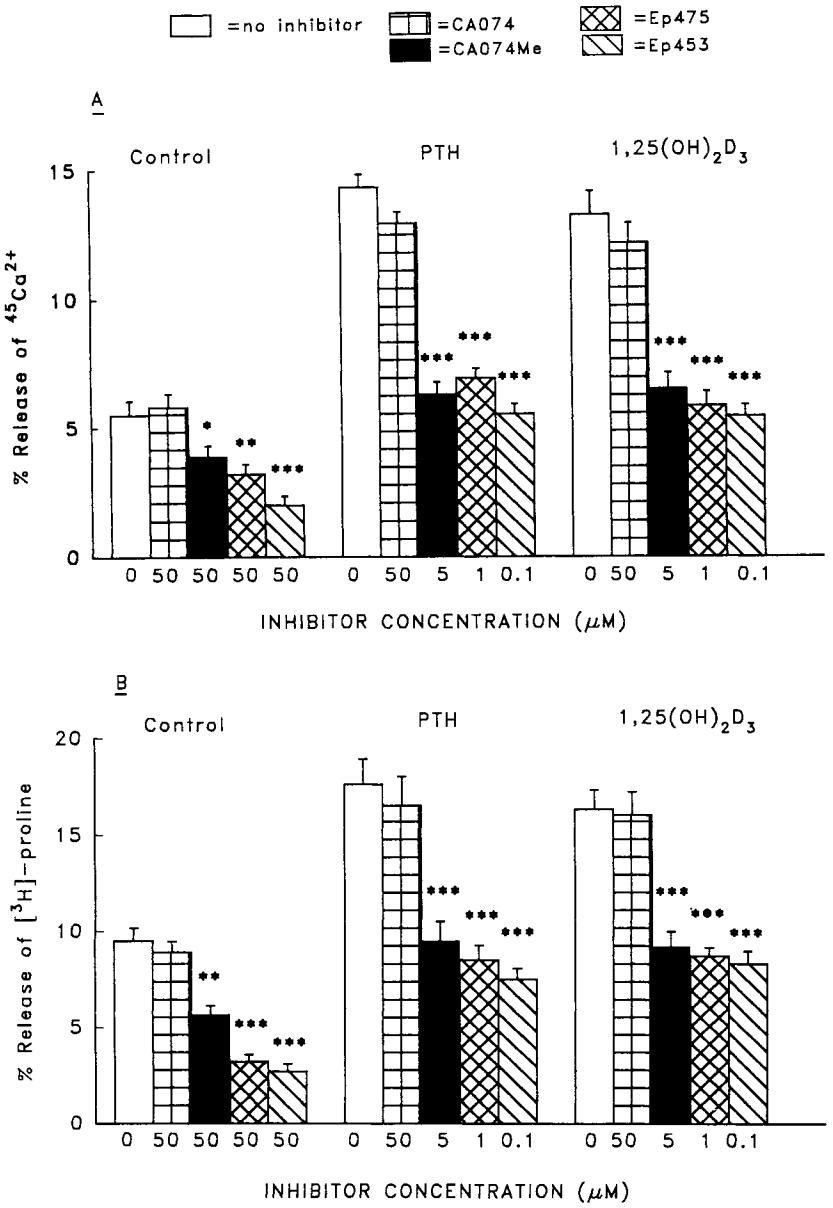

Fig. 1. Effect of the CP inhibitors on basal, PTH (20 nM), and $1,25(OH)_2D_3$ (10 nM)-stimulated release of $^{45}Ca^{2+}$ (**A**) and [^{3}H]-proline (**B**) from calvarial bones. Values are expressed as the mean percentage (± SEM) of radioisotope released from five pairs of cultured bones after 24 h of incubation. The stimulatory effect of PTH and $1,25(OH)_2D_3$ was significantly different from the untreated controls ($P < 0.001$). The inhibitory effects of CA074Me, Ep475, and Ep453 were significantly different from those of either PTH or $1,25(OH)_2D_3$ alone and on the unstimulated release of [^{3}H]-proline and $^{45}Ca^{2+}$; CA074 was without effect. $^*P < 0.05$, $^{**}P < 0.025$, $^{***}P < 0.01$.

As expected, actinomycin D (100 μM) and hydroxyurea (100 μM) completely blocked the uptake of [^{3}H]-proline and [^{3}H]-thymidine, respectively. Finally, none of the CP inhibitors enhanced the release of the cytosolic enzyme, lactate dehydrogenase from calvarial explants (Table III), and the CP inhibitors did not affect the PTH-stimulated release of the lysosomal enzymes, β-glucuronidase, and N-acetyl-β-glucosaminidase.

Isolated Osteoclast Assay

We examined the direct effect of CP inhibitors on osteoclast function in an isolated osteoclast resorption pit assay. Devitalized cortical bone slices devoid of an osteoid layer were used as a

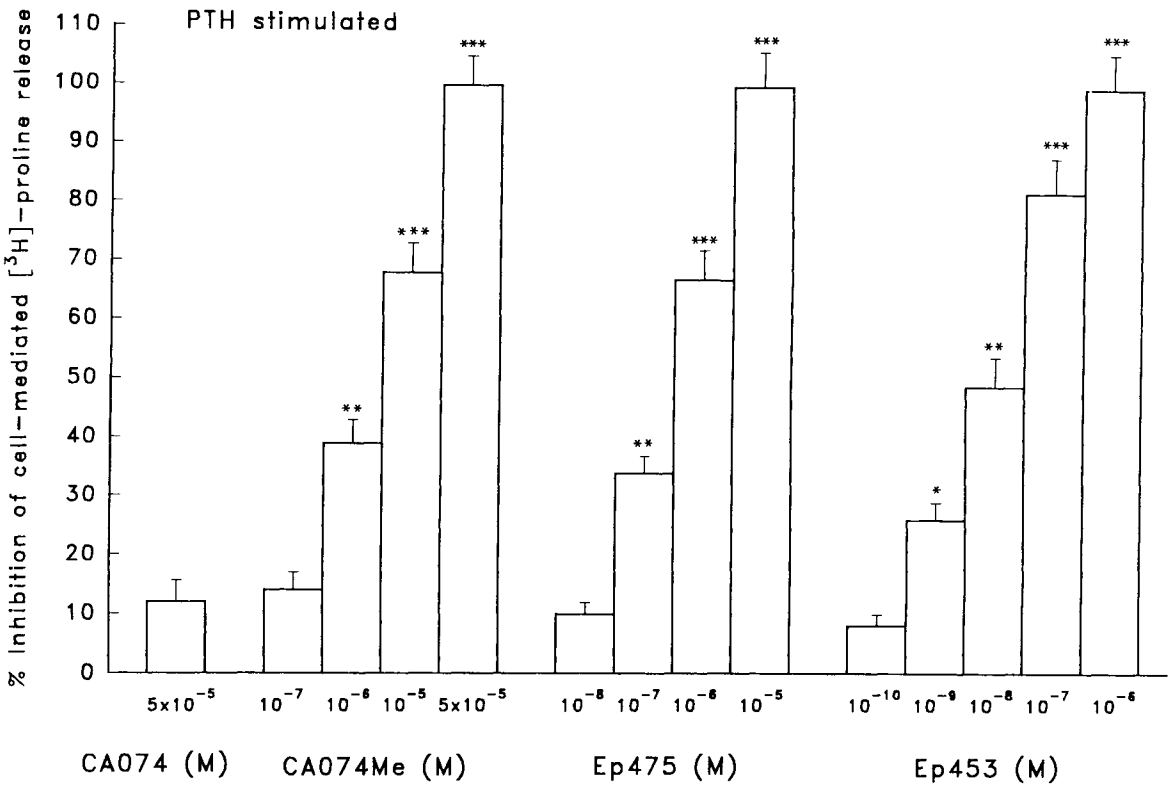

Fig. 2. Effect of the CP inhibitors at different concentrations on the PTH (20 nM)-stimulated release of [^{3}H]-proline from calvarial bones after a 48 h incubation period. The results are expressed as percentage inhibition of PTH-stimulated [^{3}H]-proline release, which was arbitrarily set to 100%. Each box is the mean ± SEM of five pairs of bones. The inhibitory effects of CA074Me (10^{-6}–5×10^{-5} M), Ep475 (10^{-7}–10^{-5} M), and Ep453 (10^{-9}–10^{-6} M) were statistically significant. *$P < 0.05$, **$P < 0.025$, ***$P < 0.01$ compared with control.

mineralized substrate, as described in the Materials and Methods section.

At the end of a 20 h culture period the cells were inspected both after neutral red staining and following fixation and staining for TRAP. In the presence of CP inhibitors multinucleated cells took up neutral red just as well as the controls, again suggesting that the viability of the cells was not affected. Morphological examination also showed that the inhibitors did not appear to alter the peripheral ruffles of the cells, indicating that the inhibitors did not interfere with the motility of the osteoclasts. Furthermore, at the maximal concentrations used (Fig. 1), none of the CP inhibitors altered the number of osteoclasts, as compared to controls. The mean number of TRAP-positive multinucleated cells per 1 cm^2 slice (nine slices per treatment) was 32.3 ± 6.1 for the control, 27.3 ± 5.4 for CA074 (50 μM), 29.3 ± 4.2 for CA074Me (50 μM), 36.1 ± 6.4 for Ep453 (50 μM), and 25.1 ± 3.1 for Ep475 (50 μM).

Three-Dimensional Analysis of the Resorption Lacunae

The analysis by confocal laser scanning microscopy of the bone surfaces on which osteoclasts had been cultured without any addition revealed typical resorption pits.

CA074Me, Ep475, and Ep453 dose-dependently inhibited the number of resorption lacunae; CA074 was again without effect (Fig. 4). At the maximum concentration (50 μM), CA074Me caused a 62 ± 3.4% reduction, Ep475 caused a 73 ± 4.6% reduction, and Ep453 caused a 79.5 ± 6.2% reduction in the number of lacunae compared to control cultures.

When the extent of bone resorption was assessed by measuring the plan surface area of the lacunae it was found that CA074Me, Ep475, and Ep453 produced a dose-dependent inhibition similar to that for the number of resorption lacunae. At the maximum concentration used (50 μM), CA074Me caused a 64.6 ± 5.1%, Ep475

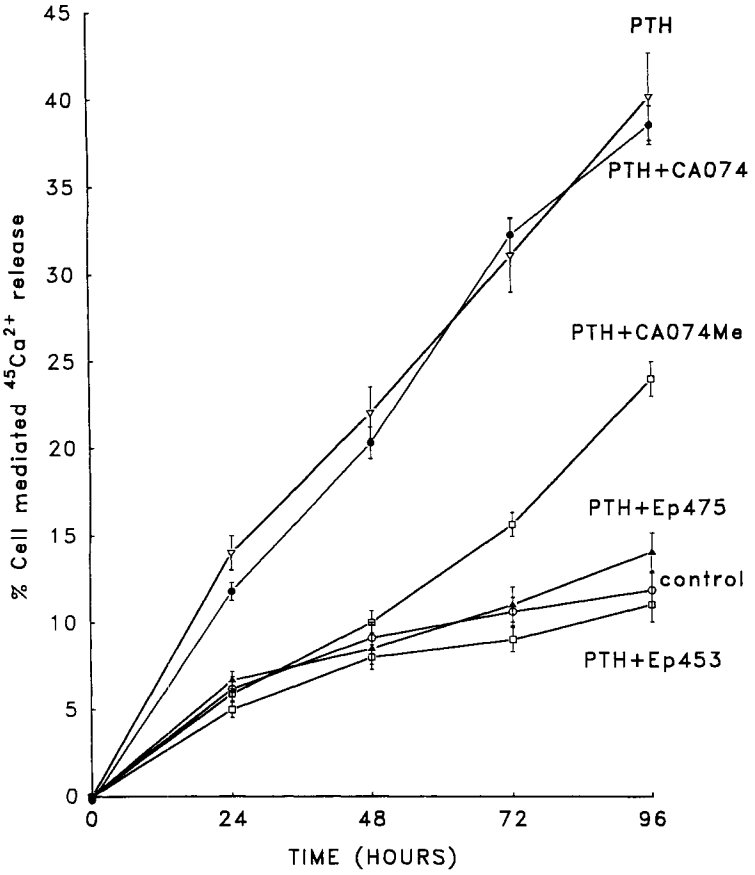

Fig. 3. Time-course of the effects of CA074 (50 μM), CA074Me (50 μM) Ep475 (50 μM), and Ep453 (5 μM) on $^{45}Ca^{2+}$ release from labelled mouse calvarial bones stimulated by PTH (20 nM). The release of $^{45}Ca^{2+}$ was determined after the removal of 50 μl of medium at each time point. Values are expressed as the mean percentage (± SEM) of radioisotope released from five bones. The stimulatory effect of PTH was significant at all time points ($P < 0.01$). CA074Me, Ep475, and Ep453 produced a significant inhibition ($P < 0.01$) of the PTH-stimulated release of $^{45}Ca^{2+}$ throughout the 96 h culture period.

TABLE I. Recovery From the Inhibitory Effects of CP Inactivators on PTH-Stimulated Release of ^{45}Ca From Mouse Calvarial Bonds*

Treatment		% Cell mediated $^{45}Ca^{2+}$ release		
0–48 h	48–144 h	0–48 h	48–96 h	96–144 h
PTH	PTH	19.9 ± 2.2	17.8 ± 1.1	14.3 ± 0.8
PTH + Ep453	PTH	5.6 ± 0.4**	9.9 ± 0.7*	16.1 ± 0.6
PTH + Ep475	PTH	6.3 ± 0.7**	10.2 ± 0.9*	16.5 ± 0.8
PTH + CA074Me	PTH	6.6 ± 0.7**	11.1 ± 0.8*	17.1 ± 0.8

*Values are mean ± SEM for five calvarial bones prelabelled with 4 μCi $^{45}CaCl_2$. PTH, Ep453, Ep475, and CA074Me were added at final concentrations of 20 nM, 1 μM, 10 μM and 50 μM, respectively. ** and * significantly different from PTH alone at $P < 0.025$ and $P < 0.05$, respectively.

a 75.9 ± 6.1%, and Ep453 a 80.5 ± 6.2% reduction in the surface area of bone resorbed compared to control cultures. This effect on the area of bone resorbed was attributable to the reduction in pit numbers as the mean surface area of each pit was similar to the control cultures (Table IV).

When bone resorption was assessed volumetrically it was found that CA074Me, Ep475, and Ep453 produced a greater degree of inhibition in the volume of bone resorbed compared to the reduction in pit numbers (Fig. 5). At the maximum concentration used (50 μM), CA074Me caused an 80.7 ± 5.8%, Ep475 caused an 87.1 ±

TABLE II. Effect of CP Inhibitors on [^{3}H]-Thymidine Uptake Into DNA and [^{3}H]-Proline Incorporation Into Proteins in Murine Calvarial Bones*

Addition	Amount (μM)	[^{3}H]-thymidine (dpm/half calvaria) DNA fraction	[^{3}H]-proline (dpm/half calvaria)
Control	—	16,556 ± 1873	27,432 ± 3101
Ep475	50	17,321 ± 2103	33,240 ± 4106
Ep453	50	19,325 ± 1980	31,144 ± 3321
CA074	50	20,036 ± 2501	27,658 ± 3161
CA074Me	50	17,639 ± 2031	30,165 ± 2976
Actinomycin D	100		1,448 ± 214
Hydroxyurea	100	1,307 ± 72	
Devitalized bone	—	847 ± 190	1,020 ± 206

*Values are means ± SEM for five calvarial halves, labelled with either 2 μCi [^{3}H]-proline (protein synthesis) or 10 μCi [^{3}H]-thymidine (DNA synthesis) for the last 6 h of a 24 h culture period.

TABLE III. Effect of Cysteine Proteinase Inhibitors on the Release of N-acetyl-β-Glucosaminidase, β-Glucuronidase, and Lactate Dehydrogenase From Mouse Calvarial Bones*

Addition	NAGase (nM/ml)	β-glucuronidase (units/ml)	Lactate dehydrogenase (units/ml)
Control	37 ± 3.0	24 ± 3	321 ± 23
PTH	70 ± 6.0**	44 ± 7**	342 ± 39
Devitalized bone	—	—	834 ± 75**
PTH + CA074	71 ± 5.0**	40 ± 5**	376 ± 41
PTH + CA074Me	63 ± 6.0**	38 ± 4**	321 ± 33
PTH + Ep475	66 ± 7.0**	45 ± 6**	296 ± 31
PTH + Ep453	79 ± 6.0**	43 ± 5**	359 ± 38

*Unlabelled calvarial explants were cultured in the presence and absence of PTH (20 nM), with or without either CA074 (50 μM), CA074Me (50 μM), Ep475 (50 μM), or Ep453 (50 μM). The results are the means ± SEM of five calvarial halves. Devitalized bones were included as controls. **Significantly different from control bones ($P < 0.025$).

7.2%, and Ep453 caused a 91.3 ± 6.4% reduction in the volume of bone resorbed compared to control cultures. This was due to the significant reduction in the mean volume of each pit as compared to the control cultures (Table IV).

Effect of the CP Inhibitors on In Vivo/In Vitro Bone Resorption

To determine whether the CP inhibitors were able to inhibit bone resorption in vivo a simple and sensitive in vivo/in vitro method of assessing bone resorption was adopted [Reynolds, 1972]. The subcutaneous injection of CA074Me, Ep475, or Ep453 into mice 3 h prior to sacrifice resulted in an approximately 20% reduction in bone resorption during a subsequent 24 h culture period (Table V): CA074 was without effect.

TABLE IV. Effects of the CP Inhibitors (1–50 μM) on the Mean Surface Area and Volume of Individual Resorption Lacunae*

Treatment	Surface area (μm^2)	Volume (μm^3)
Control	342 ± 29	951 ± 21
CA074Me	352 ± 89	662 ± 65***
Ep475	326 ± 61	656 ± 61***
Ep453	360 ± 79	521 ± 86***

*Values are mean ± S.E.M. for 914 (control) 134 (CA074Me), 104 (Ep475), and 92 (Ep453) resorption lacunea. ***Significantly different from control at $P < 0.01$.

DISCUSSION

The ability of CA074Me, but not CA074 to inhibit bone resorption is strongly suggestive of an intracellular role for cathepsin B. CA074 is a highly selective inhibitor of cathepsin B, inactivating the enzyme more than 3 orders of magnitude faster than it inactivates cathepsins H, L, S, or m-calpain [Buttle et al., 1992a,b]. CA074 is negatively charged and therefore not efficient at entering cells. Conversion to the methyl ester allows passive entry into cells and the rapid and

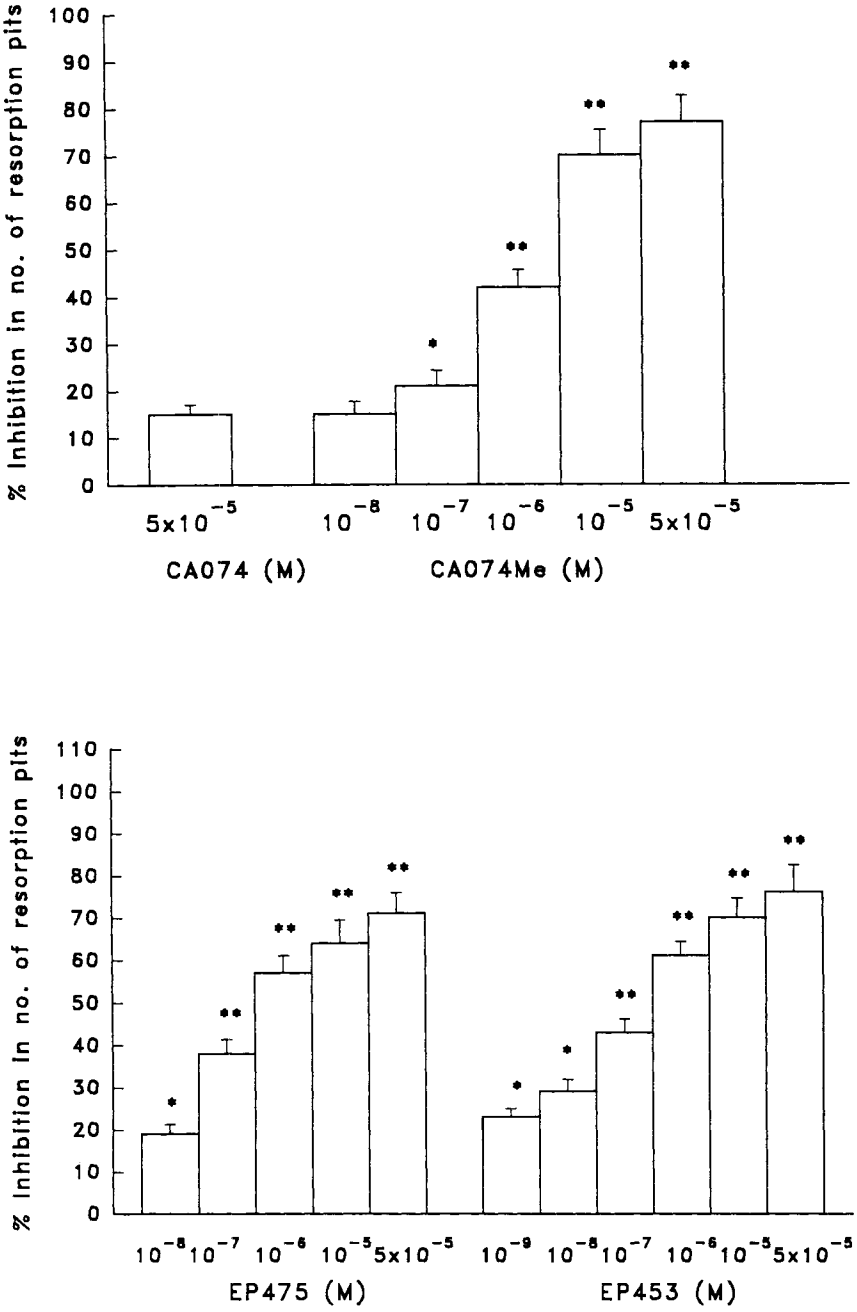

Fig. 4. Effect of the CP inhibitors on osteoclast pit formation. Each bar represents the mean ± S.E.M. from three individual experiments. The number of pits on control slices was 32 ± 5. Significantly different from control at $^{*}P < 0.05$, $^{**}P < 0.025$, $^{***}P < 0.01$.

selective inactivation of intracellular cathepsin B [Buttle et al., 1992a,b]. The implication is that cathepsin B may be responsible for the intracellular activation of a proteinase(s) which participates in the degradation of the organic matrix within the subosteoclastic resorption zone (see below).

Ep475, like CA074, is a negatively charged compound with limited membrane permeability. Unlike CA074, however, it proved to be an efficient inhibitor of bone resorption. Ep475 is a rapid inactivator of cathepsins L, S, H, and calpain, as well as cathepsin B [Buttle and Saklatvala, 1992]. Of these proteinases those with

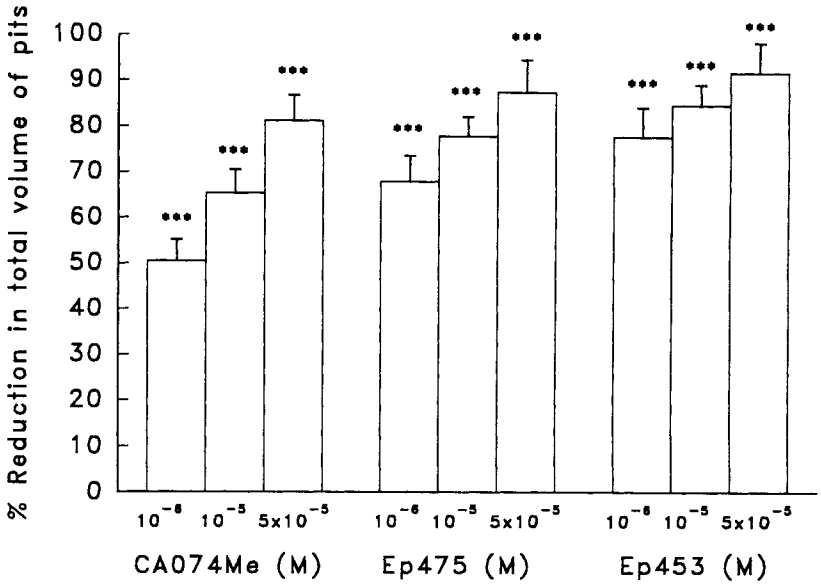

Fig. 5. Effect of the CP inhibitors on the volume of bone resorbed. Each bar represents the mean ± S.E.M. from three individual experiments. Significantly different from control at $***P < 0.01$.

TABLE V. Effects of the CP Inhibitors on Bone Resorption as Assessed by an In Vivo/In Vitro Assay*

Pre-treatment of mice in vivo	% CMR during 24 h in vitro	% inhibition of resorption
Control	9.7 ± 0.3	
CA074	10.2 ± 0.4	—
Control	12.1 ± 0.2	
CA074Me	9.7 ± 0.3	19.8*
Control	11.6 ± 0.3	
Ep475	8.9 ± 0.3	23.2*
Control	13.1 ± 0.4	
Ep453	9.9 ± 0.3	24.4*

*Values are mean ± S.E.M. for five calvarial bones prelabelled with 1 μCi $^{45}CaCl_2$. CA074, CA074Me, Ep475, or Ep453 were administered at 60 μg/g body weight. Each inhibitor was tested in a separate litter of mice. $*P < 0.05$, compared to control group.

proven collagenolytic activity include cathepsin B, L, and S [reviewed in Buttle, 1994]. The ability of Ep475 but not CA074 to inhibit bone resorption therefore implicates cathepsins L and/or S, but not cathepsin B, at an extracellular site. It is likely that Ep475 can gain access to the subosteoclastic resorption zone, probably through the bone matrix. This compartment is sealed off by the attachment of the osteoclast to the calcified matrix [Baron et al., 1988] and is the site of collagen degradation [Everts et al., 1992]. Cathepsins L and/or S are likely to be active in this compartment as both enzymes efficiently digest collagen at acidic pH [Kirschke et al., 1982, 1989] and are rapidly inactivated by Ep475 [Buttle and Saklatvala, 1992]. Further evidence for a role for cathepsin L has been provided by experiments showing that Z-Phe-Phe-CHN_2, a selective inactivator of cathepsin L compared to cathepsin B, prevented the resorptive activity of isolated avian and rodent osteoclasts [Rifkin et al., 1991]. While there is no direct evidence as yet demonstrating that cathepsin L is secreted by the osteoclast into the apical resorption zone, the enzyme has been recently immunolocalized in rodent osteoclasts [Ohsawa et al., 1993].

Ep453 was the most potent inhibitor examined. This may be because of its ability to inhibit intracellularly cathepsins B, L, and S. Thus the intracellular action of cathepsin B would be controlled, and in addition cathepsin L/S would be inactivated prior to release, totally precluding any resorptive activity.

The observation that the inhibitory effects of CA074Me gradually declined after 48 h suggests that CA074Me may be slowly metabolized by tissue explants, as previously reported [Buttle et al., 1993].

Ep453, Ep475, and CA074Me were capable of inhibiting bone resorption when administered in vivo, although the degree of inhibition was not as pronounced as that obtained in vitro. This may be attributable to their rapid clearance

from the body in a similar manner to the related compound, E-64 [Hashida et al., 1982]. The degree of inhibition observed in the present study was similar to that found when serum calcium levels were used to monitor the extent of bone resorption in hypocalcaemic rats [Kakegawa et al., 1993].

The demonstration that Ep453, Ep475, and CA074Me induced a reduction in both the number and volume of the resorptive lacunae is in accordance with the effects of a related CP inactivator, E-64 [Delaissé et al., 1987]. This suggests that the CP inactivators prevent the resorptive activity of some osteoclasts and partially inhibit that of others. Whilst time lapse video records showing osteoclast motility were not undertaken, treated cells spread on the bone slices and the surface areas of the resorption lacunae remained unaffected although the volume of individual lacunae was reduced. This suggests that osteoclasts commenced resorption in a similar manner to controls but that further cavitation was impeded.

Cathepsin B has been shown to be capable of activating the MMPs, procollagenase and prostromelysin [Eeckhout and Vaes, 1977; Murphy et al., 1992] as well as the serine proteinase, pro-urokinase-type plasminogen activator [Kobayashi et al., 1991]. Since these three enzymes have recently been detected within osteoclasts [Case et al., 1989; Grills et al., 1990; Delaissé et al., 1993], it is possible that cathepsin B has a primary role in the activation of one or more of these enzymes which participate in collagen degradation.

The finding that the CP inhibitors prevent the release of [^{3}H]-proline to a greater extent than $^{45}Ca^{2+}$ in basal resorption is in agreement with previous studies [Delaissé et al., 1980, 1984] and suggests that solubilization of the bone mineral by the acidic environment of the bone lacunae is allowed to proceed until the collagenous matrix is encountered, the degradation of which is impeded. Further evidence for this view was provided by the fact that individual resorption lacunae in the isolated osteoclast assay were reduced in volume but not in surface area when the drugs were present.

In conclusion, an intracellular role for cathepsin B and an extracellular role for cathepsins L and/or S in bone resorption in vivo and in vitro has been demonstrated. Cathepsin L and/or S are probably involved in the degradation of bone collagen in the subosteoclastic resorption zone. We suggest that cathepsin B is important in the activation of bone resorbing enzymes. These could be of the cysteine proteinase and/or matrix metalloproteinase [Delaissé et al., 1984; Heath et al., 1984; Rifas et al.; 1989 Everts et al., 1992, Hill et al., 1993]. Further detailed studies using selective MMP inhibitors with CP inhibitors should clarify the respective roles of these enzymes in bone resorption.

ACKNOWLEDGMENTS

This work has been supported by funds from the Medical Research Council (M.R.C.) and the Arthritis and Rheumatism Council (A.R.C.). P.A. Hill is an M.R.C. Clinical Training Fellow. D.J.B. is an A.R.C. Post-doctoral research fellow.

REFERENCES

Arnett TR, Dempster DW (1986): Effect of pM on bone resorption by rat osteoclasts in vivo. Endocrinology 119: 119–124.

Baron R, Neff L, Louvard D, Courtoy PJ (1985): Cell-mediated extracellular acidification and bone resorption: evidence for a low pH in resorbing lacunae and localization of a 100-kD lysosomal membrane protein at the osteoclast ruffled border. J Cell Biol 101:2210–2222.

Baron R, Neff L, Brown W, Courtoy PJ, Louvard D, Farquhar MG (1988): Polarized secretion of lysosomal enzymes: Co-distribution of cation-independent mannose-6-phosphate receptors and lysosomal enzymes along the osteoclast exocytic pathway. J Cell Biol 106:1863–1872.

Baron R (1989): Molecular mechanisms of bone resorption by the osteoclast. Anat Rec 224:317–324.

Boyde A, Ali NN, Jones SJ (1984): Resorption of dentine by isolated osteoclasts in vitro. Br Dent J 156:216–220.

Burleigh MC, Barrett AJ, Lazarus GS (1974): Cathepsin B1. A lysosomal enzyme that degrades native collagen. Biochem J 137:387–398.

Buttle DJ (1994): Lysosomal cysteine endopeptidases in the degradation of cartilage and bone. In Davies ME, Dingle JT (eds): "Immunopharmacology of Joints and Connective Tissue." New York: Academic Press, pp 225–243.

Buttle DJ, Saklatvala J (1992): Lysosomal cysteine endopeptidases mediate interleukin 1-stimulated cartilage proteoglycan degradation. Biochem J 287:657–661.

Buttle DJ, Murata M, Knight CG, Barrett AJ (1992a): CA074 methyl ester: A proinhibitor for intracellular cathepsin B. Arch Biochem Biophys 299:377–380.

Buttle DJ, Saklatvala J, Tamai M, Barrett AJ (1992b): Inhibition of interleukin 1-stimulated cartilage proteoglycan degradation by a lipophilic inactivator of cysteine endopeptidases. Biochem J 281:175–177.

Buttle DJ, Handley CJ, Illic MZ, Saklatvala J, Murata M, Barrett AJ (1993): Inhibition of cartilage proteoglycan release by a specific inactivator of cathepsin B and an inhibitor of matrix metalloproteinases. Evidence for two converging pathways of chondrocyte-mediated proteoglycan degradation. Arth Rheum 36:1709–1717.

Cabaud PG, Wroblewski F (1958): Colorimetric measurement of lactic dehydrogenase activity of body fluids. Am J Clin Pathol 30:234–238.

Case JP, Sano H, Lafyatis R, Remmers EF, Kumkumian GK, Wilder RL (1989): Transin stromelysin expression in the synovium of rats with experimental erosive arthritis. In situ localization and kinetics of expression of the transformation-associated metalloproteinase in euthymic and athymic Lewis rats. J Clin Invest 84:1731–1740.

Chambers TJ, Darby JA, Fuller K (1985): Mammalian collagenase predisposes bone surfaces to osteoclastic resorption. Cell Tissue Res 241:671–675.

Chambers TJ, McSheehy PMJ, Thomson BM, Fuller K (1985): The effect of calcium-regulating hormones and prostaglandins on bone resorption by osteoclasts disaggregated from neonatal rabbit bones. Endocrinology 116:234–239.

Delaisse J-M, Eeckhout Y, Vaes G (1980): Inhibition of bone resorption in culture by inhibitors of thiol proteinases. Biochem J 192:365–368.

Delaisse J-M, Eeckhout Y, Vaes G (1984): In vivo and in vitro evidence for the involvement of cysteine proteinases in bone resorption. Biochem Biophys Res Commun 125: 441–447.

Delaisse JM, Boyde A, Maconnachie E, Ali N, Sear CHJ, Eeckhout Y, Vaes G, Jones SJ (1987): The effects of inhibitors of cysteine-proteinases and collagenase on the resorptive activity of isolated osteoclasts. Bone 8:305–313.

Delaisse J-M, Ledent P, Vaes G (1991). Collagenolytic cysteine proteinases of bone tissue: Cathepsin B, (pro)cathepsin L and a cathepsin L-like 70 kDa proteinase. Biochem J 279:167–174.

Delaisse J-M, Vaes G (1992): Mechanism of mineral solubilization and matrix degradation in osteoclastic bone resorption. In Rifkin BR, Gay CV (eds): "Biology and Physiology of the Osteoclast." Boca Raton. CRC Press, pp 289–314.

Delaisse JM, Eeckhout Y, Neff L, Francois-Gillet C, Henriet P, Su Y, Vaes G, Baron R (1993): (Pro)collagenase (matrix metalloproteinase-1) is present in rodent osteoclasts and in the underlying bone-resorbing compartment. J Cell Sci 106:1071–1082.

Eeckhout Y, Vaes G (1977): Further studies on the activation of procollagenase, the latent precursor of bone collagenase. Effects of lysosomal cathepsin B, plasmin and kallilrein, and spontaneous activation. Biochem J 166:21–31.

Etherington DJ (1972): The nature of the collagenolytic cathepsin of rat liver and its distribution in other rat tissues. Biochem J 127:685–692.

Everts V, Beertsen W, Schroder R (1988): Effects of the proteinase inhibitors leupeptin and E-64 on osteoclastic bone resorption. Calcif Tissue Int 43:172–178.

Everts V, Delaisse J-M, Korper W, Niehof A, Vaes G, Beertsen W (1992): Degradation of collagen in the bone-resorbing compartment underlying the osteoclast involves both cysteine-proteinases and matrix metalloproteinases. J Cell Physiol 150:221–231.

Fishman WH, Kato K, Anstiss CL, Green S (1967): Human β-glucuronidase: Its measurement and some of its properties. Clin Chim Acta 15:435–438.

Goto T, Tsukuba T, Nishimura Y, Kato K, Yamamoto K, Tanaka T (1993): Immunohistochemical localization of cathepsins B, D and L in the rat osteoclast. Histochemistry 99:411–414.

Grills BL, Gallagher JA, Allan EM, Yumita S, Martin TJ (1990): Identification of plasminogen activator in osteoclasts. J Bone Min Res 5:499–505.

Hashida S, Kominami E, Katunuma N (1982): Inhibitions of cathepsin B and cathepsin L by E-64 in vivo. Incorporation of [^{3}H]E-64 into rat liver lysosomes in vivo. J Biochem 91:1373–1380.

Heath JK, Atkinson SJ, Meikle MC, Reynolds JJ (1984): Mouse osteoblasts synthesize collagenase in response to bone resorbing agents. Biochim Biophys Acta 802:151–154.

Hill PA, Reynolds JJ, Meikle MC (1993): Inhibition of stimulated bone resorption in vitro by TIMP-1 and TIMP-2. Biochim Biophys Acta 1177:71–74.

Jones SJ, Boyde A, Piper K, Komiya S (1992): Confocal microscopic mapping of osteoclastic resorption. Micros Anal 7:18–20.

Kakegawa H, Nikawa T, Tagami K, Kamioka H, Sumitani K, Kawata T, Drobnic-Kosorok M, Lenarcic B, Turk V, Katunuma N (1993): Participation of cathepsin L on bone resorption. FEBS Lett 321:247–250.

Kirschke H, Kembhavi AA, Bohley P, Barrett AJ (1982): Action of rat liver cathepsin L on collagen and other substrates. Biochem J 201:367–372.

Kirschke H, Wiederanders B, Bromme D, Rinne A (1989): Cathepsin S from bovine spleen. Purification, intracellular localization and action on proteins. Biochem J 264:467–473.

Kobayashi H, Schmitt M, Goretzki L, Chucholowski N, Calvete J, Kramer M, Gunzler WA, Janicke F, Graeff H (1991): Cathepsin B efficiently activates the soluble and the tumor cell receptor-bound form of the proenzyme urokinase-type plasminogen activator (pro-uPA). J Biol Chem 266:5147–5152.

Lerner U, Granstrom G (1984): Effect of levamisole on bone resorption in cultured mouse calvaria. Eur J Pharmacol 105:1–14.

Lerner UH (1987): Modifications of the mouse calvarial technique improve the responsiveness to stimulators of bone resorption. J Bone Min Res 2:375–383.

Lerner UH, Grubb A (1992): Human cystatin C, a cysteine proteinase inhibitor, inhibits bone resorption in vitro stimulated by parathyroid hormone and parathyroid hormone-related peptide of malignancy. J Bone Miner Res 7:433–440.

Ljunggren O, Ransjo M, Lerner UH (1991): In vitro studies on bone resorption in neonatal mouse calvariae using a modified dissection technique giving four samples of bone from each calvaria. J Bone Min Res 6:543–549.

Murata M, Miyashita S, Yokoo C, Tamai M, Hanada K, Hatayama K, Towatari T, Nikawa T, Katunuma N (1991): Novel epoxysuccinyl peptides: Selective inhibitors of cathepsin B. FEBS Lett 280:307–310.

Murphy G, Ward R, Gavrilovic J, Atkinson S (1992): Physiological mechanisms for metalloproteinase activation. Matrix Suppl 1:224–230.

Ohsawa Y, Nitatori T, Jiguchi S, Kominami E, Uchiyama Y (1993): Lysosomal cysteine and aspartic proteinases, acid phosphatase in rat osteoclasts. J Histochem Cytochem 41:1075–1083.

Osdoby P, Martini MC, Caplan AI (1982): Isolated osteoclasts and their presumed progenitor cells, the monocyte, in culture. J Exp Zool 224:331–344.

Page AE, Warburton MJ, Chambers TJ, Pringle JAS, Hayman AR (1992): Human osteoclastomas contain multiple forms of cathepsin B. Biochim Biophys Acta 1116:57–66.

Reynolds JJ (1972): A sensitive in vivo/in vitro method for studying substances that influence the resorption of bone. In Talmage RV, Munson PL (eds). "Calcium, Parathyroid Hormone and the Calcitonins." Amsterdam: Excerpta Medica, pp 454–462.

Reynolds JJ, Dingle JT (1970): A sensitive in vitro method for studying the induction and inhibition of bone resorption. Calc Tiss Res 4:339–349.

Rifas L, Halstead LR, Peck WA, Avioli LV, Welgus HG (1989): Human osteoblasts in vitro secrete tissue inhibitor of metalloproteinases and gelatinase but not interstitial collagenase as major cellular products. J Clin Invest 84: 686–694.

Rifkin BR, Vernillo AT, Kleckner AP, Auszmann JM, Rosenberg LR, Zimmerman M (1991): Cathepsin B and L activities in isolated osteoclasts. Biochem Biophys Res Commun 179:63–69.

Sasaki T, Ueno-Matsuda E (1993): Cysteine-proteinase localization in osteoclasts: An immunocytochemical study. Cell Tissue Res 271:177–179.

Tamai M, Matsumoto K, Omura S, Koyama I, Osawa Y, Hanada K (1986): In vitro and in vivo inhibition of cysteine proteinases by EST a new analog of E-64. J Pharmacobio-Dyn 9:672–677.

Tamai M, Yokoo C, Murata M, Oguma K, Sota K, Sato E, Kanaoka Y (1987): Efficient synthetic method for ethyl (+)-(2S,3S)-3[(S)-3-methyl-1-(3-methylbutyl-carbamoyl)]-2-oxiranecarboxylate (EST), a new inhibitor of cysteine proteinases. Chem Pharm Bull 35:1098–1104.

Wilcox D, Mason RW (1992): The inhibition of cysteine proteinases in lysosomes and whole cells. Biochem J 285: 495–502.

Zambonin-Zallone A, Teti A, Primavera MV (1982): Isolated osteoclasts in primary culture: First observation on structure and survival in culture media. Anat Embryol 165:405–413.