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Possible role and mechanism of action of dissolved calcium in the degradation of bone collagen by lysosomal cathepsins and collagenase

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Equilibrium experiments with bone powder, at pH values ranging from 6.3 to 3.5, show a linear relation between $\log([Ca^{2+}]/[Ca^{2+}]^0)$ (where $[Ca^{2+}]^0 = 1$ M-Ca^{2+}) and pH, indicating that $[Ca^{2+}]$ could reach levels of 25 mM at pH 5 and 90 mM at pH 4. These elevated Ca^{2+} concentrations stimulated the lysis of insoluble bone collagen *in vitro* by purified lysosomes and by mouse bone collagenase, whose activities were additive at acid pH. At neutral pH, the addition of 10–100 mM-$CaCl_2$ did not influence the susceptibility of acid-soluble skin collagen in solution towards bone collagenase, but increased it markedly towards collagen in the fibrillar form. Increasing the $[Ca^{2+}]$ did not influence the susceptibility of collagen to trypsin. Elevated $[Ca^{2+}]$ and a co-operation between lysosomal cysteine proteinases and matrix collagenase could thus participate in the osteoclastic breakdown of bone collagen.

INTRODUCTION

Osteoclasts excavate mineralized bone matrix by tightly adhering to it and isolating a subosteoclastic microenvironment wherein their specialized ruffled border membrane secretes acid (Baron *et al.*, 1985; Silver *et al.*, 1988) and collagenolytic lysosomal proteinases (Delaissé *et al.*, 1984; Blair *et al.*, 1986). Several observations (Etherington & Birkedal-Hansen, 1987; Eeckhout *et al.*, 1988; Silver *et al.*, 1988) suggest that the subosteoclastic dissolution of bone mineral generates local concentrations of dissolved calcium which are one or two orders of magnitude higher than in the other extracellular fluids, and this thereby facilitates the proteolysis of demineralized bone collagen. The purpose of the present study was (1) to determine the solubility of bone calcium at pH values presumed to occur beneath the osteoclast; (2) to examine the influence and the mechanism of action of these elevated Ca^{2+} concentrations on the degradation of bone collagen by the lysosomal proteinases and by collagenase, which have both been implicated in bone resorption (for reviews, see Eeckhout *et al.*, 1988; Eeckhout & Delaissé, 1988; Vaes, 1988); and (3) to search for a possible simultaneous co-operation of collagenase and lysosomal proteinases in the degradation of bone collagen at acid pH.

A preliminary account of this work was presented at the Symposium on 'The Control of Tissue Damage' held in Cambridge, U.K. (April 1987) and at the Seventh Meeting of the International Committee on Proteolysis held in Shimoda, Japan (May 1988).

MATERIALS AND METHODS

Materials

Bone powder was generously provided by Dr. E. Munting (Laboratory of Orthopaedic Surgery, University of Louvain). It was prepared by grinding pieces of adult bovine diaphyseal bone, free of marrow, under cooling water with a Retsch instrument, type DR88 (Haan, Germany). The powder (particle diameter < 500 μm) was defatted in acetone, washed in ethanol and dried with ether.

Insoluble bone collagen was prepared from bovine bone powder by the following procedures. Bone powder (100 g) was demineralized and cleared of soluble proteins at 4 °C by 5×24 h extractions, each in 1 litre of 0.4 M-EDTA, pH 7.5, followed by 2×24 h extractions in 1 litre of 0.5 M-acetic acid, one rinse with water and two extractions in 1 litre of 1 M-NaCl containing 0.05 M-EDTA. The final insoluble collagen was rinsed with $7 \times$ 1 litre of water and lyophilized; 1 mg of dry collagen contained 124 ± 10 (mean $\pm$ S.D.) μg of hydroxyproline.

^{3}H-acetylated acid-soluble guinea-pig skin collagen (2×10^6 d.p.m./mg) was prepared by the procedure of Cawston & Barrett (1979). The soluble fraction of purified rat liver lysosomes, kindly provided by Dr. M. P. Collard (Laboratoire de Chimie Physiologie, Université de Louvain), was prepared by the method of Trouet (1974). On the basis of the specific activity of *N*-acetyl-β-glucosaminidase (EC 3.2.1.30), the enrichment of lysosomal hydrolases was 58-fold. Trypsin-activated mouse calvaria culture fluids (Vaes, 1972) were used as a source of collagenase. Z-Arg-Arg-NHMec and Z-Phe-Arg-NHMec were from Bachem, Bubendorf, Switzerland. All other reagents were of analytical grade.

Equilibrium studies with bone powder

Samples of bone powder were equilibrated essentially as described by MacGregor & Nordin (1960). In brief, except where otherwise stated, 0.5 g samples of bone powder were suspended in 2.5 ml of the indicated equilibrium fluid, sealed in dialysis tubing and placed in screw-capped glass tubes containing a further 7.5 ml of the same fluid. All equilibrium fluids contained 0.2 mg of NaN_3/ml as a bactericide. The tubes were oscillated at 37 °C. The pH of the fluid outside the dialysis bags was determined at frequent intervals and adjusted to the indicated values by the addition of HCl, until equilibrium (stable pH and Ca^{2+} concentration) was achieved. Total diffusible Ca^{2+} concentrations at equilibrium were measured by atomic ab-

Abbreviations used: $[Ca^{2+}]^0$, 1 M-Ca^{2+}; Z-, benzyloxycarbonyl-; -NHMec, 4-methyl-7-coumarylamide; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane.

sorption spectrophotometry. Logarithmic values were expressed as $\log([Ca^{2+}]/[Ca^{2+}]^0)$, where $[Ca^{2+}]^0 = 1$ M.

Digestion of insoluble bone collagen

Except where otherwise stated, 2 mg samples of bone collagen (in triplicate) were incubated for 18–24 h at 37 °C under gentle agitation in 0.4 ml of the indicated medium. At the end of the incubations, the contents of the tubes were centrifuged at 13000 *g* for 15 min at room temperature. Hydroxyproline was determined in 100 μl of the supernatants by the method of Bergman & Loxley (1963), after hydrolysis in 6 M-HCl for 22 h at 110 °C in sealed tubes. The amount of solubilized collagen was calculated on the basis that 1 mg of hydroxyproline is equivalent to 8.06 mg of collagen. It was verified that the addition of up to 0.5 M-$CaCl_2$ or -NaCl did not influence the hydroxyproline assay.

Digestion of acid-soluble skin collagen

^{3}H-acetylated acid-soluble skin collagen (0.5 mg/ml, pH 7.5) was incubated in a final volume of 100 μl, either in solution at 25 °C or as reconstituted fibrils at 35 °C according to Vaes (1972). The reaction was stopped by the addition of 25 μl of 0.6 M-EDTA, pH 7.5, supplemented with 0.2 mg of soya bean trypsin inhibitor/ml when trypsin was present. Tubes containing collagen in solution were further incubated at 35 °C for 18–24 h to allow collagen fibril formation. All the tubes were centrifuged and the radioactivity was measured in 50 μl of supernatant.

RESULTS AND DISCUSSION

Solubility of calcium in bone powder equilibrated at acid pH values

Fig. 1 illustrates the solubility of diffusible calcium when bovine lamellar bone powder was equilibrated at 37 °C over the pH range 3.5–6.3. It was verified in preliminary experiments (results not shown) that under the experimental conditions used equilibrium was reached after 3 days or less, as indicated by the stabilization of both soluble $[Ca^{2+}]$ and pH. Neither the amount of bone powder in the range 0.20–0.75 g (solid-to-fluid ratio 1:50–1:13) nor the buffer concentration in the equilibrium fluid influenced Ca^{2+} concentrations at equilibrium. The results of 29 equilibration experiments over the pH range 3.5–6.3 are presented in Fig. 1. The logarithmic transformation of these data (insert of Fig. 1) shows a linear relationship between $\log([Ca^{2+}]/[Ca^{2+}]^0)$ and pH, according to the equation: $\log([Ca^{2+}]/[Ca^{2+}]^0) = 1.31 - 0.59$ pH (correlation coefficient = 0.96).

The influence of pH on the solubilization of bone mineral has been studied extensively by several workers (Nordin, 1957; Neuman *et al.*, 1960; MacGregor & Nordin, 1960; Christoffersen, 1981). However, measurements of soluble calcium in equilibrium with powdered bone have never been studied at pH values lower than 6, apparently because such low pH values were, until recently, considered to be unphysiological. Etherington & Birkedal-Hansen (1987) have determined the solubility of Ca^{2+} in equilibrium at room temperature with inorganic hydroxyapatite at pH values ranging from 3.2 to 5.1. Our equilibrium solubility values of bone Ca^{2+} (Fig. 1) measured at 37 °C are roughly 2-fold higher, but this might result from differences in temperature and material. The solubility of bone Ca^{2+} (4 mM) at pH 6.2 confirms the findings of Nordin (1957). A linear relationship between $\log[Ca^{2+}]$ and pH was already apparent from the data of MacGregor & Nordin (1960) over the pH range 7.2–6.2, but not at higher pH values.

These Ca^{2+} solubility values were determined at equilibrium and are therefore maximal values. Soluble Ca^{2+} concentrations in the subosteoclastic bone-resorbing compartment will depend on the rate of dissolution of the bone mineral, which will be determined by factors such as the rate of proton secretion and by the rates of diffusion, in opposite directions, of H^+ and Ca^{2+} ions. The well-documented (Gaillard, 1959) motility of the osteoclastic ruffled-border membrane may be critical in this respect.

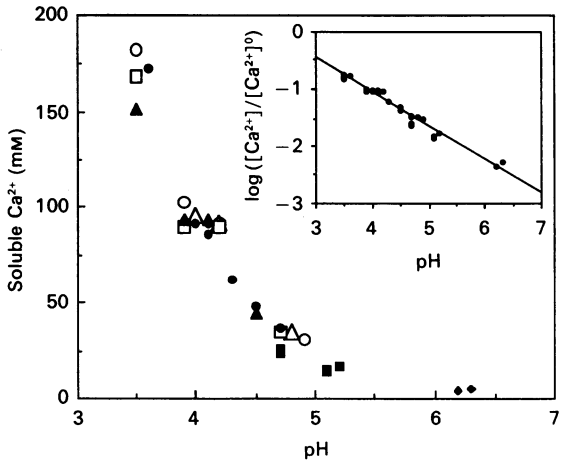

Fig. 1. Influence of pH on the solubility of bone calcium

Samples of powdered bone were equilibrated in dialysis bags (see the Materials and methods section) at the indicated pH values without buffer (○), or with 1 mM- (△), 25 mM- (□), 50 mM- (●), 100 mM- (▲) or 150 mM- (■) sodium acetate/acetic acid buffer, or with sodium cacodylate/HCl buffer (◆). Total diffusible calcium was measured at equilibrium.

The elegant microelectrode studies of Silver *et al.* (1988) revealed that osteoclasts in culture may generate pericellular pH values as low as 3 in a few minutes. According to the same authors, subosteoclastic soluble $[Ca^{2+}]$ rose *in vivo* to a maximum of 40 mM, and the pH reached a lower limit of 4.7. These values probably represent means of microenvironmental values, which depend on the distance from the H^+-secreting ruffled-border membrane and from the H^+-neutralizing dissolution of bone mineral. It is noteworthy that our measurements (see Fig. 1) predict a Ca^{2+} concentration of 35 mM at pH 4.7. Whatever the precise local concentrations of H^+ and Ca^{2+} in the intricate and moving subosteoclastic microenvironment, the data support the hypothesis that Ca^{2+} concentrations in the osteoclast-generated bone crypts are between one and two orders of magnitude higher than in the other pericellular fluids.

Influence of Ca^{2+} concentration on the digestion of insoluble bone collagen

Grant & Alburn (1960) first observed that the proteolysis of tendon collagen by neutral serine proteinases is markedly stimulated by the addition of 0.1–0.2 M-$CaCl_2$. Ca^{2+} concentrations above 2 mM are unphysiological in the neutral biological fluids, but could be relevant in acidic microenvironments such as the osteoclastic bone-resorbing compartment. Etherington & Birkedal-Hansen (1987) have shown that, at pH 4, the rate of degradation of bone collagen by lysosomal cysteine proteinases is increased 2–4-fold in the presence of 50 mM-Ca^{2+}, which might occur under the osteoclast. A similar effect of Ca^{2+} was observed independently by Eeckhout *et al.*, (1988) on the lysis of bone collagen by pepsin. As illustrated in Fig. 2, the addition of increasing Ca^{2+} concentrations (10–50 mM) stimulated the solubilization of collagen by the lysosomal extract 2–3-fold, but a similar stimulation was obtained by the addition of NaCl at equivalent ionic strengths.

About 75% of the lysosomal collagenolytic activity was inhibited by 2.8 μM-E-64, a specific inhibitor of cysteine proteinases (Barrett *et al.*, 1982), whereas only 10% was

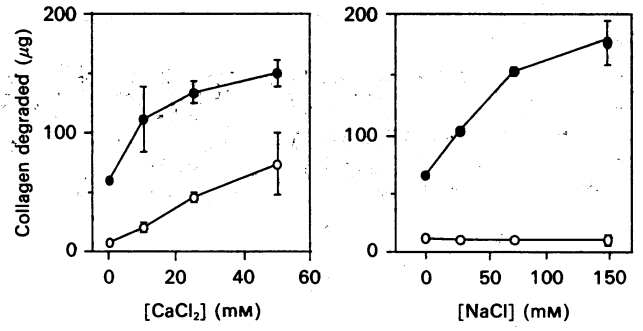

Fig. 2. Influence of $CaCl_2$ and NaCl concentrations on the degradation of insoluble bone collagen by collagenase and by purified lysosomes

Insoluble bone collagen (2 mg, in triplicate) was incubated for 23 h at 37 °C in a final volume of 0.4 ml either with 2 units of crude mouse bone collagenase/ml (○) in 50 mM-sodium acetate/acetic acid buffer, pH 5.3, containing 0.4 mM-$CaCl_2$, 30 mM-NaCl and NaN_3 (0.1 mg/ml), or with the soluble content (17 μg of protein) of purified rat liver lysosomes (●) in 50 mM-sodium acetate/acetic acid buffer, pH 4.0, containing 1 mM-dithiothreitol, 1 mM-EDTA and 1 mM-$CaCl_2$. The indicated concentrations of $CaCl_2$ (*a*) or NaCl (*b*) are those that have been added to the suspension media which contained 0.4 mM-$CaCl_2$ and 30 mM-NaCl. Soluble hydroxyproline (means ± S.D.) was measured at the end of the incubation and converted into μg of collagen. The respective control values (26 ± 5 μg of degraded collagen when lysosomes were replaced by their solvent; 31 ± 4 μg of degraded collagen when collagenase was replaced by heat-inactivated enzyme) were subtracted from the test values.

inhibited by the aspartic acid proteinase inhibitor pepstatin A (results not shown), indicating that the acid lysosomal collagenolytic activity was mainly due to cysteine proteinases. Interestingly, more than 90% of the degradation products of bone collagen could diffuse through a membrane with nominal M_r exclusion limit of 10000.

Using the synthetic substrates Z-Arg-Arg-NHMec, a specific substrate for cathepsin B (EC 3.4.22.1), and Z-Phe-Arg-NHMec, which is cleaved by both cathepsin B and cathepsin L (EC 3.4.22.15), it was observed that 50 mM-$CaCl_2$ inhibited about 50% of the lysosomal activity towards the first substrate, but exerted no effect on the activity towards the second substrate (results not shown). Therefore the stimulatory effect of $CaCl_2$ on the collagenolytic activity of the lysosomal cysteine proteinases is probably due to an effect on collagen and not on the proteinases.

Knowing that mouse bone collagenase retains significant activity at acid pH (Vaes, 1972), the influence of 10–50 mM-$CaCl_2$ on the degradation of insoluble bone collagen by mouse bone collagenase was examined at pH 5.3. Fig. 2 shows that the amount of solubilized collagen increased in direct proportion to Ca^{2+} concentration, and up to 10-fold stimulation was observed with 50 mM-$CaCl_2$. Interestingly, by contrast with its effect on lysosomal collagenolytic activity, NaCl at equivalent ionic strengths did not stimulate the degradation of bone collagen by collagenase.

Co-operation *in vitro* of collagenase and lysosomal proteinases in the degradation of bone collagen

Procollagenase seems to be associated with the mineralized bone matrix (Eeckhout *et al.*, 1986; Delaissé *et al.*, 1988) and has recently been immunolocalized in the subosteoclastic bone-resorbing compartment (Baron *et al.*, 1990). Collagenase could thus participate, together with lysosomal cysteine proteinases, in the subosteoclastic degradation of bone collagen. As shown in Table 1, the degradation of bone collagen by a mixture of collagenase and purified lysosomal extract at pH 5.5

Table 1. Co-operation *in vitro* of collagenase and lysosomal proteinases in the degradation of insoluble bone collagen

Incubation was carried out as described in the legend to Fig. 2, but at pH 5.5 in the presence of 50 mM-$CaCl_2$ and 5 mM-dithiothreitol. Enzyme preparations were 75 μl of trypsin-activated conditioned culture medium (approx. 1.5 units of collagenase) or, in the controls, trypsin-activated unconditioned medium; and 25 μl of the soluble content (20 μg of protein) of purified rat liver lysosomes or, in the controls, water. Values are means ± S.D. ($n = 4$).

Enzyme preparation	Collagen degraded (μg)
(*a*) Solvent of (*d*)	37 ± 5
(*b*) Bone collagenase + solvent of (*c*)	115 ± 14
(*c*) Lysosomal extract + solvent of (*b*)	120 ± 14
(*d*) Bone collagenase and lysosomal extract	219 ± 15

corresponded approximately to the sum of the degradations caused by the separate enzyme preparations. These additive effects of collagenase and lysosomal cysteine proteinases, the possible activation of procollagenase by cathepsin B (Eeckhout & Vaes, 1977) and recent microscopic studies of the effects of inhibitors of collagenase or cysteine proteinases (Everts *et al.*, 1990), support the hypothesis that both types of proteinase co-operate in the subosteoclastic degradation of bone collagen.

Mechanism of action of Ca^{2+} on collagen breakdown

The experiment illustrated in Fig. 3 shows that, whereas collagenase activity on collagen in solution at 25 °C was not

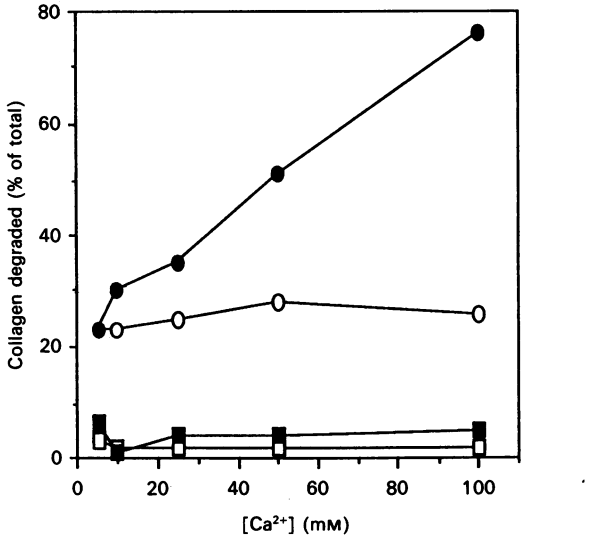

Fig. 3. Effect of Ca^{2+} concentration on the degradation of acid-soluble collagen

^{3}H-acetylated acid-soluble skin collagen (0.5 mg/ml, pH 7.5) was incubated (see the Materials and methods section) for 2 h, either in solution at 25 °C (○ and □) or as reconstituted fibrils at 35 °C (● and ■), with either bone collagenase (1 unit/ml) (○ and ●) or trypsin (12.5 μg/ml) (□ and ■), in the presence of the indicated $CaCl_2$ concn. The reaction was stopped by the addition of 25 μl of 0.6 M-EDTA, pH 7.5, supplemented with soya bean trypsin inhibitor (0.2 mg/ml) when trypsin was present. Tubes containing collagen in solution were further incubated at 35 °C for 18–24 h to allow collagen fibril formation. All of the tubes were centrifuged and the radioactivity was measured in 50 μl of supernatant; 100% degradation of collagen corresponded to 15000 c.p.m. in the supernatant.

influenced by the increase in [Ca^{2+}] from 10 to 100 mM, this increased Ca^{2+} concentration markedly stimulated the action of the same enzyme preparation on reconstituted collagen fibrils at 35 °C. The susceptibility of collagen to trypsin was, however, unaffected under these conditions. Similar results (not shown) were obtained when both incubations were performed at 30 °C, demonstrating that these high Ca^{2+} concentrations influence the susceptibility of collagen in fibrillar form, but not in solution, without inducing the unfolding of the triple-helical structure.

The precise mechanism of action of high Ca^{2+} concentrations on the susceptibility of collagen to proteolysis will only be resolved by structural studies on purified proteinases. Yet the present observations (Fig. 3) suggest that 10–100 mM-Ca^{2+} exerts its effect on the supramolecular structure of the collagen fibrils, but, as previously noticed by Seltzer *et al.* (1976), it does not act either on collagenase or on the unfolding of the individual collagen molecules.

The data presented in the present paper thus confirm the hypothesis that locally elevated concentrations of H^+ and Ca^{2+}, as well as a co-operation between lysosomal cysteine proteinases and matrix collagenase, could play important roles in the breakdown of collagen that is carried out by the osteoclast in a specialized bone-resorbing microenvironment.

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