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MEDULLARY BONE AND AVIAN CALCIUM REGULATION

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Summary

Medullary bone forms in egg-laying birds in response to gonadal steroids and is the most overtly oestrogen-dependent of all bone types. It acts as a labile reservoir for the supply of eggshell calcium. Previous studies indicate that feeding calcium- and vitamin-D-deficient diets to chickens results in resorption of cortical rather than medullary bone. More recent studies in calcium-stressed quail hens question this hypothesis and suggest that during the first 2 weeks of dietary calcium depletion the medullary bone is resorbed while cortical bone volume remains intact. The role of the osteoclast in bone resorption is the focus of much research that has recently included studies of medullary bone osteoclasts. The functional morphology of the avian cells, i.e. changes from quiescent to active osteoclasts with ruffled borders, reflects the rapid changes in medullary bone turnover that occur during the egg-laying cycle. Unlike mammalian osteoclasts, those from avian sources generally appear refractory to inhibitory factors such as calcitonin or raised extracellular calciumconcentration. However, medullary bone osteoclasts cultured *in vitro* for several days recover their ability to respond to the latter factor by increasing their levels of free cytosolic Ca^{2+}, reducing tartrate-resistant acid phosphatase secretion and reducing their cell spread area. It is suggested that factors such as ambient calcium levels and prostaglandins may form part of a system of rapid local control for medullary bone osteoclast activity.

Introduction

Birds and reptiles are distinguished from other vertebrate classes by their ability to lay eggs with calcified eggshells. It is only in birds, however, that the massive development of the endosteal cavities of the long bones provides a reservoir for the minerals required for shell calcification. This endosteal bone, known as medullary bone (Dacke, 1979, 1989), is a non-structural type of woven bone (Bonucci and Gherardi, 1975) and is normally found only in long bones of female egg-laying birds. It consists of a system of bone spicules that grow out from endosteal surfaces and may completely fill the marrow spaces (Simkiss, 1967; Dacke, 1979). First described by Foote and rediscovered by Kyes and Potter (see Dacke, 1979) in pigeons, medullary bone is formed in female birds shortly before the onset of egg laying and persists throughout the egg-laying period. Thus, it is

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more or less continuously present in domesticated species such as chickens and Japanese quail which, under appropriate lighting conditions, will lay eggs throughout the year. Members of the crocodile and alligator family, the closest living relatives of birds, do not apparently form medullary bone (Elsey and Wink, 1985).

Medullary bone formation

In female birds, medullary bone formation is stimulated by the synergistic action of androgens and oestrogens, accompanying the maturation of the ovarian follicles, while in adult male birds it can be induced artificially by administration of these hormones (see Dacke, 1979; Miller and Bowman, 1981).

In adult male Japanese quail, oestrogen induces differentiation of endosteal cells to form osteoblasts and decreases the number of osteoclasts on the endosteal surface (Kusuhara and Schraer, 1982; Ohashi *et al.* 1987). Conversely, the anti-oestrogen tamoxifen suppresses oestrogen-induced medullary bone formation and reduces bone matrix (Ohashi *et al.* 1990). Ohashi *et al.* (1987) also reported increases in osteoclast numbers in these animals following oestrogen and tamoxifen treatment and suggested that the role of oestrogen in the endosteal cellular response is to accelerate the differentiation of osteoblasts and to inhibit osteoclast activity. Oestrogen receptors have been localised in Japanese quail medullary bone osteoblasts and immature osteocyte nuclei and also in the nuclei of preosteoblasts from oestrogen-treated male quails (Ohashi *et al.* 1991), but these authors were unable to identify nuclear oestrogen receptors in osteoclasts. Oursler *et al.* (1991) reported that pure freshly isolated and cultured avian osteoclasts also possess nuclear oestrogen-binding sites and that the steroid directly inhibited osteoclastic bone resorption *in vitro.*

Structure of medullary bone

The mineral phase of medullary bone is similar to that of cortical bone and consists of a hydroxyapatite lattice (Ascenzi *et al.* 1963). In cortical bone, the hydroxyapatite crystals are oriented with respect to the organic matrix (Neuman and Neuman, 1958), while medullary bone has apatite crystals randomly distributed throughout the matrix. Medullary bone is more heavily calcified than cortical bone, although its collagen fibril content is much lower, while the apatite to collagen ratio is higher (Taylor *et al.* 1971). Non-collagenous proteins, proteoglycans and carbohydrates are also more abundant in medullary bone. The proteoglycan component of medullary bone matrix has been investigated with emphasis on its time of appearance after bone induction by oestrogen and on the nature of its glycosaminoglycan side chains (Schraer and Hunter, 1985). The glycosaminoglycan component in chicken and quail medullary bone was identified as keratin sulphate (Candlish and Holt, 1971; Fisher and Schraer, 1982). Proteoglycans bind calcium, and Stagni *et al.* (1980) extracted a proteoglycan from medullary bone with high calcium affinity and phosphatase activity which hydrolysed ATP, GTP and pyrophosphate. The precise role of proteoglycans in the calcification process is

undefined, but Blumenthal (1989) proposed that their primary role is to inhibit hydroxyapatite formation.

Medullary bone and the egg-laying cycle

The eggshell cycle of the pigeon is accompanied by intense osteoclastic activity resulting in total removal of medullary bone, while osteoblastic activity, resulting in a high degree of medullary bone formation, predominates when no egg is calcifying (Bloom *et al.* 1958). However, van de Velde *et al.* (1984) observed that, in chickens, when bone resorption was low, bone formation was also low and when bone resorption was high, bone formation was also high. In both phases, numbers of nuclei per osteoclast and numbers of osteoclasts were similar. They also reported that, during the active period, the resorbing surface, i.e. the ruffled border, per active osteoclast increased and, although the total number of osteoclasts remained constant, the percentage of active osteoclasts was increased. This agrees with results from Holtrop and King (1977) and Miller (1981). van de Velde *et al.* (1984) presumed that matrix formation and osteoclastic resorption were in phase such that, during eggshell formation, fully calcified medullary bone is replaced by an organic matrix low in calcium, after which the organic matrix is recalcified during the subsequent inactive period. Hence, the total medullary bone volume does not change during the egg-laying cycle, only the degree of calcification. Kusuhara (1976) also reported that, during the inactive period, medullary bone contains more calcium than during the active period. Hence, the mineralization rate appears to be high when the rate of osteoclastic resorption is low and *vice versa*. Since osteoblastic and osteoclastic activities are so well balanced in medullary bone, a very efficient coupling mechanism must exist. The differences between pigeons and chickens may be attributed to the fact that pigeons only lay two eggs in a clutch over a 44h period, while the chicken lays anything from 2 to 20 or more eggs in a sequence at intervals of 24h. Skeletal metabolism in the chicken is, therefore, even more intense than in the pigeon. This high rate of bone turnover would also apply to the Japanese quail, which has a similar egg-laying cycle to the chicken (Taylor and Dacke, 1984).

Function of medullary bone

More than four decades ago, it was proposed that medullary bone acts as a labile calcium store which can be used to form the eggshell. Medullary bone has a large surface area, is well vascularized, is better mineralised and can be metabolised at a rate at least 10–15 times faster than cortical bone (Hurwitz, 1965; Simkiss, 1967). The high number of osteoclasts in avian medullary bone compared with cortical bone emphasises the fact that avian medullary bone represents a very active bone remodelling system (van de Velde *et al.* 1984).

Tracer experiments indicate that osteoclastic resorption of chicken medullary bone deposits supplies as much as 40% of the calcium for eggshell formation (Mueller *et al.* 1964, 1969). However, the concept that medullary bone is a reservoir from which calcium is withdrawn in times of need does not always hold. Taylor and Moore (1954) observed

that medullary bone osteoclast activity and the quantity of medullary bone in egg-laying hens maintained for 7 days on a calcium-deficient diet were relatively unaffected, but that cortical bone was depleted while showing clear signs of osteoclastic activity. The major histological change in medullary bone of calcium-deficient hens was a considerable increase in osteoblast numbers (Zambonin-Zallone and Mueller, 1969), while the osteoclasts were found to be substituted in the trabecular surface by active osteoblasts. The osteoclasts withdraw from the medullary bone surface, appearing to have temporarily exhausted their function (Zambonin-Zallone and Teti, 1981). The percentage of medullary bone in the skeleton actually increases during egg-laying on a calcium-deficient diet because the rest of the skeleton is depleted in order to maintain it. To explain this, Simkiss (1967) proposed that medullary and cortical bone are calcium reservoirs which differ in their lability or ease of calcium mobilisation. On normal-calcium diets, and possibly also on the first day of depletion when there is increased osteoclastic activity in medullary bone, medullary reserves are adequate to buffer the cyclic changes in calcium demand caused by egg formation. During prolonged calcium deficiency, the hen responds by increasing the size of the medullary reservoir at the expense of the less labile cortical bone.

We recently re-examined this question by feeding Japanese quail hens diets deficient in either calcium or calcium and vitamin D_3 for up to 23 days. The total quantity of medullary *versus* cortical bone area in decalcified tibial sections was quantified by image analysis. Surprisingly, after 16 days on these diets, the apparent volume of medullary bone was reduced by more than 50% in the deficient birds compared with controls, while the quantity of cortical bone remained more or less constant. However, after 23 days, the volume of medullary bone had begun to recover, with some accompanying loss of cortical bone (Fig. 1). Whether this result reflects a difference in response according to the species of bird used or whether it is simply a matter of timing remains to be seen. However, if medullary bone is either resistant to, or able to recover from, a severe and continuing calcium deficiency, this presents an intriguing series of questions as to the nature of the mechanisms involved in this remarkable response.

The hormonal stimulus for medullary bone mobilisation is probably increased parathyroid hormone (PTH) secretion stimulated by hypocalcaemia during shell formation (Taylor and Belanger, 1969). Dacke (1976) demonstrated that PTH injection into quail hens within 4h of ovulation resulted in eggshell thinning but also enhanced uptake of a chronic $^{45}Ca^{2+}$ label from bone to eggshell. The hormone had no such effect if injected later in the cycle at a stage when endogenous PTH secretion would be high. Taylor (1970) proposed that differences exist in sensitivities of cortical and medullary bone to PTH and also in the circulating concentrations of PTH on high- and low-calcium diets. He suggested that normally there are only small fluctuations in circulating PTH levels and that these mainly stimulate medullary bone resorption but, under severe calcium deficiency, they are greatly increased (de Bernard *et al.* 1980), producing substantial responses in cortical bone with only slight further effects in medullary bone. This was demonstrated practically by Bannister and Candlish (1973), who measured the collagenolytic response to PTH of medullary and cortical bone in laying hens. Although medullary bone was not destroyed by placing a chicken on a calcium-deficient diet, its

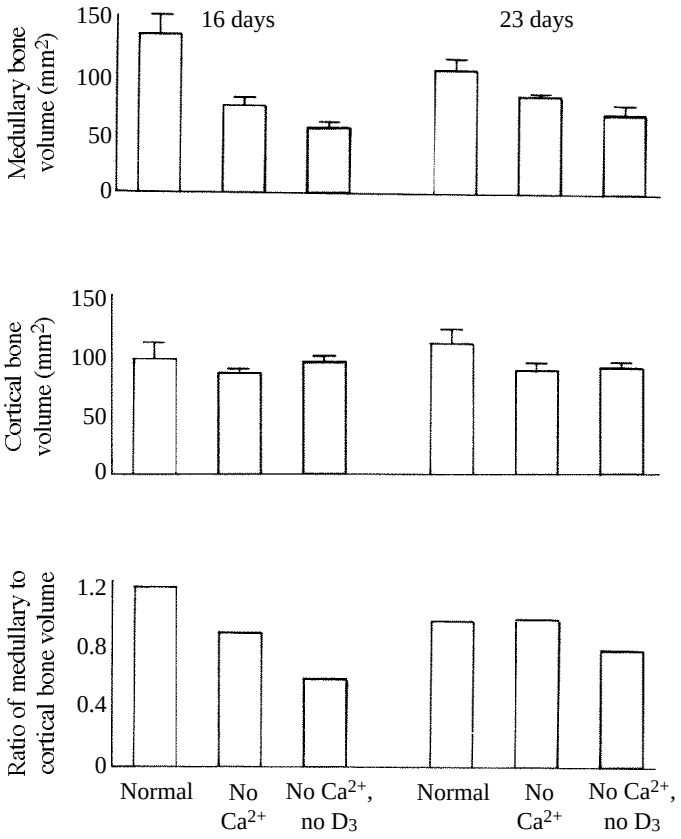

Fig. 1. Effect of feeding diets deficient in either calcium or calcium and vitamin D_3 on medullary and cortical bone volume in egg-laying Japanese quail hens. Cross-sectional bone volumes in decalcified tibiae were estimated and averaged by image analysis. Data are means + S.E.M. for groups of four hens per treatment.

composition changed more than that of any other bone. The magnesium, sodium, potassium and phosphorus contents of bone ash were increased, while those of calcium, CO_2 and citrate decreased (Simkiss, 1967). Hence, the medullary bone trabeculae were constituted of poorly calcified osteoid matrix. These changes suggest that, although medullary bone appears to maintain its substance, it is a most labile type of bone and that medullary bone osteoclasts are highly active and responsive to changes in extracellular fluid.

In vitro **studies**

There have been relatively few attempts to study medullary bone in organ culture. In recent years Harrison and Clark (1986) succeeded in culturing medullary bone harvested from egg-laying hens. They demonstrated the viability of these cultures by the normal histological and histochemical characterisations and by demonstrating inhibition of [^{3}H]proline uptake by 1,25 dihydroxyvitamin D_3 after 72h in culture.

Medullary bone osteoclasts

Osteoclasts in general possess several unique ultrastructural characteristics. They are extremely large cells containing up to 100 nuclei (Mundy and Roodman, 1987), abundant mitochondria, lysosomes, free ribosomes and extensive Golgi complexes (Vaes, 1988). The most outstanding feature, which is one of a few specific markers for osteoclast identification, is the ruffled border (Miller, 1977), a complex system of plasmalemmal infoldings extending deep within the cell's cytoplasm and entirely surrounded by an annular (clear or sealing) zone, rich in contractile actin-like filaments (King and Holtrop, 1975) and in vinculin (Teti *et al.* 1991*a*). This region corresponds to the adhesive structures, podosomes, which are short microfilament-containing protrusions that act as cellular adhesive feet (Marchisio *et al.* 1984). The clear zone and ruffled border are considered to constitute the resorptive apparatus of the cell. Miller (1977), using egg-laying Japanese quails at different points in the egg-laying cycle, observed clear zones and ruffled borders in actively resorbing medullary bone osteoclasts, while inactive osteoclasts, although still adhering to the bone surface, were not polarised and failed to display specialised membrane areas. Osteoclasts are highly motile and are thought to exist in alternating states of motility and immotility corresponding to the stages of migration and resorption respectively (Teti *et al.* 1991*a*).

Osteoclasts are activated by contact with exposed mineralised bone. They closely adhere to the mineralised matrix *via* the clear zone with the aid of podosomes and are believed to recognise and bind to bone surfaces *via* cellular adhesion molecules, integrins, present on the podosomes (Horton and Davies, 1989; Teti *et al.* 1991*a*; Chambers and Hall, 1991). Immunofluorescence studies demonstrated that vinculin, F-actin and α-actinin are localised as a ring of dot-like structures in chick osteoclasts, such that their intracellular organisation parallels the circumferential structure of the sealing zone (Marchisio *et al.* 1984). Several reports indicate that osteoclastic podosomes are localised at the sealing zone in both avian and human osteoclasts (Marchisio *et al.* 1984; Teti *et al.* 1989*a*). Podosomes may also be present in the clear zone of resorbing cells *in vivo* (Zambonin-Zallone *et al.* 1988). The organisation of these adhesion sites *in situ* appears to be similar to that *in vitro*. Zambonin-Zallone *et al.* (1988) also reported the presence of small indentations on the bone surface (*in vivo* and *in vitro*) coinciding with podosomes on the osteoclasts. This suggests the possibility that podosomes may be capable of excavating small depressions on the bone surface. Marchisio *et al.* (1984) showed that each podosome in an avian osteoclast appears as a ventral, discrete, foot-like conical structure, consisting of a short membrane protrusion, often cup-shaped. Each podosome presents a core of microfilaments associated with α-actinin, fimbrin and gelsolin linked through vinculin and talin to the integrin receptor. It is proposed that this receptor ultimately recognises and binds to components of the extracellular matrix (Teti *et al.* 1991*a*). Podosomes are able to assemble and disassemble within a few minutes (see Teti *et al.* 1991*a*). Their lack of stability could provide an important point at which to regulate osteoclastic bone resorption and, when antibodies are raised against integrins associated with osteoclastic podosomes, inhibition of bone resorption results (Davies *et al.* 1989).

Osteoclast attachment to bone, and its subsequent cytoplasmic spreading and formation of ruffled borders, gives rise to the resorptive hemivacuole, a sealing zone, between the bone-opposed surface of the osteoclast and the bone substratum. This provides a microenvironment favourable to the local concentration of the resorbing agents secreted by the osteoclast (Vaes, 1988). Bone resorption depends on acidification of the resorbing microenvironment. Osteoclasts transport protons, made available through the action of carbonic anhydrase (Gay *et al.* 1984), into the resorbing site by a polarised vacuolar proton pump (Blair *et al.* 1989), thus creating an acidic microenvironment in the resorptive hemivacuole (Baron *et al.* 1985). Using pH microelectrodes, Silver *et al.* (1988) showed the pH to be as low as 3.0 in the attachment zone between the cell and the base of the culture dish. This allows for dissolution of the bone mineral as well as providing an optimal pH for lysosomal acid hydrolases, e.g. cysteine proteinases and acid phosphatases, that are secreted by the osteoclast. Cysteine proteinases degrade collagen (Vaes, 1988), while acid phosphatase is believed to degrade pyrophosphate, which is a natural inhibitor of bone dissolution and formation (Russell and Fleisch, 1970). Removal of the inorganic and organic constituents of bone results in excavations known as resorption pits (lacunae) on the bone surface (Chambers *et al.* 1984; Ali *et al.* 1984), following which the osteoclast moves away and resorbs at another site, thus repeating the cycle.

Therefore, bone resorption is the result of individually controlled but successive steps; substratum recognition and attachment *via* podosomes, cell spreading, ruffled border formation, secretion of protons and enzymes (resulting in removal of inorganic and organic components), termination of resorption and, finally, migration to another site in order to repeat the cycle. Interference with any of these processes could alter the resorption capability of the osteoclast.

Reorganisation of the cytoskeleton plays an important role in cell shape changes and, since podosomes contain cytoskeletal proteins, they would also be affected. Teti *et al.* (1991*a*) suggest that the cytoskeleton is controlled by cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and intracellular pH levels, and that these variables are sensitive to extracellular messages and might act as mechanisms of signal transduction. Teti *et al.* (1989*a*) reported that metabolic acidosis stimulated cultured avian osteoclasts to polarise and to form podosome-rich clear zones, and that this increased bone resorption, while alkalisation induced an inhibition of bone particle resorption associated with osteoclasts devoid of clear zones and lacking podosomes. This suggests that local acidic conditions may stimulate osteoclast adhesion to bone surfaces. This is substantiated by reports that rat (Arnett and Dempster, 1986) and, to a lesser extent, chick (Arnett and Dempster, 1987) osteoclastic resorption was affected by the pH of the culture medium. The modifications in osteoclastic function observed in response to altered intracellular pH may be mediated *via* changes in $[Ca^{2+}]$. Teti *et al.* (1989*a*) observed that changes in $[Ca^{2+}]_i$ are coupled to changes in intracellular pH, so that cell acidification leads to a reduction in $[Ca^{2+}]_i$.

The process of bone resorption results in dissolution of the hydroxyapatite matrix, thus generating calcium ions as well as inorganic phosphate (P_i) and, ultimately, organic components of the matrix. Silver *et al.* (1988), using microelectrodes, showed that osteoclasts become exposed in their acidic microcompartment to high concentrations of

Ca^{2+} that can rise to 40mmoll^{-1}. There have been several investigations of the fate of the calcium solubilized from bone mineral and its role in regulating osteoclastic bone resorption. Treatments that increase $[Ca^{2+}]_i$ (increased extracellular $[Ca^{2+}]$, opening of Ca^{2+} channels, Ca^{2+} ionophores and Ca^{2+}-ATPase inhibitors) disrupt the microfilamentous core of podosomes and convert avian osteoclasts into cells devoid of the clear zone and lacking podosomes and with a reduced capacity for bone resorption (Miyauchi *et al.* 1990; Malgaroli *et al.* 1989; Teti *et al.* 1989*a*).

One of the distinguishing features of osteoclasts is their ability to form resorption pits when settled onto slices of devitalised substratum such as bovine cortical bone or whale dentine (Chambers *et al.* 1984; Ali *et al.* 1984). Indeed, Chambers (1991) considers pit formation to be a key identification feature for osteoclasts. Oreffo *et al.* (1990) have reported that osteoclasts harvested from medullary bones of egg-laying chickens are able to form resorption pits *in vitro*, although we are not aware of any photographic evidence for this claim and the authors did not report whether the pits formed by avian cells have similar topographical characteristics to those produced by neonatal rat cells. We recently carried out studies of isolated quail medullary bone osteoclasts settled onto slices of devitalised bovine cortical bone (Bascal and Dacke, 1992). Fig. 2A,B shows scanning electron micrographs of these cells. The cell in Fig. 2A, which appears to be in a relatively retracted condition with typical spicules appearing from the edge, could represent a closely adhering actively resorbing cell, while that in Fig. 2B appears to be well spread with pseudopodia reaching out over the bone surface, this being typical of a motile cell that may have completed its resorption cycle.

Fig. 3 shows a typical resorption pit formed by medullary bone osteoclasts settled onto bovine bone slices for periods of up to 48h. These pits are usually very limited in number compared with those that we can obtain from cultured neonatal rat osteoclasts. The pits that are formed often have irregular shapes (Fig. 3) and we frequently observe shallow depressions suggestive of incomplete pit formation.

Motility and cell spread area

Alterations in osteoclast motility and spread area are associated with changes in osteoclastic bone resorption. Calcitonin (CT), which inhibits bone resorption in mammalian species, is reported to induce cellular contraction and inhibit migration of rat osteoclasts (see below). This peptide may also reduce the spread area of chick osteoclasts in organ culture (Pandalai and Gay, 1990). Conversely, pro-absorptive stimuli, such as parathyroid hormone (PTH), vitamin D_3 metabolites and prostaglandin E_2 (PGE_2), increase osteoclast ruffled borders, clear zones and cell size in mammalian and avian species (see below).

Cytoplasmic spreading is related to functional status in the osteoclast. In the rat

Fig. 2. Scanning electron micrographs of a quail medullary bone osteoclast-like cells settled onto devitalised bovine cortical bone slices. (A) The cell is tightly compacted with typical spicule formation at the edges; it may represent an actively resorbing cell. Scale bar, 10 μm. (B) This cell appears to be well spread and may represent a motile cell at the end of its resorbing cycle migrating to a new location on the bone surface. Scale bar, 5 μm.

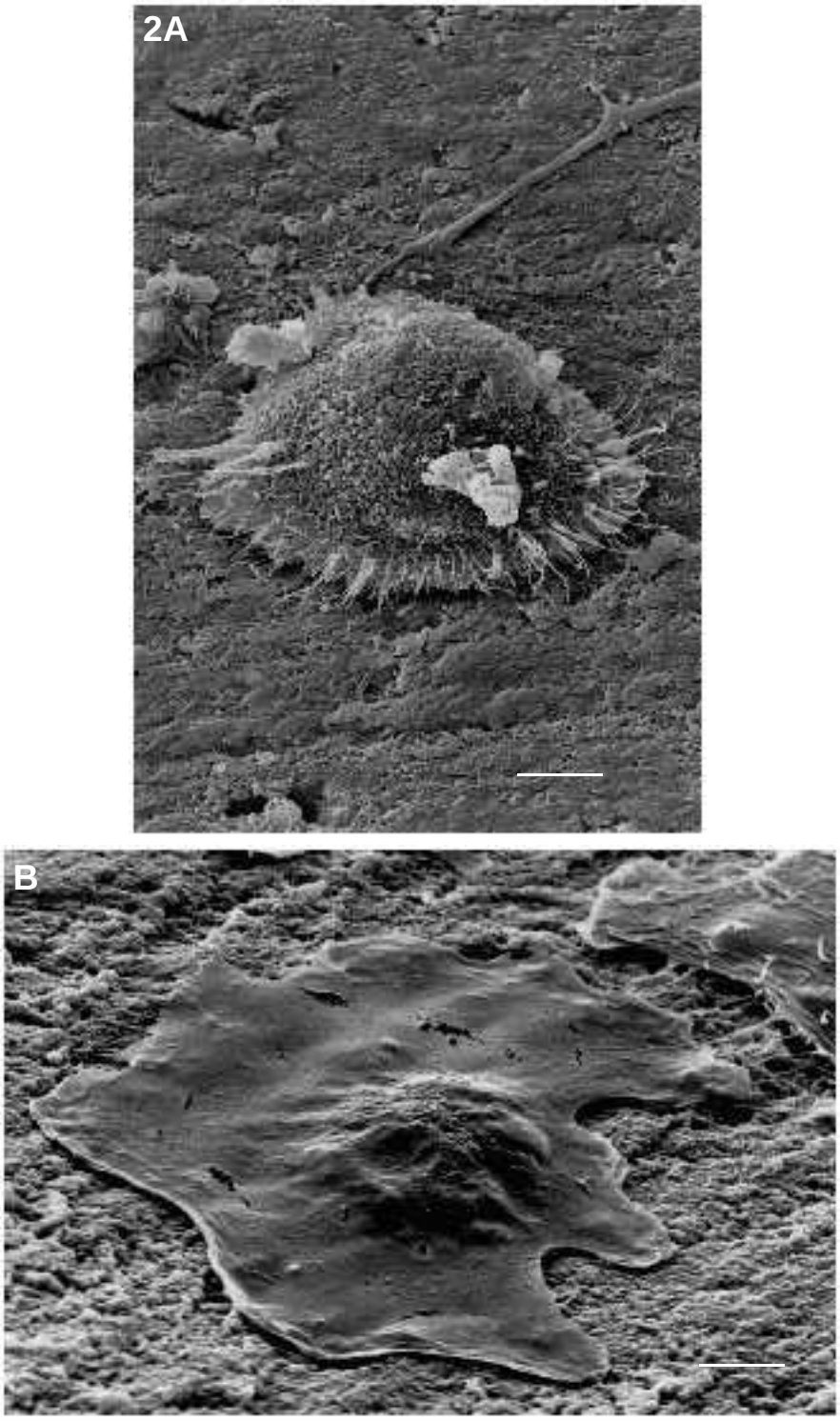

Fig. 2

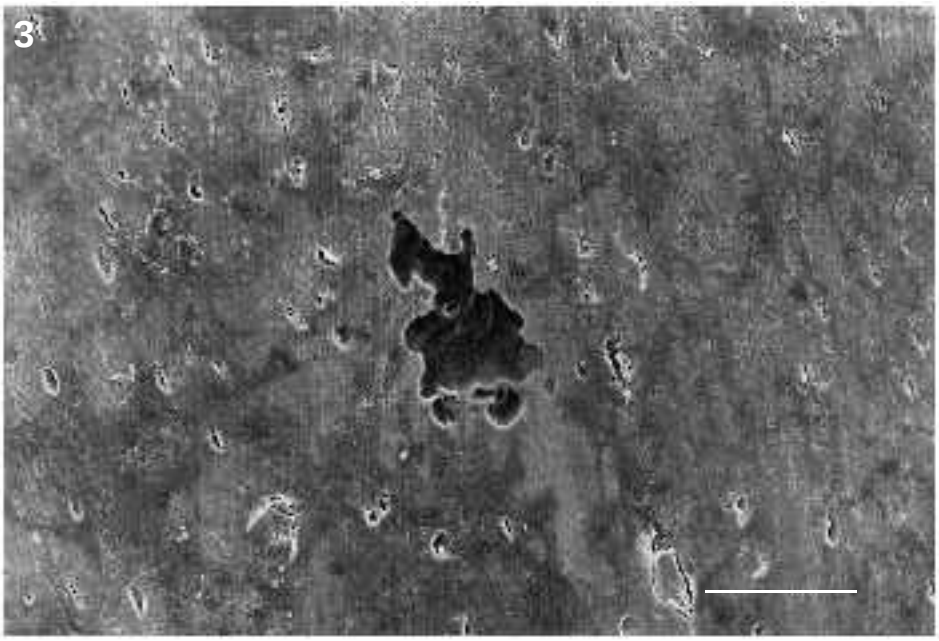

Fig. 3. Scanning electron micrograph of a typical resorption pit formed by a quail medullary bone osteoclast settled for 48h onto a devitalised bovine cortical bone slice. This pit is relatively large and has a more irregular shape than the equivalent pit formed by a mammalian osteoclast. Scale bar, 0.1mm.

osteoclast, inhibition of bone resorption, e.g. by CT, is associated with loss of cytoplasmic folds from the osteoclast surface followed by detachment of the osteoclast from the bone surface (Holtrop *et al.* 1974; Pandalai and Gay, 1990), a sequence likely to cause a reduction in osteoclast surface area. The effects of CT on osteoclast cell spreading are also observed in pure osteoclast cultures (Chambers and Magnus, 1982). Holtrop *et al.* (1974), using rat foetal long bones in organ culture, reported that stimulators of bone resorption, e.g. PTH, 1,25-dihydroxyvitamin D_3 and PGE_2, also altered the bone cells morphologically. They increased osteoclastic ruffled borders, clear zones and cell size. Pandalai and Gay (1990), using chick tibia organ culture, also observed an increase in osteoclast cell spreading in response to PTH. These effects were faster in onset (2–4min) than in rat bones, suggesting differences in sensitivity to PTH between avian and mammalian osteoclasts in organ culture.

Not all agents that inhibit motility reduce mammalian osteoclast cell spread area and *vice versa*, e.g. elevation of extracellular $[Ca^{2+}]$ drastically reduces cell spreading with no apparent effect on cell motility (Datta *et al.* 1989), while CT gene products, amylin and calcitonin gene-related peptide (CGRP) reduce osteoclastic motility but not cell spread area in mammalian osteoclasts (Zaidi *et al.* 1990*b*).

There is mounting evidence that cyclic AMP is the second messenger inhibiting cytoplasmic motility, while $[Ca^{2+}]_i$ levels mediate cellular contraction. Thus, theophylline, which inhibits cyclic AMP degradation, potentiates CT-induced osteoclast

quiescence, while imidazole, which increases cyclic AMP degradation, reduces osteoclast sensitivity to CT (Chambers and Ali, 1983). Agents increasing $[Ca^{2+}]_i$, e.g. ionomycin, CT, elevated extracellular calcium ($[Ca^{2+}]_e$) and perchlorate, are known to inhibit cytoplasmic spreading (Zaidi *et al.* 1990*b*).

We recently reported that osteoclasts freshly derived from medullary bone of Japanese quail hens, unlike those from neonatal rats, do not respond to increases in $[Ca^{2+}]_e$ by reducing their cell spread area, although they do show modest responses to ionomycin (Bascal *et al.* 1992*b*). However, when quail cells are cultured for several days, they apparently regain an ability to respond to $[Ca^{2+}]_e$ with a similar sensitivity to the rat cells, suggesting a down-regulation of the freshly isolated avian cells (Bascal *et al.* 1993).

Tartrate-resistant acid phosphatase

Acid phosphatase has been localised in osteoclasts using light and electron microscopy (see Doty, 1991). Kelly *et al.* (1991) found acid phosphatase within extracellular channels of osteoclast ruffled borders, in the bone-apposed cytoplasm and extracellularly on the bone surface, suggesting secretion of the enzyme. There are at least six different acid phosphatase isoenzymes; the most basic is distinguished from the others because it is not inhibited by D-tartrate (Lam and Yam, 1977) and it is referred to as tartrate-resistant acid phosphatase (TRAP). TRAP has been located biochemically and histochemically in bone (Vaes and Jacques, 1965; Wergedal, 1970). Minkin (1982) suggested that TRAP could be used as a relatively specific marker for osteoclasts, although the enzyme also exists in other differentiated cells of the monohistocytic series (Efstratidies and Moss, 1985). However, the enzyme remains a useful marker, especially for osteoclasts on the bone surface. Tartrate-resistant ATPase has been localised to the ruffled border region of osteoclasts and to the bone surfaces facing the ruffled border zone in rat (Reinholt *et al.* 1990) and chicken bone (Fukushima *et al.* 1991*a*).

A role for acid phosphatases in bone resorption has been suspected since the discovery of increased levels of enzyme activity in plasma during bone remodelling (Lau *et al.* 1987). Additionally, Taylor *et al.* (1965) demonstrated cyclical changes in the activities of acid and alkaline phosphatases during eggshell calcification in the domestic fowl. *In vitro* bone cultures have provided evidence for the potential role of calciotropic hormones in TRAP release and a correlation between bone resorption and enzyme secretion. Minkin (1982) reported that stimulation of bone resorption with PTH or 1,25-dihydroxyvitamin D_3 increased both TRAP and tartrate-sensitive acid phosphatase (TSAP) activity in the medium. He suggested that TRAP was released from osteoclasts, while TSAP was released from other bone cells as well as from osteoclasts.

Miller (1985), using egg-laying Japanese quail, provided *in vivo* evidence that activated medullary bone osteoclasts secrete acid phosphatase only 3h after oviposition, i.e. before commencement of eggshell calcification and intensive bone resorption. The osteoclasts lacked ruffled borders but stained intracellularly for acid phosphatase. There was no evidence of extracellular acid phosphatase activity associated with these cells; however, 20min after PTH administration, the enzyme was present in the matrix and extracellular space adjacent to the ruffled border of active osteoclasts associated with bone resorption.

Fig. 4. Cultured quail medullary bone osteoclasts forming a confluent monolayer stained specifically for tartrate-resistant acid phosphatase (TRAP). Areas of enzyme concentration stain a purple-brown colour. Scale bar, 0.2mm.

Until recently, it was difficult to demonstrate directly that osteoclasts secrete TRAP. A major impediment to the investigation has been the absence of an experimental system whereby osteoclasts can be isolated from bone and incubated in culture. Recently, Chambers *et al.* (1987) developed simple techniques to investigate TRAP release from mammalian osteoclasts. They observed that osteoclasts incubated on a plastic substratum released a considerable amount of the enzyme into the culture supernatant and that CT inhibited TRAP secretion. They also reported that prostaglandin E_1 (PGE_1) transiently inhibited TRAP activity, while PTH and 1,25-dihydroxyvitamin D_3 had no influence in pure osteoclast cultures. Braidman *et al.* (1986) reported that PTH raised TRAP activity in osteoclast-like cells only when they were co-cultured with osteoblast-like cells, suggesting that the effects of PTH on osteoclasts are mediated *via* osteoblasts. Using isolated chicken medullary bone osteoclast cultures, Oreffo *et al.* (1988) observed increases in TRAP activity following retinoic acid or retinol treatment; retinol also inhibited bone resorption.

$[Ca^{2+}]_i$ is also believed to play a second-messenger role in regulating TRAP secretion. However, unlike the situation in most cells, where an increase in $[Ca^{2+}]_i$ leads to an increase in exocytosis, the situation is reversed in osteoclasts. The calcium ionophore ionomycin and elevated $[Ca^{2+}]_e$ both increase $[Ca^{2+}]_i$ and inhibit TRAP secretion in isolated neonatal rat osteoclasts (Datta *et al.* 1989; Moonga *et al.* 1990).

We recently investigated the effects of a variety of agents on TRAP secretion in medullary bone osteoclast monolayers from Japanese quail, cultured for a week (Z. A. Bascal, D. J. Cook and C. G. Dacke, unpublished data). The osteoclasts stained specifically for TRAP (Fig. 4) and also secreted significant amounts of the enzyme. Raised $[Ca^{2+}]_e$ significantly decreased TRAP secretion over a period of 24h, while ionomycin had a similar effect although, at low doses of the ionophore, a small stimulatory effect was seen. As in rodent osteoclasts, levels of TRAP secretion were also reduced by PGE_2 and the phosphodiesterase inhibitor 1-isobutyl 3-methylxanthine, the latter implicating a role for cyclic AMP in the release of TRAP by these cells. The enzyme levels were also reduced by raised ambient $[P_i]$. This latter response may be physiologically as important as that to ambient $[Ca^{2+}]$, since local P_i levels are likely to be markedly raised in the vicinity of the resorbing bone surface. CT and CGRP, however, failed to influence TRAP production by these cells.

Controlling factors

Calcium

Levels of both plasma total and ionised calcium are known to fall as eggshell secretion proceeds, indicating a drain on calcium reserves which is presumably not fully compensated for by increased bone mineral mobilisation (Dacke *et al.* 1973). The mechanism by which mobilised calcium reaches the bloodstream is unknown. It could be

actively transported through the osteoclast cytoplasm, diffuse through a paracellular route between bone surface and osteoclast membrane or diffuse into extracellular fluid upon the migration of the osteoclast from part or all of the resorption site as a result of an increase in $[Ca^{2+}]$ in the microevironment (Bronner and Stein, 1992). There is indirect evidence for the existence of a Na^{+}/Ca^{2+} exchanger in the basolateral membrane of osteoclasts (Baron *et al.* 1986), which could be responsible for the extrusion of calcium ions entering the cell passively at the apical ruffled border membrane. The Ca^{2+}-ATPase in chick osteoclast basolateral membranes (Akisaka *et al.* 1988) may also be involved in the transcellular movement and extrusion of calcium ions from these cells.

The effects of elevated $[Ca^{2+}]_e$ on the morphology and function of osteoclasts has been examined by a number of laboratories. All suggest that high $[Ca^{2+}]_e$, comparable to that found in the resorbing microenvironment, can inhibit mammalian and avian osteoclastic bone resorption (Malgaroli *et al.* 1989; Zaidi *et al.* 1989; Miyauchi *et al.* 1990). Datta *et al.* (1989), using freshly isolated rat osteoclasts, observed that increased $[Ca^{2+}]_e$ dramatically reduced cell size, accompanied by a marked reduction in TRAP release and inhibition of bone resorption, while Miyauchi *et al.* (1990) showed that the expression of podosomes was significantly decreased in avian osteoclasts cultured on glass surfaces when they were exposed to a high $[Ca^{2+}]_e$ (Fig. 5). This potentially represents a physiologically important negative feedback mechanism for osteoclast control, in which extracellular osteoclastic activity would generate calcium levels locally capable of limiting further bone resorption. Thus, contraction of the osteoclast would expose the pit to extracellular fluid and lead to

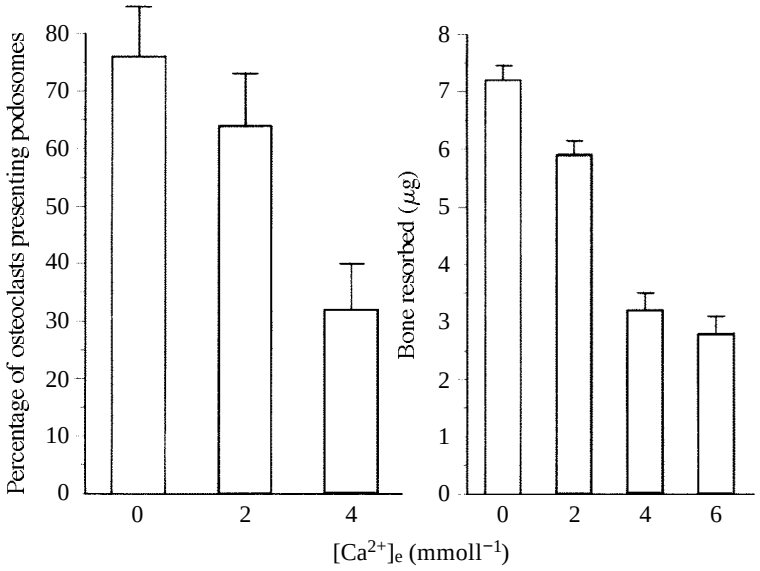

Fig. 5. Effects of raised extracellular calcium concentration on podosome expression (left-hand histogram) and resorption of radiolabelled bone particles (right-hand histogram) by cultured chicken medullary bone osteoclasts. Redrawn from Miyauchi *et al.* (1990). Values are mean + S.E.M. (N=3).

the diffusion of calcium from the microenvironment. The reduced calcium levels would allow the osteoclast to commence resorbing or to migrate to a new site.

The effects of $[Ca^{2+}]_e$ on cell spread area, enzyme release and ultimately bone resorption are believed to be mediated by raised $[Ca^{2+}]_i$. Elevated $[Ca^{2+}]_e$ is associated with increased $[Ca^{2+}]_i$ (Malgaroli *et al.* 1989; Zaidi *et al.* 1989) and treatments which increase $[Ca^{2+}]_i$ decrease TRAP activity, reduce cell spread area and inhibit bone resorption (Moonga *et al.* 1990; Zaidi *et al.* 1989). Teti *et al.* (1991*a*) have hypothesised that the increase in $[Ca^{2+}]_i$ activates gelsolin which, in turn, depolymerises actin filaments, causing rearrangement of adhesion proteins located in the podosomes and detachment of the osteoclast from the bone.

The mechanism of $[Ca^{2+}]_e$-activated $[Ca^{2+}]_i$ elevation is unclear. It may be due to Ca^{2+} influx into the osteoclast *via* a special type of receptor-operated (or Ca^{2+}-activated) Ca^{2+} channel or to intracellular redistribution of Ca^{2+} consequent on the activation of a low-affinity Ca^{2+}-binding protein or receptor. Alternatively, a greater proportion of Ca^{2+} channels might be open in the actively resorbing osteoclast (Zambonin-Zallone and Teti, 1991). Zambonin-Zallone and colleagues have characterised Ca^{2+} channels in isolated avian and mammalian osteoclasts and their role in osteoclastic regulation. They showed that osteoclasts possessed both Ca^{2+}-operated (CaOCC) (Malgaroli *et al.* 1989) and dihydropyridine-sensitive voltage-dependent Ca^{2+} channels (VDCC) (Miyauchi *et al.* 1990), although VDCCs may not be involved in $[Ca^{2+}]_e$-stimulated elevation of $[Ca^{2+}]_i$ (Datta *et al.* 1989). The increase in $[Ca^{2+}]_e$, together with the opening of the CaOCCs, also stimulates Ca^{2+} release from intracellular stores, leading to a transient spike of cytosolic Ca^{2+} followed by a sustained phase at a higher than basal level (Miyauchi *et al.* 1990). CaOCCs appear to operate during bone resorption, while VDCCS are down-regulated when the cells are incubated in the presence of bone fragments.

In a recent study, we found that, unlike freshly prepared individual neonatal rat osteoclasts, those freshly obtained from quail medullary bone do not exhibit a rise in $[Ca^{2+}]_i$ in response to an increase in $[Ca^{2+}]_e$ to $20 mmol\,l^{-1}$ (Bascal *et al.* 1992*a*). However, in subsequent experiments, we found that medullary bone osteoclast monolayers cultured for 7 days were able to respond to extracellular Ca^{2+} levels ranging from 5 to $40 mmol\,l^{-1}$ with dose-dependent increases in $[Ca^{2+}]_i$ (Fig. 6) (S. A. Arkle, I. M. Wormstone, Z. A. Bascal and C. G. Dacke, unpublished data). Given this relationship and the increased expression of VDCCs in osteoclasts during tissue culture (Teti *et al.* 1989*b*), it is of interest to speculate that responsiveness to $[Ca^{2+}]_e$ may be related to VDCC expression.

It is not known how changes in $[Ca^{2+}]_e$ are transduced into an intracellular signal to give a functional response. Calcium may have a direct effect on plasmalemmal Ca^{2+} channels, it may enter the cytosol by a non-selective process or other second-messenger pathways may be involved. The osteoclast is among a number of cells able to respond to changes in $[Ca^{2+}]_e$. Research, in particular on PTH-secreting cells and to a lesser extent on osteoclasts and other cell types, is providing evidence for a divalent-cation-sensitive receptor on the cell surface. This is popularly termed the 'putative Ca^{2+} receptor'. Although the transduction process for this putative receptor in avian osteoclasts is unknown, a comparison may be drawn with PTH-secreting cells, where the receptor is

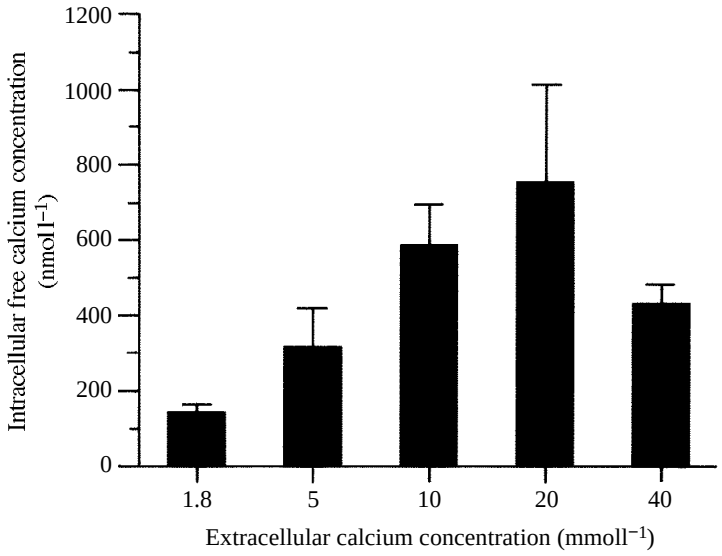

Fig. 6. Effect of raised extracellular calcium concentration on intracellular free calcium levels in medullary bone osteoclast monolayers cultured for 7 days. Intracellular calcium concentration was estimated by fluorimetry following incubation with Fura-2. Note the dose-dependent response up to 20mmol l^{-1} Ca^{2+} after which the response begins to decline. Values are mean + S.E.M. (N=3–5).

thought to be linked to phosphatidylinositol turnover and to the generation of an inositol trisphosphate (InsP_3)-dependent Ca^{2+} signal (Brown, 1991). Thus, osteoclasts may monitor changes in $[Ca^{2+}]_e$ by means of a Ca^{2+} receptor coupled, *via* G-proteins and subsequent phosphatidylinositol turnover, to the inhibition of bone resorption.

Calcitonin

Calcitonin, a 32 amino acid polypeptide hormone, is secreted from the ultimobranchial bodies in birds in response to increased plasma calcium levels (Dacke, 1979). During the ovulatory cycle of egg-laying quail, fluctuations in plasma CT levels occur, with higher values 7h after ovulation and lower levels during shell calcification, suggesting that the hormone levels are inversely correlated with total plasma calcium concentration in the egg-laying hen (Dacke *et al.* 1972). CT levels in female Japanese quail also show a transient surge before the commencement of ovulation, i.e. at the time of initial formation of medullary bone and when steroidal biogenic pathways are becoming activated. In the adult male quail, plasma levels of hormone are three times higher than in laying hens or chicks, indicating a possible regulatory role for plasma androgens (Boelkins and Kenny, 1973; Dacke *et al.* 1976).

There is convincing evidence that CT inhibits mammalian bone resorption through direct effects on osteoclasts. The hormone dramatically alters the morphology of isolated neonatal rat osteoclasts by rapidly (in minutes) inducing cessation of cell motility followed by pseudopodial retraction (Chambers and Magnus, 1982). It also inhibits rat

osteoclast-induced pit formation on bone slices (Chambers *et al.* 1985; Arnett and Dempster, 1987) and release of TRAP from osteoclast cultures (Chambers *et al.* 1987). The effects of CT appear to be mediated by rises in intracellular cyclic AMP concentration (Nicholson *et al.* 1986) and $[Ca^{2+}]_i$ (Malgaroli *et al.* 1989; Zaidi *et al.* 1990*a*,*b*).

Effects of CT on bone resorption in mammals are transient both *in vitro* and *in vivo*, with CT-inhibited PTH-stimulated bone resorption in organ culture lasting 24–48h, followed by an increase in bone resorption despite the continued presence of CT (Wener *et al.* 1972). This phenomenon, known as 'escape from inhibition', is also observed *in vivo*; it may be due to down-regulation of CT receptors on osteoclasts in the continued presence of CT (see Dacke, 1979), to the emergence of receptors unresponsive to CT or to modulation of second messengers or their effectors such as protein kinase C (Skjodt and Russell, 1991).

Submammalian species, including birds (Boelkins and Kenny, 1973), generally have extremely high circulating levels of CT compared with mammals and there is relatively little evidence that they are able to respond to exogenous doses of the hormone in terms of hypocalcaemia or enhanced bone resorption, suggesting that these functions may be down-regulated under normal physiological conditions (Dacke, 1979). Chronic CT deficiency after ultimobranchiolectomy in chickens does not alter serum $[Ca^{2+}]$ or alkaline phosphatase levels and does not affect the chemical composition of bone (Brown *et al.* 1969). However, chronic administration of CT to chickens is reported to increase bone mass, although this effect is somewhat variable and appears to be age- and dose-dependent (Belanger and Copp, 1972). These authors found that CT dosage in laying hens affected the quality and quantity of medullary bone by inhibiting bone resorption, while cortical bone showed evidence of an osteoporotic breakdown. This indicates that medullary bone is more sensitive than cortical bone to CT. Luck *et al.* (1980) reported that a large dose of salmon CT reduced plasma ionised, but not total, calcium levels by about 18% when injected into hens about 15–16h after ovulation. Immunohistochemical examination of metatarsi from chicks injected with CT indicated less carbonic anhydrase expression than in controls (Anderson *et al.* 1982). Additionally, their osteoclasts were altered morphologically, with the ruffled borders being dramatically shortened and the osteoclasts being smaller and rounder, both features indicative of cell inactivity.

In contrast to its effects on rat osteoclasts, CT failed to alter mobility or to inhibit bone resorption by freshly isolated chick osteoclasts (Arnett and Dempster, 1987) nor did it increase cyclic AMP accumulation by avian medullary or cortical bone in organ culture (Miyaura *et al.* 1981), by medullary bone in culture (Nicholson *et al.* 1986) or by freshly isolated embryonic (Nicholson *et al.* 1987) or 15-day-old chick (Ito *et al.* 1985) osteoclasts. Similarly, it failed to enhance adenylate cyclase activity from the plasma membrane of medullary bone osteoclasts (Felix *et al.* 1983). Radioiodine-labelled salmon CT (sCT) did not bind to avian osteoclasts, indicating the absence of CT receptors on these cells (Nicholson *et al.* 1987). To eliminate the possibility that the native peptide, chicken CT (cCT), is required to produce an inhibitory effect, Dempster *et al.* (1987) examined the effects of cCT on chick osteoclastic activity, where it failed to inhibit bone resorption or motility. However, CT rapidly and drastically reduced the area of chick

osteoclasts on endosteal bone surface in organ culture (Pandalai and Gay, 1990). This effect was augmented by dibutyryl cyclic AMP, suggesting a cyclic-AMP-mediated response. Rifkin *et al.* (1988) also observed that CT increases cyclic AMP accumulation in chick osteoclasts. The hormone exerted similar morphological effects on single isolated chick osteoclasts cultured for 3–5 days. The cells responded to CT within 30min by rounding up and contracting. Immunocytochemical studies showed an alteration of the cytoskeletal protein elements of these cells (Hunter *et al.* 1989). The same group also demonstrated a reduction in the intracellular acidity of chick osteoclasts cultured in the presence of CT, indicating that CT may mediate its effects by alterations in pH (Hunter *et al.* 1988). CT has also been reported to inhibit avian osteoclastic bone resorption by decreasing the amount of $^{45}Ca^{2+}$ released in culture by isolated cells (de Vernejoul *et al.* 1988).

Generally, studies indicating a positive effect of CT on avian osteoclasts involved chicks that were maintained on diets deficient in calcium or calcium and vitamin D, while those showing a lack of response to CT used osteoclasts from chicks fed normal diets. This has been confirmed in a recent report by Eliam *et al.* (1988). In calcium-deficient chickens, autoradiography revealed CT binding sites, and CT increased cyclic AMP accumulation and induced cellular retraction in isolated osteoclasts. However, osteoclasts from chickens fed normal diets showed none of these responses. Additionally, plasma Ca^{2+} and CT levels were markedly reduced in Ca^{2+}-deficient chickens. It is therefore possible that the high circulating levels of CT in normal chickens caused CT receptor down-regulation, whereas when chicks are placed on calcium-deficient diets, the fall in both Ca^{2+} and CT levels in the plasma up-regulates osteoclastic CT receptors. For this to occur, the chicks have to be hypocalcaemic (and possibly vitamin-D-deficient) for several weeks. The effect of diet also appears to regulate other parts of the CT receptor–effector system. Fukushima *et al.* (1991*b*) compared the distribution of adenylate cyclase in osteoclasts from chicks fed both normal and calcium-deficient diets. No adenylate cyclase was detected in osteoclasts from chicks on a normal diet, while abundant enzyme activity was reported in cells from chicks fed deficient diets.

Parathyroid hormone

Parathyroidectomy in birds causes hypocalcaemia, while injection of PTH into birds causes a transient rise in plasma $[Ca^{2+}]$. The effects of PTH are greater in laying hens than in cockerels. It has been suggested that this is due either to binding of calcium to yolk proteins or to the possible presence of extra PTH receptors, e.g. in medullary bone and avian oviduct (Dacke, 1979). Immature birds show a rapid and sensitive reaction to intravenous PTH, e.g. Japanese quail respond within 15–60min (Dacke and Kenny, 1973; Kenny and Dacke, 1974). This response is widely used as basis for a PTH bioassay method.

There is evidence that circulatory levels of PTH vary during the ovulatory cycle of hens (Singh *et al.* 1986). The primary role of the hormone during eggshell formation is probably to protect plasma $[Ca^{2+}]$ at the expense of bone and calcifying eggshell. As mentioned previously, medullary and cortical bone are utilised at different rates depending upon dietary calcium requirement. Taylor (1970) suggested that, under normal

circumstances, medullary bone, by virtue of its rich vasculature, large surface area and the different nature of its mineral phase, may be more sensitive to small changes in circulating PTH than is cortical bone, but that, under periods of dietary calcium deficiency, levels of endogenous hormone are greatly elevated. These would tend to have more profound effects on cortical bone than on medullary bone, which rapidly achieves its maximum response to PTH.

PTH stimulates bone resorption acutely and, depending on dose, can inhibit or stimulate bone formation, acting indirectly as a consequence of enhanced remodelling and directly as a stimulator of osteoblasts (Wong, 1984; Dacke *et al.* 1993). However, the main effect of PTH on bone is undoubtedly to increase resorption. The effects of PTH on bone appear to be a net result of indirect stimulation of osteoclastic bone resorptive activity and an accompanying inhibition of osteoblastic bone formation. In organ culture, PTH stimulates bone resorption in both mammalian (Raisz, 1977) and avian (Ramp and McNeil, 1978) species. It enhances the destruction of bone matrix that is associated with an increase in proteolytic, lysosomal and acid-producing enzymes in bone, including acid phosphatase, β-glucuronidase and carbonic anhydrase (Vaes, 1988).

PTH induces rapid morphological changes in osteoblasts and osteoclasts *in situ*. Scanning electron microscopy reveals retraction of osteoblasts after treatment with PTH from a flattened to a stellate shape (Ali *et al.* 1990; Pandalai and Gay, 1990). Dramatic changes are seen in osteoclast morphology within 10–60min of PTH exposure, both in organ culture and *in vivo* (Holtrop *et al.* 1974; Pandalai and Gay, 1990; Miller, 1978; Miller *et al.* 1984).

Jones and Boyde (1976) were the first to suggest that the initial target of PTH action in bone may not be the osteoclast. They hypothesised that the primary effect of PTH may be to cause retraction of osteoblast or lining cells, allowing the osteoclasts to develop their processes and to give ruffled borders access to bone substratum. The hypothesis is supported by a number of observations indicating that PTH does not act directly on osteoclasts, but that its stimulatory effects on bone resorption are mediated by osteoblasts (see Chambers, 1991). The second messengers reported to be involved in osteoblastic PTH responses are cyclic AMP and $[Ca^{2+}]_i$ (for a review, see Peck and Woods, 1988). Lowik *et al.* (1985) have proposed that separate PTH receptor subtypes may signal to each of these second messengers, although the receptor pathways may interact and both pathways may need to be activated for a full osteoblastic response to ensue.

Most workers report the absence of PTH receptors on osteoclasts. However, Teti *et al.* (1991*b*) have reported PTH binding to cultured medullary bone osteoclasts. PTH is generally reported to have no direct effect on second messenger generation in osteoclasts. Thus, PTH has no effect on cyclic AMP levels in rat and avian (including medullary bone) osteoclasts (Nicholson *et al.* 1986; Rifkin *et al.* 1988) and the reported elevation of cyclic AMP levels in osteoclast-rich cultures may be attributable to contaminating osteoblasts (Ito *et al.* 1985; Nicholson *et al.* 1986). Other more recent reports have indicated direct effects of PTH causing a decrease in carbonic anhydrase activity and a decrease in cellular pH in avian osteoclasts. Additionally, May *et al.* (1993) have also observed direct effects of PTH on osteoclasts, believed to be mediated by cyclic AMP.

Prostaglandins

Prostaglandins (PGs) are stongly implicated in the bone loss associated with pathological conditions such as rheumatoid arthritis and malignant osteolysis and have also been proposed as mediators of hypercalcaemia (Seyberth *et al.* 1975). Santoro *et al.* (1977), using mice injected with PGE_2 analogue, demonstrated a loss of trabecular bone and an increase in the area of bone lined by osteoclasts with a corresponding depletion of bone calcium.

PGs are also postulated to play a role in avian calcium metabolism. In laying hens, circulating PGE levels are drastically elevated 6–8h post-ovulation and remain elevated between 6 and 16h post-ovulation (i.e. during eggshell calcification) (Hertelendy, 1980). Plasma PGE levels fall after oviposition and are lower on non-laying than on laying days in hens, suggesting that PGs, in particular PGE_2, may play a physiological role in the avian calcium-mobilising process during active shell calcification (Hertelendy and Biellier, 1978). Intravenous injection of a PGE_2 analogue, 16,16-dimethyl PGE_2, into young chicks causes profound hypercalcaemia (Kirby and Dacke, 1983). Later studies suggested that this response, in common with that to PTH in chicks, could be partly explained in terms of decreased calcium clearance from the blood compartment to the skeleton (Shaw and Dacke, 1985; Dacke and Shaw, 1987). There are no reports of any PG actions on medullary bone either *in vivo* or *in vitro.*

Further evidence of a role for PGs in avian calcium metabolism stems from the hypocalcaemic responses reported in Japanese quail hens injected with indomethacin 4–6h after ovulation (Dacke and Kenny, 1982). Indomethacin inhibits prostaglandin and thromboxane synthesis. They postulated that indomethacin was acting directly on either medullary bone and/or the shell gland to limit the supply of calcium in the blood through a PG synthesis mechanism.

Chambers and colleagues examined the effect of PGs on osteoclast function. Ironically, they found that the same PGs, i.e. PGE_1, PGE_2 and PGI_2, that stimulated bone organ culture resorption strongly inhibited resorption on bone slices by isolated rabbit (Chambers *et al.* 1985) and chick osteoclasts (Arnett and Dempster, 1987; de Vernejoule *et al.* 1988). In isolated rat osteoclasts, PGs cause cessation of motility followed by cytoplasmic retraction (Chambers and Ali, 1983; Arnett and Dempster, 1987), possibly mediated *via* cyclic AMP, since their effect on motility is potentiated by phosphodiesterase inhibitors (Chambers and Ali, 1983). PGs also cause rat osteoclasts to produce cyclic AMP (Nicholson *et al.* 1986). PGI_2 and, to a lesser extent, PGE_2 also inhibit TRAP release by isolated rat osteoclasts (Chambers *et al.* 1987), suggesting that the PGs inhibit bone resorption *via* decreased enzyme secretion as well as inhibiting cell motility.

Although Arnett and Dempster (1987) reported an inhibitory effect of PGE_2 on chick osteoclastic resorption, unlike the effect in rat osteoclasts, PGs had no apparent effect on the motility of avian osteoclasts. However, PGE_2 is reported to increase cyclic AMP levels in avian osteoclasts from a variety of sources, including medullary osteoclasts from chicken (Nicholson *et al.* 1986; Rifkin *et al.* 1988; Miyaura *et al.* 1981), and Felix *et al.* (1983) reported that PGE_1 stimulated adenylate cyclase activity in the plasma membranes

of cultured medullary bone osteoclasts. This discrepancy between motility, cyclic AMP levels and bone resorption observed with avian osteoclasts, but not with mammalian osteoclasts, highlights the fact that the relationship between these cellular activities is not completely understood. Using quail medullary bone osteoclasts settled onto plastic, we recently found that PGE_2 stimulated production of cyclic AMP by these cells, whereas other calciotrophic factors, such as CT, CGRP and PTH, were without effect (Z. A. Bascal and C. G. Dacke, unpublished data).

Conclusions

Avian medullary bone represents probably the most oestrogen-sensitive of all known vertebrate bone types in that gonadal steroids are absolutely essential for its induction and maintenance in the egg-laying bird. As such, it has potential for research into mechanisms of human and chicken osteoporosis. Post-menopausal osteoporosis is, in economic terms, one of the most costly of all human diseases, but the precise mechanisms by which declining oestrogen levels bring about the loss of bone density associated with this disease are not well understood. Further studies into the biology of medullary bone are likely to yield useful insights into this problem. Our knowledge of the mechanisms controlling medullary bone formation and turnover is still sparse. It is clear that, with a cell cycle time about 25% that of cortical bone, medullary bone represents an extremely active remodelling system. However, the control mechanisms remain obscure. The classical calcium-regulating hormone PTH probably plays an important role, as does the vitamin D_3 system. CT appears to have less influence, although the high circulating levels of this hormone reported in birds is an enigma. Birds appear to be almost permanently down-regulated to CT, except possibly during times of extreme calcium deficiency. Evidence is emerging for an important regulatory role for calcium and possibly inorganic phosphate using medullary bone osteoclasts, settled onto either plastic or bone slices, as well as for PGs. It seems likely that these may function as part of a local system to regulate osteoclast activity in this bone type, possibly allowing for the very rapid switching that is required to change from a state of net mineral deposition to mobilisation at different points in the hen's egg-laying cycle. However, the precise nature of the signals that control this function is still largely obscure and will require much additional research for its elaboration.

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