Osteoclast function:
role of extracellular pH and ATP

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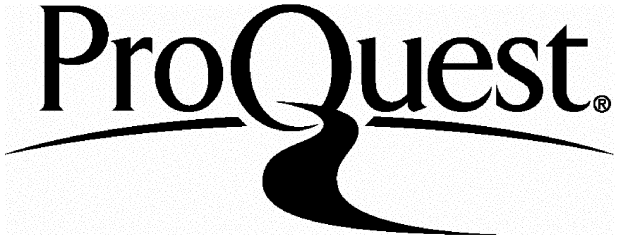

ABSTRACT

Osteoclasts are multinucleated cells responsible for the resorption of both the organic and inorganic components of bone. The molecular mechanisms by which these cells are activated to resorb bone are still poorly understood. Previous work has shown that mature rat osteoclasts in short term cultures are extremely sensitive to small shifts in extracellular pH ($[H^+_{out}]$) and are strongly stimulated in acidified conditions (pH 6.8 - 7.0). The aim of the work presented in this thesis was to further investigate the actions of $[H^+_{out}]$ on osteoclast function and to study the interactions of $[H^+_{out}]$ with other stimulators of resorption.

Experiments with mature rat osteoclasts indicated that the acid-activation effect does not abate over time but may become even more pronounced. Conversely, osteoclast formation in long-term mouse marrow cultures was inhibited at low pH and stimulated in more alkaline conditions; however, after formation in marrow cultures, the mature mouse osteoclasts exhibited the same acid-activation characteristics as freshly isolated mature rat osteoclasts. Experiments with cultured mouse calvarial bones showed similar effects: osteoclastic resorption was strongly activated below pH 7.0, but acidified conditions resulted in a reduction in the number of osteoclasts visible in bones. Acid-stimulated resorption in calvaria occurred with HCO_3^- rather than CO_2 acidosis and was blocked by inhibitors of prostaglandin and leukotriene

synthesis. In contrast, prostaglandin inhibitors stimulated pit formation by cultured rat osteoclasts. I also found that resorption pit formation by isolated chick osteoclasts was very sensitive to small changes in $[H^+_{out}]$, although maximum pH sensitivity occurs over a more alkaline pH range than is the case for rodent osteoclasts. The data from these diverse systems provide strong support for the critical role of acid-base balance in modulating osteoclast function, despite apparent differences in the role of prostaglandins. The results show how pH can be manipulated to optimise resorption assays, and emphasise the importance of controlling this key variable.

I also discovered that extracellular ATP, now recognised as an important signalling molecule in many tissues, stimulated both the activation and formation of rodent osteoclasts. There was a marked synergy between the stimulatory effects of $[H^+_{out}]$ and low dose ATP on the resorptive activity of rat osteoclasts. Acid-activated resorption was blocked by apyrase (an ecto-ATPase) and by suramin (an ATP antagonist). Thus, extracellular ATP and low pH both appear to be necessary for osteoclast activation. The findings suggest an important new mechanism for the local control of osteoclast function which may be relevant to, for example, the bone loss associated with inflammation.

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FIGURES AND TABLES

FIGURE NUMBER AND BRIEF DESCRIPTION:

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ACKNOWLEDGEMENTS

Before starting my Ph.D. studies a friend mentioned to me that it would be the quickest three years of my life. I laughed then, but not now! However, I think that one of the reasons my time has passed so quickly has been the excellent supervision I have received from Dr Tim Arnett. As my supervisor, he has always provided me with expert advice and guidance throughout my work. I would therefore like to take this opportunity to thank him for all of his help over the last three years. I would also like to thank Paul Martin and everyone in his laboratory for putting up with me, and especially Richard Grose for his time and patience when I have been using and abusing the computer, and being generally hopeless on Adobe photoshop! I would also like to thank Dr Sajeda Meghji and Kyle Heron for their help with the mouse calvarial assay. I would also like to express my gratitude to Charlotte Lader and Martin Meyer for reading through my thesis.

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Chapter 1

General Introduction

In this general introduction I will endeavour to give a brief overview of bone biology, with a description of the cell types involved, before focusing on the osteoclast and the role this cell plays in bone.

The skeletal system includes the osseous tissues of the body and the connective tissues that stabilise or interconnect the individual bones (*e.g.* cartilage). The skeletal system has several functions, summarised as follows: (i) provides structural support for the entire body, with individual bones or groups of bones providing a framework for the attachment of soft tissues and organs; (ii) protects delicate tissues and organs (*e.g.* ribs protect the heart and lungs, skull encloses the brain); (iii) haemopoietic system within bone produces red blood cells and other blood elements; (iv) major reservoir of body calcium and phosphate.

The high mechanical strength of the skeleton is determined by the complex mixture of mineral and organic components of bone. The predominant protein product in the organic matrix is type I collagen, with other non-collagenous products such as osteocalcin and bone sialoproteins also present. The major inorganic components of bone are hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), brushite ($CaHPO_4$), and calcium carbonate ($CaCO_3$). A number of osteotropic factors have been isolated from the mineralised bone matrix, including transforming growth factor β (TGF-β), insulin-like growth factors I and II, and fibroblast growth factors.

Bone remodelling is a coupled process between resorption and formation occurring at discrete locations, and consists of osteoclast-mediated resorption followed by osteoblast-mediated bone formation. During early life, bone formation exceeds bone resorption, with a net increase in bone mass, while later in life (*e.g.* postmenopausally) bone resorption exceeds bone formation, with net loss of bone, which may lead to osteoporosis. Remodelling not only maintains the mechanical integrity of bone, but also enables the regulation of body calcium homeostasis. Within bone three different cell types can be distinguished; osteoblasts, osteocytes and osteoclasts.

Osteoblasts

Bone is formed by osteoblasts, which are mononuclear cells of mesenchymal origin. Osteoblasts produce and secrete the major part of the organic bone matrix, type I collagen, as well as other non-collagenous proteins such as osteocalcin, osteopontin, bone sialoproteins, and osteonectin (for reviews see Aubin *et al.*, 1993 & Martin *et al.*, 1993). Osteoblasts also secrete growth factors (*e.g.* insulin-like growth factors -I and II), which stimulate the proliferation and differentiation of bone cells (Alsina *et al.*, 1996). Some osteoblasts remain on the surface of the mineralising bone, while others remain behind the advancing mineralising bone surface and become entrapped in lacunae, where they are termed osteocytes.

Osteocytes

Osteocytes embedded in bone are able to communicate with each other, and possibly other cells such as osteoblasts, by means of gap junction-coupled cytoplasmic processes which extend through canaliculi in the mineralised matrix. The exact role of the osteocyte is unclear, however evidence suggests that the extensive cell network may facilitate the co-ordinated response of bone to mechanical stress and deformation (Skerry *et al.*, 1989; Lanyon, 1993; Mullender and Huiskes, 1997). It has also recently been reported that osteocytes undergoing apoptosis in discrete areas of bone may attract or be permissive to osteoclastic bone resorption (Noble *et al.*, 1997).

Osteoclasts - *Morphology and Phenotype*

The role of the osteoclast in bone resorption was first described by Kolliker (1873), who also provided the cell's descriptive name. Osteoclasts are large multinucleated cells that can range in size up to 100 μm in diameter. Osteoclasts are located on endosteal surfaces within the Haversian system and on the periosteal surface.

Contact with the bone surface induces a rapid reorganisation of the osteoclast cytoskeleton, causing cell polarisation and organisation of the resorbing apparatus, the most characteristic feature of which is the presence of the ruffled border and clear zone (Baron, 1989). The ruffled border is comprised of a number of finger-like projections of the plasma membrane adjacent to bone. The importance of the ruffled border in resorption is demonstrated by c-*src* gene "knockout" mice, which develop osteopetrosis, as the osteoclasts present lack ruffled borders and do not form resorption lacunae (Boyce *et al.*, 1992). Recent work has suggested that phosphatidylinositol-3 (PI-3) plays an important role in ruffled border formation, probably in the fusion of membrane vacuoles with the plasma membrane (Nakamura *et al.*, 1997a).

The ruffled border is surrounded by the clear zone, a region of cytoplasm which is devoid of cellular organelles but has a characteristic organisation of F-actin microfilaments into a ring-like structure, enclosed by a double circle of vinculin. Actin ring formation appears to be dependent on matrix substrates,

proteins and integrins (Nakamura *et al.*, 1996a). This type of microfilament organisation is seen only in resorbing osteoclasts, and can therefore be used as a marker for resorbing cells (Lakkakorpi and Vaananen, 1996). It is now generally accepted that the clear zone (or sealing zone) serves for the attachment of osteoclasts to the bone surface, and separates the resorption lacunae underneath the ruffled border from the extracellular fluid.

At this sealing zone area, the mechanism by which osteoclasts attach to bone is unknown. However, one way that cells can interact with the extracellular matrix is by a family of heterodimer transmembrane glycoproteins known as integrins. These are composed of noncovalently associated α and β subunits. Osteoclasts express the integrins $\alpha 2\beta 1$ and $\alpha v\beta 1$, which are thought to be involved in the adhesion to native type I collagen (Helfrich *et al.*, 1996), but the predominant osteoclast integrin is the vitronectin receptor, $\alpha v\beta 3$ (Horton, 1997). The $\alpha v\beta 3$ integrin mediates a promiscuous recognition of many arg-gly-asp (RGD)-containing bone matrix proteins, such as osteopontin and bone sialoprotein. Peptides containing this motif have been shown to inhibit osteoclast attachment (Flanagan and Lader, 1997; Mercer *et al.*, 1998) and resorption (Sato *et al.*, 1990). The anti-vitronectin receptor antibody 23c6 also inhibits resorption and cell spreading by osteoclasts (Horton *et al.*, 1991; Flanagan and Lader, 1997).

However, the question as to whether integrins actually play a role in the sealing zone is still disputed (Lakkakorpi *et al.*, 1991; Nakamura *et al.*, 1996b). For example, Vaananen & Horton (1995) suggest that the distance between the osteoclast cell membrane and the bone matrix is simply too small to allow an integrin-mediated event. One essential role for integrins could be the podosomal attachment during migration, for example, the β3 integrin subunit is known to play a crucial role in the movement of osteoclasts from the periosteum to bone (Holt and Marshall, 1998). Recently, it has been shown that a peptidomimetic antagonist (SC56631) of the αvβ3 integrin is able to inhibit bone resorption *in vitro*, and prevent the rapid bone loss which accompanies oestrogen withdrawl *in vivo* (Engleman *et al.*, 1997). This may represent a novel approach to treating bone diseases characterised by excessive osteoclastic resorption.

Monoclonal antibody 23c6 recognises the αvβ3 subunit of the vitronectin receptor, and thus preferentially stains osteoclasts (Horton *et al.*, 1984; Davies *et al.*, 1989). It is routinely used to identify human osteoclasts from a variety of sources, such as osteoclastoma (Horton *et al.*, 1985) and osteoclast-rich tumours (Flanagan *et al.*, 1988; Flanagan and Chambers, 1989), as well as freshly isolated human osteoclasts (Flanagan *et al.*, 1992) and osteoclasts generated from human bone marrow (Sarma and Flanagan 1996; Lader and Flanagan 1998).

In addition to morphological markers and vitronectin expression, osteoclasts also express several phenotypic features that help to distinguish them

from other multinucleated giant cells (*e.g.* macrophages). These include tartrate - resistant acid phosphatase (TRAP), matrix metalloproteinase 9 (MMP-9) and calcitonin receptors. TRAP is widely used as a specific histochemical marker of osteoclasts in bone tissue (Minkin, 1982; Asotra *et al.*, 1994a). However its precise role in osteoclasts is not known. Various studies have suggested that TRAP may be important in osteoclastic resorption, since TRAP activity / secretion is increased by both parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), (Minkin, 1982) and is inhibited by calcitonin (Chambers *et al.*, 1987). TRAP may also be involved in the regulation of osteoclast attachment to bone via matrix protein dephosphorylation, generation of oxygen free radicals, or the normal mineralisation of cartilage in developing bone (Zaidi *et al.*, 1989; Ek-Rylander *et al.*, 1994; Hayman and Cox, 1994; Hayman *et al.*, 1996). However, TRAP is not an exclusive marker for osteoclasts, since in bone marrow cultures and pathological conditions macrophages can become TRAP-positive (Modderman *et al.*, 1991). Therefore, care should be taken when assessing TRAP stained cultures, (*e.g.* the mouse marrow assay), where many non osteoclastic TRAP-positive multinucleate cells may be present (Hattersley and Chambers, 1989a).

MMP-9 (also known as gelatinase B) is a matrix metalloproteinase that cleaves denatured type I collagen and collagen types III, IV, VII and XI. It is produced by osteoclasts during resorption and may also be important in osteoclast migration from the periosteum to bone (Blavier and Delaissé, 1995;

Rice *et al.*, 1997). MMP-9 is also released from osteoblasts, where it may act with other collagenases to remove the non-mineralised osteoid layer which covers the bone surface prior to osteoclastic resorption (Chambers *et al.*, 1985a; Hill *et al.*, 1995). Rice *et al.*, (1997) suggest that MMP-9 expression can be used as a marker for osteoclasts, especially during early bone development, as MMP-9 is expressed during human osteoclast differentiation before TRAP (Roodman, 1996).

Another marker for osteoclasts is the calcitonin receptor (CTR), which is unique among bone and bone marrow cells (Hattersley and Chambers, 1989b). Results have shown a strong and specific correlation between the generation of bone-resorptive cells and CTR positive cells, and suggest that CTR expression may be a reliable marker for the identification of osteoclastic differentiation *in vitro* (Hattersley and Chambers, 1989b). CTRs are expressed at the protein and mRNA levels in late precursor cell differentiation (Lee *et al.*, 1994; Massey *et al.*, 1998). Numerous specific binding sites for calcitonin (as many as 10^6 per cell) have been demonstrated by autoradiographic studies on isolated rat osteoclasts (Nicholson *et al.*, 1986), osteoclasts isolated from human foetal bone (Flanagan *et al.*, 1992), osteoclasts generated from murine and human bone marrow (Hattersley and Chambers, 1989a; Sarma and Flanagan, 1996) and on osteoclasts generated in the peripheral blood UMR co-culture system (Massey *et al.*, 1998). However, not all osteoclasts express CTRs. Avian osteoclasts do not respond to calcitonin (Arnett and Dempster, 1987) and do not express CTRs

(Nicholson *et al.*, 1987). Consequently, CTR expression is not an absolute feature of resorbing osteoclasts, but does represent one of the best markers for distinguishing mammalian osteoclasts from macrophage polykaryons (Roodman, 1996).

However, analysis of histochemistry and immunohistochemistry is subjective and interpretation is likely to differ between observers. Reports of large numbers of "osteoclast-like" cells in cultures in which there was virtually no bone resorption (Takahashi *et al.*, 1989; Flanagan *et al.*, 1992) suggest that the presence of stained multinucleate cells is not a reliable marker for osteoclasts generated *in vitro*. Therefore, only bone resorption provides unequivocal evidence that osteoclasts are present *in vitro* (Arnett, 1990; Lader and Flanagan, 1998).

Osteoclasts - *Resorption*

Bone resorption is a multistep process which includes the proliferation and activation of osteoclastic mononuclear precursor cells, attachment of fully differentiated osteoclasts to mineralised bone surfaces and the actual resorption process itself. Although it is not known how resorption sites are determined, it is known that the first sign of a forthcoming resorption site on the endosteal surface is the retraction of bone lining cells (Jones and Boyde, 1976). This retraction uncovers non-mineralised osteoid, which Chambers *et al.*, (1985a) have suggested is degraded by osteoblast-derived collagenase (MMP-1), after

which osteoclasts can attach to the mineralised matrix and initiate bone resorption.

Osteoclasts have the capacity to resorb both the inorganic and organic components of bone (Blair *et al.*, 1986), and are also able to resorb a wide variety of mineralised substrates, such as avian egg shell and enamel, which have a sparse organic matrix with no collagen content (Jones *et al.*, 1984). Although it was suggested thirty years ago (Vaes, 1968) that osteoclasts resorb bone through the formation of an extracellular acid compartment, direct evidence of a low pH in the resorption lacuna has only been presented within the last few years. Baron *et al.*, (1985) used the fluorescent weak base acridine orange, which accumulates in acidic compartments, to show that the osteoclast actively acidifies the zone beneath the ruffled border. Silver *et al.*, (1988) placed pH electrodes beneath osteoclasts on plastic substrates and found that the pH in the contact zone fell to a value of pH 3 or less within a few minutes of attachment. Indeed, in order to solubilize bone mineral and facilitate degradation of the organic matrix of bone, osteoclasts must secrete 1-2 protons for every Ca^{2+} ion liberated (Schlesinger *et al.*, 1994).

It is generally accepted that the organic constituents of bone are degraded by lysosomal enzymes secreted by osteoclasts into the resorption lacunae. An acidic pH may provide optimal conditions for the action of some of these enzymes secreted by the osteoclast (Blair *et al.*, 1988). However, the roles of

the different enzymes are controversial and are not fully understood. For example, there is a significant decrease in the activity of collagenases below pH 6 (Vaes, 1972).

Lysosomal enzymes belong to two major families: the matrix metalloproteinases (*e.g.* MMP-9) and the cysteine proteinases. A number of studies have suggested a specific cysteine protease involved in bone resorption. Drake *et al.*, (1996) demonstrated the abundant and selective expression of cathepsin K in osteoclasts, suggesting a specialised role in bone resorption, while cathepsins S, L and B, which have been proposed to be involved in resorption (Hill *et al.*, 1994), were undetectable. Cathepsin K appears to undergo autocatalytic activation in the low pH environment of the resorption lacunae (McQueney *et al.*, 1997). Recently, it was demonstrated that in foetal mouse bone, cathepsin K expression by osteoclasts occurred at the onset of osteoclast differentiation, suggesting cathepsin K as an early marker for osteoclasts (Dodds *et al.*, 1998).

Acidification of the resorption lacuna requires the active transport of protons (H^+) across the ruffled border area of the cell membrane. This transport involves an H^+-ATPase of the plasmalemmal vacuolar type (V-ATPase), which has been shown to be located within the ruffled border (Blair *et al.*, 1989; Vaananen *et al.*, 1990). PTH and acidosis can enhance the number or activity of V-ATPases present at the ruffled border of osteoclasts (Vaananen *et al.*, 1990;

Nordstrom *et al.*, 1997), and may provide a mechanism for increased resorption. Bafilomycin A_1 specifically inhibits vacuolar H^+-ATPase, and can abolish the resorptive activity of osteoclasts *in vitro*, and trigger apoptosis (Sundquist *et al.*, 1990; Okahashi *et al.*, 1997). The importance of V-ATPase in the resorptive process is illustrated by the osteosclerosis seen in *oc/oc* mice. Studies have shown this to be due to a lack of H^+-ATPases in the ruffled border area, although H^+-ATPases were present throughout the rest of the osteoclast cytoplasm (Nakamura *et al.*, 1997b). Additionally, the gene encoding the vacuolar H^+-ATPase 16 kd subunit is one of the most abundantly expressed by the osteoclast, which is consistent with very active H^+ secretion (Sakai *et al.*, 1995).

In osteoclasts the majority of protons are generated via the enzyme carbonic anhydrase II, which is found in abundance in the osteoclast cytoplasm (Vaananen and Parvinen, 1983). Carbonic anhydrase II (CA II) catalyses the hydration of CO_2 to H_2CO_3, which dissociates to produce H^+ and HCO_3^-. CA II is known to play a critical role in osteoclastic resorption, since inhibitors, *e.g.* acetazolamide, inhibit resorption *in vitro* (Minkin and Jennings, 1972; Raisz *et al.*, 1988) and *in vivo* (Kenny, 1985). Recent work has also demonstrated that acetazolamide can decrease the 1,25-dihydroxyvitamin D_3-induced formation of multinucleated TRAP-positive osteoclasts in a dose-dependent manner *in vitro* (Lehenkari *et al.*, 1998). This suggests that CA II is essential not only for osteoclastic bone resorption, but also osteoclast differentiation. Patients with congenital CA II deficiency form osteoclasts, but these osteoclasts are unable to

produce protons, leading to osteopetrosis (Sly *et al.*, 1985). During active resorption, secretion of H^+ ions by osteoclasts generates an equal amount of HCO_3^-, which may be exchanged for chloride to maintain intracellular pH (Teti *et al.*, 1989a). Inhibition of this Cl^- / HCO_3^- exchanger abolishes bone resorption *in vitro* (Hall and Chambers, 1989).

Regulation of osteoclast intracellular pH

The ability to regulate intracellular pH (pH_i) within a tight physiological range is crucial for normal cellular function. Along with the Cl^- / HCO_3^- exchanger mentioned above, osteoclasts *in vitro* are able to maintain intracellular pH via a Na^+ / H^+ exchanger (Gupta *et al.*, 1996). Nordstrom *et al.*, (1995) also demonstrated the presence of a pH and membrane potential sensitive H^+ conductance in osteoclast membranes, and suggested that this conductance contributes significantly to pH_i regulation. In osteoclasts *in vitro*, the dominant current may be an inwardly rectifying K^+ current, which permits the rapid switching of membrane potential between two stable levels (Sims and Dixon, 1989). It is possible that osteoclastic resorption and secretion can be modulated by these changes in membrane potential. It has also been reported that the osteoclast outward rectifying K^+ current can be altered by changes in extracellular cation concentrations. For example, lowering pH reduced the outward K^+ current at all voltages, suggesting a change in the surface charge. This may help to explain how H^+ functions as an extracellular signal that regulates bone resorption (Arkett *et al.*, 1994).

Recent work by Lehenkari *et al.*, (1997) using isolated rat osteoclasts cultured on bovine bone slices, glass, or plastic loaded with a pH-sensitive indicator demonstrated that osteoclasts have different mechanisms of pH_i regulation depending on the phase of activity. During resorption, osteoclast pH_i is mainly regulated by H^+-ATPase activity, and while resting / migrating, pH_i is regulated by the Na^+ / H^+ exchanger. Osteoclast pH_i also varies according to the substrate, with a much higher pH_i (*i.e.* more alkaline) when cultured on bone compared to glass. The significant differences in osteoclast pH_i regulation depending on the substrate (Lehenkari *et al.*, 1997) cast some doubt on the relevance of work examining osteoclasts cultured on glass or plastic. Additionally, the majority of studies examining osteoclast ion flows are conducted using non-physiologically buffered media (*i.e.* HEPES), which may perturb intracellular pH regulation and therefore alter osteoclast function (Arnett *et al.*, 1994). These results therefore demonstrate the importance of an appropriate environment when studying the function of osteoclasts.

Elimination of degraded bone material

Nesbitt & Horton (1997) and Salo *et al.*, (1997) recently demonstrated that during the resorptive process, while osteoclasts are tightly sealed to the bone matrix, the products of bone degradation (*e.g.* type I collagen) are endocytosed from the ruffled border membrane and transported in vesicles through the cell to the basolateral membrane, where they are released into the extracellular space.

This helps to explain how resorbing osteoclasts can remove large amounts of degraded matrix products while simultaneously penetrating deeper into bone, and suggests a regulatory mechanism for the control of tissue degradation within osteoclasts. These products of bone resorption can be used as clinical markers of bone turnover, the most promising of which are all based on collagen degradation products, particularly cross-linking amino acids and cross-linked telopeptides (Calvo *et al.*, 1996). Recent work has demonstrated that resorbing osteoclasts cultured on human bone degrade bone collagen to release cross-linked collagen N-telopeptides (NTx), but not free pyridinolines into the culture medium. NTx concentration is highly correlated to the area of bone surface resorbed. This suggests that by analysing the NTx content in urine, an accurate measure of bone resorption can be obtained (Apone *et al.*, 1997).

Osteoclast formation

It is now generally accepted that osteoclasts are formed by the fusion of mononuclear precursors of haemopoietic origin (Figure 1.1). This was first established by parabiosis and transplantation experiments *in vivo* (Walker, 1973; Gothlin and Ericsson, 1976). Baron *et al.*, (1986) demonstrated that the formation of multinucleated osteoclasts *in vivo* is preceded first by mononuclear cells containing a non-specific esterase (an enzyme present in monocytes and not mature osteoclasts) and then by mononuclear cells which attach to bone and become TRAP-positive. These mononuclear cells eventually form multinucleated osteoclasts.

Work with human cells has also demonstrated that the osteoclast precursor circulates in the monocyte fraction of blood. Culture of these precursor cells with the rat osteoblastic UMR106 cell line, $1,25(OH)_2D_3$ and Macrophage-Colony Stimulating Factor (M-CSF) resulted in numerous TRAP, calcitonin and vitronectin positive multinucleated cells, which were capable of resorbing bone (Fujikawa *et al.*, 1996a; Massey *et al.*, 1998). The UMR106 cell line synthesises murine M-CSF and supports osteoclast formation from murine peripheral blood in the absence of exogenous M-CSF (Quinn *et al.*, 1994). However, as murine M-CSF has no effect on human cells, addition of human M-CSF is required in order to induce human osteoclast formation (Roussel *et al.*, 1988).

The majority of studies indicate that the osteoclast precursor is a cell in the monocyte-macrophage lineage, with the CFU-GM (Colony-Forming Unit-Granulocyte - Macrophage) proposed as the immature osteoclast progenitor cell (Roodman, 1995). This progenitor cell gives rise to an early cell in the monocytic lineage, which is at least bipotent, in that it can form monocytes capable of differentiating into tissue specific macrophages such as Kupffer cells and alveolar macrophages (Johnston, 1988), or it can differentiate to a more committed progenitor for the osteoclast (Roodman, 1995). Additionally, Athanasou *et al.*, (1986) using immunocytochemical staining, demonstrated the

presence of macrophage-associated antigens in human osteoclasts, indicating that they are phenotypically similar to macrophages.

Recently, it has been reported that mature monocytes and macrophages isolated from patients with rheumatoid arthritis have the ability to differentiate into functional osteoclasts, demonstrating the overlapping phenotypes of these cells (Fujikawa *et al.*, 1996b). Indeed, Tondravi *et al.*, (1997) demonstrated that "knocking out" the haematopoietic transcription factor PU.1 in mice resulted not only in an absence of osteoclasts, causing osteopetrosis, but also a lack of macrophages, demonstrating their shared precursor origins. Similarly, mice lacking *c-fos* (a component of the AP-1 transcription factor complex) develop severe osteopetrosis due to a dearth of osteoclasts. However, this absence of osteoclasts in *Fos* "knock out" mice was accompanied by an increase in bone marrow macrophages (Grigoriadis *et al.*, 1994), suggesting the lack of *Fos* causes a block in differentiation at the branch point between monocyte-macrophages and osteoclasts. Recently, the NF-κB proteins have also been suggested to be required for normal osteoclast-macrophage development in a similar manner to *c-fos* (Iotsova *et al.*, 1997).

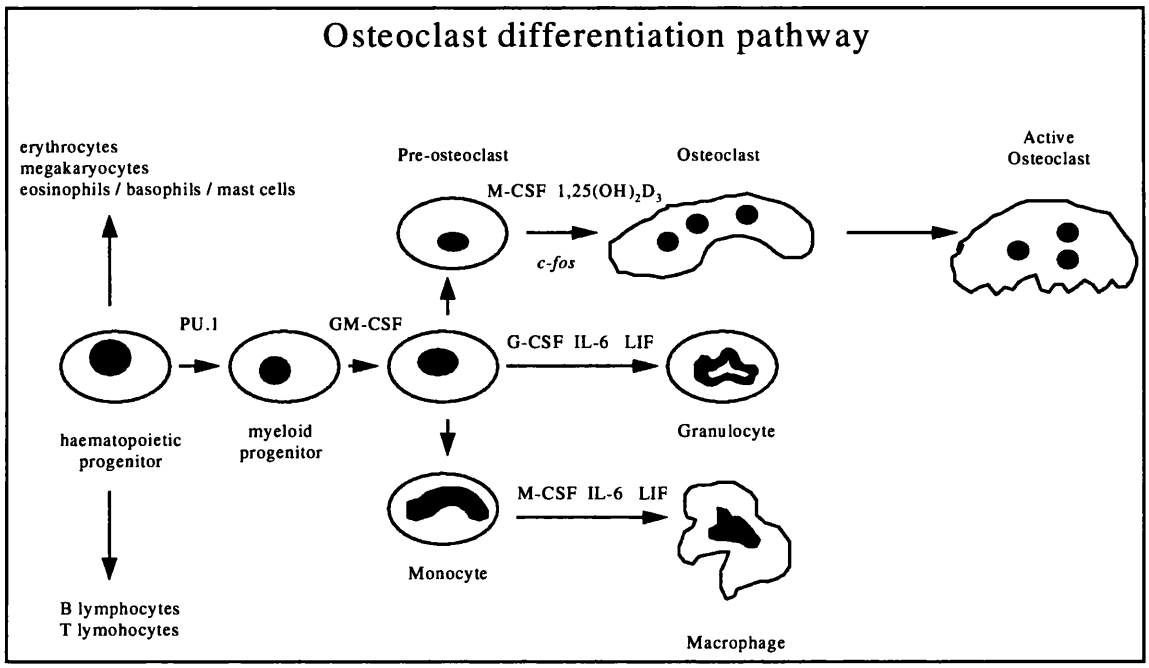


Figure 1.1 Haematopoietic lineages showing principal factors involved. Adapted from Liebermann *et al.*, (1998).

Several *in vitro* model systems have now been developed to study osteoclast proliferation and differentiation, the majority using spleen cells or bone marrow. Feline, primate, canine, murine, rabbit and human bone marrow have been used for osteoclast generation (Testa *et al.*, 1981; Allen *et al.*, 1981; Roodman *et al.*, 1985; Ibbotson *et al.*, 1987; Takahashi *et al.*, 1988a; Fuller and Chambers, 1989a; Hattersley and Chambers, 1989a, and Sarma and Flanagan., 1996).

Bone marrow assay

The long term mouse bone marrow assay developed in 1988 by Takahashi *et al* provides a convenient method of *in vitro* osteoclast generation. Osteoclasts generated from marrow cells cultured for 7 to 10 days with $1,25(OH)_2D_3$,

dexamethasone, and bone stromal cells produce multinucleated cells with typical osteoclast characteristics, such as TRAP-positivity, calcitonin receptors, multinuclearity and the presence of resorption pits. [^{3}H]-thymidine incorporation studies have revealed that osteoclast progenitors proliferate primarily during the first 4 days, and then differentiate into multinucleated cells, predominantly during the final days of culture (Tanaka *et al.*, 1993).

The precise role of glucocorticoids (*e.g.* dexamethasone) in osteoclast development is unknown. Glucocorticoids may stimulate the differentiation of stromal cells into cells capable of promoting osteoclast differentiation (Kaji *et al.*, 1997). However, results from other groups suggest that dexamethasone may directly affect bone marrow cells to stimulate osteoclast formation by inhibition of endogenous GM-CSF (Granulocyte - Macrophage-Colony Stimulating Factor) production, which may function as a negative regulator of osteoclast formation (Shuto *et al.*, 1994). Osteolytic hormones (*e.g.* 1,25$(OH)_2D_3$ and PTH) induce the differentiation of immature precursors (TRAP and calcitonin receptor negative) into mature precursors (TRAP and calcitonin receptor positive), and hence increase the numbers of osteoclasts formed (Roodman *et al.*, 1985).

The effect of prostaglandins on osteoclast differentiation, an important point of control for bone resorption, is poorly understood, and has proved to be controversial. Some groups maintain that prostaglandins stimulate bone resorption and calcitonin receptor-positive cell formation (Collins and Chambers,

1991; 1992), as well as TRAP-positive cell formation in murine bone marrow cultures. (Akatsu *et al.*, 1989). However, it has been suggested that PGE_2 exerts the opposite effect on human osteoclast formation, having been found to be a potent inhibitor of 23c6-positive cell formation (Chenu *et al.*, 1990). Flanagan *et al.*, (1995) reported that PGE_2 enhanced osteoclastic bone resorption in human bone marrow cultures, suggesting that PGE_2 induces osteoclast formation in both human and murine species. Following on from these results, Lader and Flanagan, (1998) demonstrated that addition of indomethacin to M-CSF treated cultures virtually abolished osteoclast parameters (*i.e.* 23c6-positive cells and bone resorption), indicating that prostaglandins are essential for both human and murine osteoclast formation.

Addition of calcitonin to mouse marrow cultures markedly inhibits the formation of TRAP-positive multinucleated cells, but not the appearance of TRAP-positive mononuclear cells (Takahashi *et al.*, 1988a), indicating that calcitonin selectively inhibits the fusion of preosteoclasts. Indeed, osteoclasts formed from cultures treated with calcitonin from the outset express only very low levels of CTR or CTR mRNA. This suggests that the "escape" phenomenon seen with prolonged CT administration may be due to a reduced synthesis of CTR, and to the appearance in bone of CTR-deficient functional osteoclasts (Ikegame *et al.*, 1996).

Bone marrow-derived osteoblastic stromal cells appear to play an important role in modulating the differentiation of osteoclast progenitors in two different ways: the production of soluble factors, and cell-to-cell recognition between osteoclast progenitors and osteoblastic stromal cells. M-CSF is probably the most important soluble factor, appearing to be necessary for not only the proliferation of osteoclast progenitors, but also differentiation into mature osteoclasts and their survival (Suda *et al.*, 1995a). Identification of a mutation within the coding region of the M-CSF gene in the osteopetrotic op/op mouse (Yoshida *et al.*, 1990), along with *in vivo* experiments which showed that op/op mice could be partially cured by administration of recombinant human M-CSF demonstrated the essential nature of this cytokine in osteoclast formation (Felix *et al.*, 1990). M-CSF has also been shown to be essential in op/op murine osteoclast formation *in vitro* (Tanaka *et al.*, 1993), but inhibits resorption and osteoclast formation in normal murine haemopoietic tissue (Hattersley *et al.*, 1991).

However, until recently, the formation of bone-resorbing human osteoclasts from human bone marrow had been difficult to achieve (Flanagan *et al.*, 1992; Pacifici, 1995). The problem was solved by the finding that addition of recombinant human M-CSF to human bone marrow cultures caused substantial bone resorption and osteoclast formation in a dose and time dependent manner, above that induced by 1,25 dihydroxy D_3 (Sarma and Flanagan, 1996). These results suggest a critical role for M-CSF in the formation of human osteoclasts,

and are consistent with the report that M-CSF is essential for murine osteoclast formation (Yoshida *et al.*, 1990). Interestingly, M-CSF mRNA expression is decreased in bone marrow cultures exposed to increased hydrostatic pressure, suggesting a mechanism by which load-bearing can affect osteoclast formation and therefore skeletal integrity (Rubin *et al.*, 1997).

M-CSF is able to stimulate both motility and spreading of isolated rat osteoclasts, as well as support osteoclast survival by preventing apoptosis (Fuller *et al.*, 1993). Paradoxically, M-CSF inhibits bone resorption by isolated rat osteoclasts. This suggests that apart from the established role of M-CSF in the provision of precursors for osteoclastic formation, a major role in bone resorption is the enhancement of osteoclast survival, migration, and chemotaxis (Fuller *et al.*, 1993). However, bone resorption by mature human foetal osteoclasts, including pit area, depth and volume was increased in the presence of M-CSF. Osteoclast numbers were maintained in the presence of M-CSF, whereas they were considerably reduced in its absence, suggesting that, as for isolated rat osteoclasts, M-CSF prolongs the life span of isolated human osteoclasts, rather than promoting the formation of new osteoclasts (Edwards *et al.*, 1998).

The importance of cell-cell contact for osteoclastogenesis was demonstrated by Burger *et al.*, (1984), who showed that live bone-forming cells were required for osteoclast formation. This finding was confirmed by later work culturing spleen and primary osteoblastic cells separated by a membrane,

which inhibited osteoclastogenesis (Takahashi *et al.*, 1988b). Adhesion molecules are likely to be important during the process of osteoclast formation, for example, $1,25(OH)_2D_3$ induces the expression of vascular adhesion molecule 1 (VCAM-1), which may be involved in the interaction between stromal cells and osteoclastic precursors (Feuerbach and Feyen, 1997).

Additionally, the expression of osteopontin by osteoclast and osteoblast progenitors in murine bone marrow cultures suggest that its cell adhesion properties are required for osteoclastogenesis (Yamate *et al.*, 1997). These findings indicate that osteoclast-inducing activity is tightly associated with osteoblasts, and may explain the dependence of osteoclast formation in the mouse marrow assay on a high cell density. Using this assay various workers, including myself, have found that TRAP-positive osteoclasts generally always form near colonies of osteoblasts / stromal cells, lending credence to the suggestion that osteoblastic cells are somehow involved in osteoclast formation (Suda *et al.*, 1992). However, these results contrast with the work of Owens *et al.*, (1996), who suggested that osteoblasts may not be essential for osteoclast formation, but rather are required to activate and regulate the resorptive function of mature osteoclasts.

Although the mouse marrow assay is a useful model of osteoclast formation, it does have several limitations. For example, the assay generally has

a significant amount of variability within groups, meaning that only factors which produce very large effects will be identified.

Over the last two years, a novel secreted glycoprotein that regulates bone resorption has been identified by several laboratories, and named osteoprotegerin (OPG) (Simonet *et al.*, 1997) and osteoclastogenesis-inhibitory factor (OCIF) (Yasuda *et al.*, 1998). OPG / OCIF is a member of the tumour necrosis factor receptor (TNFR) superfamily, and is able to act in a soluble manner to regulate bone mass. In vitro, OPG / OCIF blocks osteoclastogenesis by inhibiting osteoclast differentiation. The ligand for OPG / OCIF is a TNF-related cytokine that can replace the *in vitro* requirement for stromal cells, $1,25(OH)_2D_3$ and glucocorticoids for osteoclast formation, and is termed osteoprotegerin ligand (OPGL) or osteoclast differentiation factor (ODF) (Lacey *et al.*, 1998; Matsuzaki *et al.*, 1998). OPGL / ODF binds to a unique haematopoietic progenitor cell which is committed to the osteoclast lineage, and stimulates the rapid induction of genes that typify osteoclast development. OPGL / ODF is also able to directly activate isolated osteoclasts *in vitro*. OPGL / ODF was found to be identical to TRANCE / RANKL, which enhances T-cell growth and dendritic cell function (Yasuda *et al.*, 1998). These findings suggest that OPG / OCIF and OPGL / ODF are likely to interact in the bone microenvironment to regulate osteoclastogenesis and indicate that OPGL / ODF may be a crucial coupling factor produced by osteoblasts (Lacey *et al.*, 1998).

Mature osteoclast isolation

Progress in the understanding of the events controlling osteoclast differentiation and resorption has been hampered for several reasons: osteoclasts are few in number relative to other cell types; are difficult to isolate because they are embedded in bone; and furthermore no osteoclast cell line exists, although several groups have produced cell lines in which a proportion of the cells formed are functional osteoclasts (Chambers *et al.*, 1993; Miyamoto *et al.*, 1998).

Before the marrow assays described above were in general use, techniques were developed for isolating mature osteoclasts from long bones. Boyde *et al* and Chambers *et al* simultaneously developed the bone slice assay in 1984, which was then adopted as an assay of bone resorption by isolated osteoclasts. By varying the time for which the bone cell population is allowed to adhere to the bone slice before rinsing off non-adherent cells, osteoclasts may be "functionally purified", as demonstrated by McSheehy and Chamber (1986a) who showed that isolated osteoclasts did not respond to PTH. However, one of the most important factors in this assay system is the level of basal resorption. This may be achieved by using low pH, as first described by Arnett & Dempster (1986), and is discussed in more detail in chapter 2.

Assessment of resorption is typically achieved by simply counting the number of resorption pits present using the technique of reflected light microscopy (Walsh *et al.*, 1991). Reflected light microscopy can provide a high

quality image and can be used on unstained specimens as well as stained. However, other groups have suggested that measuring the volume of each individual pit is a more accurate method of assessing resorption (Boyde and Jones, 1991).

Systemic factors affecting osteoclast formation and activation

Osteoclast differentiation and activation are regulated by many systemic factors. Among these, the principal calcium-regulating hormones, parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and calcitonin are probably the best known.

PTH and $1,25(OH)_2D_3$ stimulate resorption *in vivo*, in organ culture and in the isolated osteoclast assay, as well as stimulating osteoclast formation from osteoclast progenitors in the murine and human marrow system (Raisz and Kream, 1983; McSheehy and Chambers, 1986a; Takahashi *et al.*, 1988a; Flanagan *et al.*, 1995; Uy *et al.*, 1995). PTH is an 84 amino acid polypeptide hormone which is synthesised and secreted by the chief cells of the parathyroid glands. The 1-34 amino-terminal fragment is the biologically active fragment (Segre et al., 1981). PTH is commonly considered to act on mature osteoclasts via receptors expressed by osteoblasts (McSheehy and Chambers, 1986a). It is also unlikely that $1,25(OH)_2D_3$ acts on mature osteoclasts directly, since mature osteoclasts do not express vitamin D receptors (Narbaitz *et al.*, 1983).

Calcitonin is able to act at multiple stages in the mammalian osteoclast lineage, including inhibiton of osteoclast formation (Takahashi *et al.*, 1988a), inhibition of mature osteoclast resorption (Chambers *et al.*, 1985b; Arnett and Dempster, 1987) and inhibition of apoptosis (Selander *et al.*, 1996). Calcitonin receptors are expressed on committed osteoclast precursors and appear to be a differentiation marker for mature osteoclasts (Hattersley and Chambers, 1989b). Calcitonin inhibits mammalian osteoclasts by stimulating adenylcyclase activity and cAMP accumulation (Chambers and Dunn, 1983), which results in immobilisation and contraction of the osteoclast (Chambers and Magnus, 1982).

The deleterious effects of systemic acidosis on the skeleton have been recognised since the early part of this century (Goto, 1918). More recent *in vivo* studies suggested that the bone loss associated with acidosis was not due to passive physico-chemical processes, but involved enhanced osteoclastic resorption (Barzel and Jowsey, 1969).

Oestrogen is one of the major inhibitors of osteoclast formation and activation. *In vivo*, oestrogen appears to antagonise bone resorption and remodelling (Turner *et al.*, 1994). *In vitro*, oestrogen inhibits bone resorption in murine marrow cultures by reducing the number of osteoclasts formed (Harganani, Morrison and Arnett, unpublished). In human bone marrow cultures, 17β-oestradiol reduces osteoclast number (as assessed by calcitonin receptor and 23c6-positive cells) and bone resorption in a dose-responsive

manner, possibly via the down regulation of the membrane bound form of M-CSF (Sarma *et al.*, 1998). However, controversy surrounds the presence of oestrogen receptors on osteoclasts. Oursler *et al.*, (1994) demonstrated that osteoclastic cells purified from human giant cell tumours expressed the oestrogen receptor; however, other groups have failed to detect oestrogen receptors in avian or human osteoclasts (Colston *et al.*, 1989). Oestrogen may also cause an increase in osteoclast apoptosis via a TGF-β mediated mechanism (Hughes *et al.*, 1996). This work contrasts with the findings of Arnett *et al.*, (1996) who found that 17β-oestradiol exerted no significant effects on osteoclast viability or resorptive function.

For a review of the actions of oestrogen, androgens, growth hormone and glucocorticoids on bone see Peck, (1984); Turner *et al.*, (1994 & 1995), Vanderschueren and Bouillon, (1995) and Ohlsson *et al.*, (1998).

Local factors affecting osteoclast formation and activation

Besides the systemic factors mentioned above, several locally released (*i.e.* paracrine and autocrine) factors such as prostaglandins, cytokines, growth factors and factors released from the bone matrix during resorption have been identified over the last few years which have powerful effects on both osteoclasts and osteoblasts.

Bone cells, particularly osteoblasts, are abundant producers of prostaglandins (Norrdin *et al.*, 1990), which are potent, multifunctional regulators of bone cell metabolism. Their effects are complex: *in vivo*, regardless of the route of administration, PGE_1 or E_2 stimulate bone resorption and formation (reviewed by Jee and Ma, 1997), in bone organ culture they stimulate resorption (Klein and Raisz, 1970), and when added to isolated osteoclasts they are inhibitory (Arnett and Dempster, 1987; Fuller and Chambers, 1989b). Prostaglandins may also be responsible for the localised bone loss associated with inflammation *in vivo* (Robinson *et al.*, 1975). The effects of prostaglandins on bone cells therefore appear to be dependent on the dose administered and the assay used.

The identification of a large number of growth factors and cytokines in skeletal tissue has opened a new approach to the understanding of bone formation and resorption. The cytokines important in bone metabolism include interleukin-1, tumour necrosis factor α and β and TGF-β. For general reviews on osteoclast regulation by local factors see Suda *et al.*, (1995a); Manolagas, (1995); Alsina *et al.*, (1996); Roodman, (1996) and Pacifici, (1998).

Local changes in extracellular pH are also known to alter osteoclast formation and activity (Shibutani and Heersche, 1993; Arnett and Spowage, 1996), indeed, the hydrogen ion is the only presently known direct stimulator of osteoclastic bone resorption (Arnett and Dempster, 1990). Tissue injury and

inflammation often result in localised acidosis along with the release of adenosine 5' triphosphate (ATP) and other substances (Steen *et al.*, 1992). For example, the extracellular pH at sites of inflammation can be as low as pH 5.4, and may contribute to the pain and hyperalgesia in disease states, such as rheumatoid arthritis (Steen *et al.*, 1995). Protons are unusual in that they are able to drive nociceptors continuously without apparent tachyphyllaxis or adaptation (Steen *et al.*, 1992), a role they also play in stimulating mature rat osteoclasts. The role of extracellular pH is discussed in more detail in Chapter 2. Clearly, the effects of pH on osteoclasts could interact with those of other osteolytic agents. In areas of inflammation, local ATP release from damaged cells, mast cells or platelets (Gordon, 1986) could give a concentration as high as 20μM (Bowler *et al.*, 1995), which results have shown would be more than sufficient to stimulate both osteoclastic bone resorption and osteoclast formation. The role of extracellular ATP is discussed in more detail in Chapter 4.

Nitric oxide (NO) has recently been identified as a potent multifunctional signalling molecule with widespread actions within bone. It is produced in response to diverse stimuli such as pro-inflammatory cytokines, mechanical strain and sex hormones, and has biphasic effects on osteoclast function: (high doses inhibit and low doses stimulate resorption), as well as affecting the growth and differentiation of osteoblasts (Evans and Ralston, 1996). Results indicate that like ATP, NO may be involved in the pathogenesis of bone disease and tissue

damage associated with inflammatory conditions such as rheumatoid arthritis (Ralston, 1997).

General aims of present work

The general aim of my work has been to examine the effects of various factors on osteoclast formation and mature osteoclast activity. Additionally, work performed in collaboration with Dr S Meghji at the Eastman Dental Institute, London, is included in this thesis.

Chapter 2

Modulation of osteoclast formation and activation by changes in extracellular pH

"Life is a struggle, not against sin, not against the Money Power, not against malicious animal magnetism, but against the hydrogen ions"
H.L. Mencken, (1919).

Introduction

Acid-base balance - general considerations

Evolutionary selection has produced several systems in the body which serve to maintain the pH of the body fluids within a narrow range (pH 7.36 to 7.44). This is important because enzyme catalysed reactions are usually strongly affected by changes in pH. For example, the activity of phosphofructose, the key enzyme regulating the rate of glycolysis, increases 20-fold with a pH increase of 0.1 unit (7.1 to 7.2) (Kitajima *et al.*, 1983). Changes in pH also have important effects on the nervous system. In extreme acidosis, the activity of the

central nervous system can be depressed leading to unconsciousness (Rosner and Becker, 1984). There is also evidence that neuronal function (Saadoun *et al.*, 1998) and the development of neurofibrillary degeneration in Alzheimer's disease (Kenessey *et al.*, 1997) are pH-dependent.

Most chemical reactions in the body produce or absorb protons. Dietary fats and proteins are converted to fatty acids and amino acids which circulate in the blood, while glucose from dietary carbohydrate is converted into pyruvic acid by the glycolitic pathway. These are known as non-volatile acids, and are excreted from the body via the kidneys. Sulphuric acid and phosphoric acid are the most important of the so called "fixed" acids. They are generated in small amounts during the catabolism of amino groups and compounds containing phosphate groups, including phospholipids and nucleic acids. However, much larger amounts of H^+ can be formed indirectly from the carbon dioxide (CO_2) generated by metabolism, which reacts with water to give carbonic acid and ultimately H^+ and HCO_3^-. CO_2 is known as a volatile acid because it readily diffuses out of solution. Roughly 10-20 moles must be removed by the lungs each day. Extra acid loads in the form of lactic acid are added to the blood by strenuous exercise . Dietary alkali is found in fruits and vegetables, as the sodium and potassium salts of weak acids. On balance, people eating a typical western meat-containing diet generate a residue of about 100 mEq of H^+ daily (Barzel, 1995), which must be excreted to maintain a neutral acid-base balance. In the face of renal insufficiency, humans cannot excrete this normal daily

endogenous acid production. In order to avoid a significant reduction in systemic pH the excess-retained hydrogen ions must be buffered.

Buffering

The excess CO_2 and H^+ generated in the body are ultimately removed by the lungs and kidneys, but in the short term various buffering mechanisms in the blood (*e.g.* haemoglobin), extracellular fluid and intracellular fluid (*e.g* bicarbonate buffering), prevent any large fluxes in H^+ concentration. *In vivo* and in physiologically buffered media *in vitro*, the following equilibrium exists:

$$H^+ + HCO_3^- \leftrightarrow H_2CO_3 \leftrightarrow H_2O + CO_2$$

The Henderson-Hasselbalch equation (see below) can be used to assess different buffers *in vivo* or *in vitro*.

$$pH = pK + \log_{10} \frac{[A^-]}{[HA]}$$

(HA = weak acid; A^- = weak acid salt)

In vitro, physiologically buffered tissue culture media formulated with Earle's salts, containing 2.2g $NaHCO_3$ / l (DMEM contains 3.7g $NaHCO_3$ / l), are normally used in combination with a 5% CO_2 atmosphere to give an operating pH at equilibrium close to 7.25. Addition of protons (in the form of concentrated HCl) to the medium reduces the HCO_3^- concentration and the

equilibrium operating pH at constant PCO_2. H^+ reacts with HCO_3^- to form H_2CO_3 (carbonic acid) and then CO_2. This reduces pH, thus mimicking *in vivo* metabolic acidosis. Conversely, increasing PCO_2 reduces pH, while the HCO_3^- concentration remains more or less constant, a model of respiratory acidosis.

Acidosis and the skeleton

The skeleton has long been recognised as being important in the maintenance of acid-base balance in the body (Goto, 1918; Jaffe *et al.*, 1932). The skeleton contains 80% of body carbonate and 35% of body sodium, (as well as being a reservoir of labile calcium), which is available to buffer protons if the kidneys and lungs are unable to maintain acid-base balance within narrow physiological limits (Sebastian and Morris, 1994; Barzel, 1995). The role of the skeleton in acid-base homeostasis in adults may contribute to the progressive decline in bone mass that occurs with age, and ultimately lead to osteoporosis (Wachman and Bernstein, 1968; Barzel, 1978).

Studies *in vivo* (Barzel and Jowsey, 1969; Kraut *et al.*, 1984) and *in vitro* using bone organ cultures (Bushinsky *et al.*, 1985; Bushinsky, 1989) have implicated enhanced osteoclastic resorption as the mediator of the bone loss associated with acidosis. Goldhaber & Rabadjija (1987) demonstrated that acidification of culture media with HCl produced large dose-dependent increases in stable calcium release from mouse calvaria over 7 days. The effect was

blocked by calcitonin (an osteoclast inhibitor) and by indomethacin, suggesting prostaglandin release was involved (Goldhaber and Rabadjija, 1987).

Experiments by Bushinsky and colleagues have shown that in short term cultures (3 hours) of neonatal mouse calvaria, the net calcium efflux observed when the pH was decreased (either by reducing HCO_3^- or by increasing CO_2) appeared to be due to physicochemical dissolution of bone calcium carbonate (Bushinsky and Lechleider, 1987).

However, in long-term calvaria cultures (96-99 hours) there was evidence for proton stimulation of osteoclast-mediated calcium release, and that metabolic acidosis (*i.e.* decreasing HCO_3^-) was more potent at stimulating osteoclastic resorption than respiratory acidosis, suggesting that decreased medium bicarbonate, and not just a fall in pH, is necessary to enhance net calcium efflux from live calvaria (Bushinsky, 1989, 1994; Bushinsky *et al.*, 1996). However, Arnett *et al.*, (1994) suggested that in the short term (24 hours), rat osteoclasts were more sensitive to stimulation by CO_2 acidosis than by HCO_3^- acidosis. CO_2 acidosis not only increased the number of resorption pits, but also their depth and width, whilst decreasing HCO_3^- increased the number of pits resorbed per osteoclast, but resulted in a progressive reduction in pit size. Paradoxically, Dominguez & Raisz (1979) reported that in bone organ culture, H^+, CO_2 and HCO_3^- concentrations had no influence over cell-mediated resorption, and non-cell-mediated mineral loss was linearly related to H^+ concentration, but not to

CO_2 and HCO_3^- concentrations. As with the effects of prostaglandins (see Chapter 3), these disparities could be accounted for to some extent by the differences of the various *in vitro* assay systems and their time courses. Thus, the potential different effects of metabolic and respiratory acidosis on osteoclastic resorption are unclear, and warrant further investigation.

Experiments using the disaggregated rat osteoclast assay have provided the most direct evidence for the stimulatory action of protons on bone resorbing cells. In culture media which is buffered non-physiologically (using HEPES only), resorption pit formation increased progressively as medium pH was reduced from 7.4 to 6.8 (Arnett and Dempster, 1986). Subsequent work using media buffered physiologically with HCO_3^- / CO_2, demonstrated that osteoclasts are stimulated to resorb when the pH is changed within a more limited range, by either decreasing the concentration of HCO_3^- or by increasing CO_2 (Arnett *et al.*, 1994). The remarkable sensitivity of rat osteoclasts to extracellular protons when cultured in HCO_3^- / CO_2-buffered media was recently demonstrated (Arnett and Spowage, 1996). Small shifts in extracellular pH (from 7.25 to 7.00) resulted in a steep sigmoidal response curve, suggesting that very slight alterations in ambient hydrogen ion concentration could "switch on" or "switch off" rat osteoclasts *in vitro*. Time course studies over 30 hours indicated that exposure to low pH conditions (7.06) did not activate rat osteoclasts to continue resorbing after transfer to more alkaline media (7.34), and that resorption pit formation is closely related to time spent at low pH (Spowage and Arnett, 1995).

This suggests that extracellular H^+ can act in a continuous and reversible manner. However, in longer term mature rat osteoclast cultures the effects of low pH are unclear (Murrills *et al.*, 1993; Arnett and Spowage, unpublished) The stimulatory effects of low pH have been observed in osteoclasts derived from numerous species, including mouse (Tamura *et al.*, 1993), human (Matayoshi *et al.*, 1996) and rabbit (Shibutani and Heersche, 1993). Arnett and Dempster (1987) and Walsh *et al.*, (1990) observed that embryonic chick osteoclasts are pH sensitive, although differences were reported in the optimal pH for osteoclast activity.

During acidosis, along with osteoclasts being stimulated to resorb, osteoblasts are also affected. For example, osteoblastic collagen synthesis and alkaline phosphatase activity are decreased (Bushinsky, 1995). This suggests that the cell-mediated component of the calcium efflux during acidosis in the calvarial culture system *in vitro* results from a combination of inhibited osteoblastic and stimulated osteoclastic activity (Krieger *et al.*, 1992). As with osteoclasts, osteoblasts were affected to a greater degree by metabolic acidosis than respiratory acidosis (Bushinsky, 1995). Using *in vitro* bone nodule formation as a model system, Sprague *et al.*, (1994) demonstrated that both metabolic and respiratory acidosis inhibited nodule formation, with metabolic acidosis again causing a greater effect than respiratory acidosis. Supporting these results, Kaysinger and Ramp (1998) demonstrated that the activity of cultured human osteoblasts (*e.g.* collagen synthesis, alkaline phosphatase activity

and mitosis) increased as pH increased from 7.0 to 7.6. Changes in external pH also alter the expression of genes critical to the function of mouse calvarial bone cells, predominantly osteoblasts. For example, decreasing pH to 6.8 led to a parallel reduction in egr-1 stimulation (an immediate early response gene), and an increase in pH to 7.6 led to an increase in egr-1 stimulation. RNA for type 1 collagen was also stimulated over a similar range (Frick *et al.*, 1997). Therefore, *in vivo*, metabolic acidosis may be doubly destructive for bone: not only does it inhibit osteoblastic bone formation, it also stimulates the activation of osteoclasts.

In short-term experiments, the effects of extracellular pH on mammalian osteoclastic resorption are well documented. However, the effects of extracellular pH in short-term cultures of chick osteoclasts (which show important differences to mammalian osteoclasts) are less clear. Additionally, in long-term osteoclast cultures, the effects of extracellular pH are unknown. The aim of the present study was therefore to examine the effects of extracellular protons on both short term and long term osteoclast cultures, and to examine whether the effects of pH interact with those of other osteotropic agents (*e.g.* $1,25(OH)_2D_3$ and indomethacin).

Methods

Disaggregated rat osteoclast resorption assay

The effects of various factors on resorption pit formation by mature rat osteoclasts were studied using modifications of an assay originally described by Chambers *et al.*, (1984) and Boyde *et al.*, (1984). Elephant ivory was prepared by cutting 200-300 μm thick transverse sections on a low speed saw with a diamond wafering blade (Buehler, Coventry, West Midlands) (see Figure 2.1). The ivory wafers so produced were soaked for 2 hours in distilled water to reduce brittleness and 5 mm diameter discs were then punched out using a standard, single paper hole punch (Rexel, Aylesbury, Buckinghamshire) These discs fit neatly into the wells of 96-multiwell plates. The discs were cleaned by sonication in multiple changes of distilled water and stored dry at room temperature. Before use, discs were sterilised by immersion in ethanol, allowed to air dry completely, and finally rinsed in sterile phosphate-buffered saline.

All experiments were performed using minimum essential medium (MEM) supplemented with Earle's salts, 10% foetal calf serum, L-glutamine and penicillin / streptomycin / amphotericin B. Culture medium was acidified by the direct addition of small amounts of concentrated hydrochloric acid (5-10 mEq/l H^+), as described by Goldhaber and Rabadjija (1987). This has the effect of

reducing HCO_3^- concentration and producing an operating pH close to 7.0 in a 5% CO_2 environment, which is optimal for resorption pit formation (Arnett and Spowage, 1996); further acidification in CO_2 / HCO_3^- -buffered media does not enhance resorption greatly and may ultimately reduce cell survival (Murrills *et al.*, 1998). Culture medium can also be acidified to give an operating pH close to 7.0 by increasing the concentration of CO_2 to 10% (equivalent to a partial pressure of 85mmHg), while HCO_3^- remains constant (see Figure 2.2). Clearly, it is difficult to monitor pH during the course of incubations, though PCO_2 can be measured (± 0.5%) using a Fyrite combustion kit (Jencon Instruments Ltd., Leighton Buzzard, Bedfordshire).

Mixed cell populations containing osteoclasts were obtained by rapidly mincing the pooled long bones of five, two day old rat pups killed by cervical dislocation (Sprague-Dawley strain, Biological Services, UCL) in 5 ml MEM in a non-tissue culture-treated plastic dish, triturated 20 times through a wide mouth polyethylene transfer pipette, followed by brief vortexing (10-20 seconds) in a 7 ml "bijou" tube. The resulting cell suspension was allowed to sediment for 45-50 minutes onto sterile 5mm diameter ivory discs in 96-well plates (100 µl / disc). The discs were then removed, washed in two changes of PBS before transfer to the pre-equilibrated test culture media in a 6-well plate. Each test or control well contained 4ml of MEM and 5 replicate ivory discs.

The Radiometer ABL330 blood gas analyser (Copenhagen, Denmark, see Figure 2.1) enables accurate measurement of medium pH. The ABL330 blood gas analyser is an automated micro blood gas and acid-base balance analyser. It uses a three electrode system to measure quantitatively pH, PCO_2 and PO_2 in the injected sample. The ABL330 automatically equilibrates two buffer solutions (high and low pH) by means of air and pure CO_2. The equilibrated solutions are used for electrode calibration, which was set to occur every 2 hours. However, in order to maintain the high degree of accuracy of the blood gas analyser achieved via internal calibration, the regular use of known quality control standards is also necessary. Typically, the measured pH, PCO_2 and PO_2 values are within 0.2% of the expected values.

At the end of the culture period, culture plates were sealed in a polyethylene box to prevent CO_2 loss before medium pH and PCO_2 were measured. The first blood gas measurement taken immediately after opening the box was assumed to provide a PCO_2 value valid for all test and control groups. Measured PCO_2 typically falls by ~2-3 mmHg for each subsequent reading, with a corresponding increase in measured pH, due to the rapid diffusibility of CO_2. The pH values so determined were corrected downwards to the initially measured PCO_2 value using pH - PCO_2 calibration curves recorded for the appropriate media (see Figure 2.2). After 26 hours incubation, the discs were fixed in 2% glutaraldehyde, and stained for tartrate-resistant acid phosphatase (TRAP) using Sigma kit 387-A.

The numbers of TRAP-positive multinucleated osteoclasts (two or more nuclei), were assessed 'blind' using transmitted light microscopy. Following staining with 1% toluidine blue in 1% sodium borate for 5 minutes, total cell numbers on each disc were estimated from three randomly selected 20X fields. Cells were then stripped from the discs by sonication in 0.25M ammonium hydroxide. Discrete resorption pits were counted by scanning the entire surface of each disc using reflected light microscopy (Nikon Labophot 2A, with 100W epi-illumination and metallurgical objectives, Nikon, Kingston upon Thames, UK) after restaining in 1% toluidine blue in 1% sodium borate for 2 minutes.

Disaggregated chick osteoclast resorption assay

Ivory slices were prepared as described above. All experiments were performed using minimum essential medium (MEM) supplemented with Earle's salts, 10% foetal calf serum, L-glutamine and penicillin / streptomycin / amphotericin B. Culture medium was modified by the direct addition of small amounts of concentrated hydrochloric acid (5-10 mEq/l H^+), as described by Goldhaber and Rabadjija, (1987) or hydroxyl ions as NaOH (5-15 mEq/l OH^-). Mixed cell populations containing osteoclasts were obtained by rapidly mincing the pooled long bones of four day 17 embryonic White Leghorn chicks (Poyndon Farm, St Albans, Hertfordshire), killed by decapitation in 5 ml MEM in a non-tissue culture-treated plastic dish, titrated 20 times through a wide mouth polyethylene transfer pipette, followed by brief vortexing (10-20 s) in a 7 ml "bijou" tube. The resulting cell suspension was allowed to sediment for 30-40

minutes onto sterile 5mm diameter ivory discs in 96-well plates (100 µl / disc). The discs were then removed, washed in two changes of PBS before transfer to the pre-equilibrated test culture media in a 6-well plate. Each test or control well contained 4ml of MEM and 5 replicate ivory discs. At the end of the experiment, medium pH and PCO_2 were measured using a blood gas analyser as above. Discs were fixed in 2% glutaraldehyde, and stained for TRAP. The numbers of TRAP-positive multinucleated osteoclasts (two or more nuclei), total cell number and discrete resorption pits were assessed 'blind' using transmitted and reflected light microscopy as described above.

Osteoclast formation assay

The effects of various factors on osteoclast formation were studied using modifications of an assay described previously (Takahashi *et al.*, 1988a). The long bones of two 6-8 week old mice, killed by cervical dislocation (MF1-strain, Harlan Ltd., Bicester, Oxfordshire) were fragmented in 5ml unmodified MEM, followed by vortexing for 1 minute.

The resulting cell suspension was allowed to sediment for 24 hours onto sterile 5mm diameter ivory discs, pre-wetted with 50µl MEM, in 96-well plates. Total viable cell counts were calculated using trypan blue stain and a hemocytometer. Generally, for a density of 5 - 6 x 10^6 cells, 100µl cell suspension / disc was used. Ivory discs were then removed and placed in test or

control medium in a 6-well plate. Each test or control well contained 4ml of MEM supplemented with Earle's salts, 10% foetal calf serum, L-glutamine, penicillin / streptomycin / amphotericin B, 10nM $1,25(OH)_2D_3$, 10nM dexamethasone, and 5 replicate ivory discs.

The cultures were incubated in a humidified atmosphere of 5% CO_2 / 95% air, with medium changes every 2 to 3 days. Medium pH and PCO_2 were monitored during and at the end of experiments using a blood gas analyser, as above. After 10 to 14 days incubation, the discs were fixed in 2% glutaraldehyde, and stained for TRAP. A control group of ivory discs was also removed, fixed and stained after 3 days incubation to check for the presence of any mature osteoclasts that might have been released during the initial cell preparation.

The total area occupied by TRAP-positive multinucleated osteoclasts and resorption pits was assessed 'blind' by transmitted and reflected light microscopy, via output from a colour video camera (DXC-151A; Sony Corporation, Tokyo, Japan), using standard 'dot count' morphometry (Lennox, 1975). Area measurements, rather than discrete cell and pit counts, were necessary because this assay system requires high cell densities to function: large semi-contiguous groups of TRAP-positive osteoclasts are then generated, associated with extensive and often conjoined areas of resorption.

Calvarial resorption assay

Bone resorption can be conveniently measured by the release of calcium from explants of neonatal mouse calvaria in culture. Calvaria were dissected from five day old mice (MF1-strain, Harlan Ltd., Bicester, Oxfordshire) killed by cervical dislocation. The dissected calvaria were washed in Hanks Balanced Salt Solution (HBSS), before being divided along the sagittal suture and placed in fresh HBSS. Each half calvarium was cultured separately on a stainless steel grid (1cm^2 Minimesh) in 1.5ml of Biggers, Gwatkin and Heyner medium (BGJ) medium with 5% heat inactivated FCS. The bones were incubated for 24 hours at 37°C in a humidified atmosphere of 5% CO_2 / 95% air. The 24 hour pre-incubation is included to enable the pre-equilibration of bones with the medium, and to permit calcium exchange to reach an equilibrium. Bones killed by three freeze-thaw cycles in liquid nitrogen were used to indicate physicochemical Ca^{2+} release during the culture period.

Following the 24 hour pre-incubation, medium was removed and replaced with fresh medium containing the test substances. Media were acidified by adding 0-20 mEq/l H^+ as HCl. Cultures were incubated for a further 48 hours and resorption was measured as the release of calcium into the culture medium over this period. Resorption in test cultures was compared to that in control cultures containing BGJ alone. Each experimental group contained five separate calvarial cultures. Incubator CO_2 was monitored during the experiment using a Fyrite combustion kit as previously mentioned. Culture medium pH and PCO_2

were measured in test and blank media at the end of experiments using a blood gas analyser, as above.

At the end of the incubation period, calcium concentrations in the culture medium were measured colorimetrically on an autoanalyser (Chem Lab Instruments, Hornchurch, Essex), by using the metal complexing dye cresolphthalein complexone. The absorbance of the resultant purple-coloured solution was measured at 570nm. Calcium concentrations were calculated from the absorbance peak heights measured against a standard curve. Calvaria were fixed for 10 minutes in 95% ethanol / 5% acetic acid before being stained for TRAP (Holt and Marshall, 1998), and mounted in melted glycerol jelly. The numbers of TRAP-positive multinucleated osteoclasts (two or more nuclei), were assessed 'blind' on coded samples using transmitted light microscopy. Dissection of calvaria and calcium measurements were performed by Dr S Meghji (Eastman Dental Institute, London, U.K.).

Solutions

Fresh stock solutions of ATP, bovine parathyroid hormone fragment 1-34 (PTH) and salmon calcitonin (sCT) were prepared in phosphate buffered solution (PBS) for each experiment. Stock solutions of dexamethasone, $1,25(OH)_2D_3$, prostaglandin E_2 (PGE_2) and indomethacin were prepared in ethanol and stored for short periods at -20ºC. The final concentration of ethanol in test and control culture medium was constant at 0.2%. ATP solutions were titrated to pH 7.0

with NaOH immediately before use to avoid unwanted pH effects on osteoclast function (Arnett and Spowage, 1996).

Statistics

Statistical comparisons were made by one-way analysis of variance using the statistical computer package 'Minitab'. Data are presented as mean ± SEM for 5 or 6 replicates.

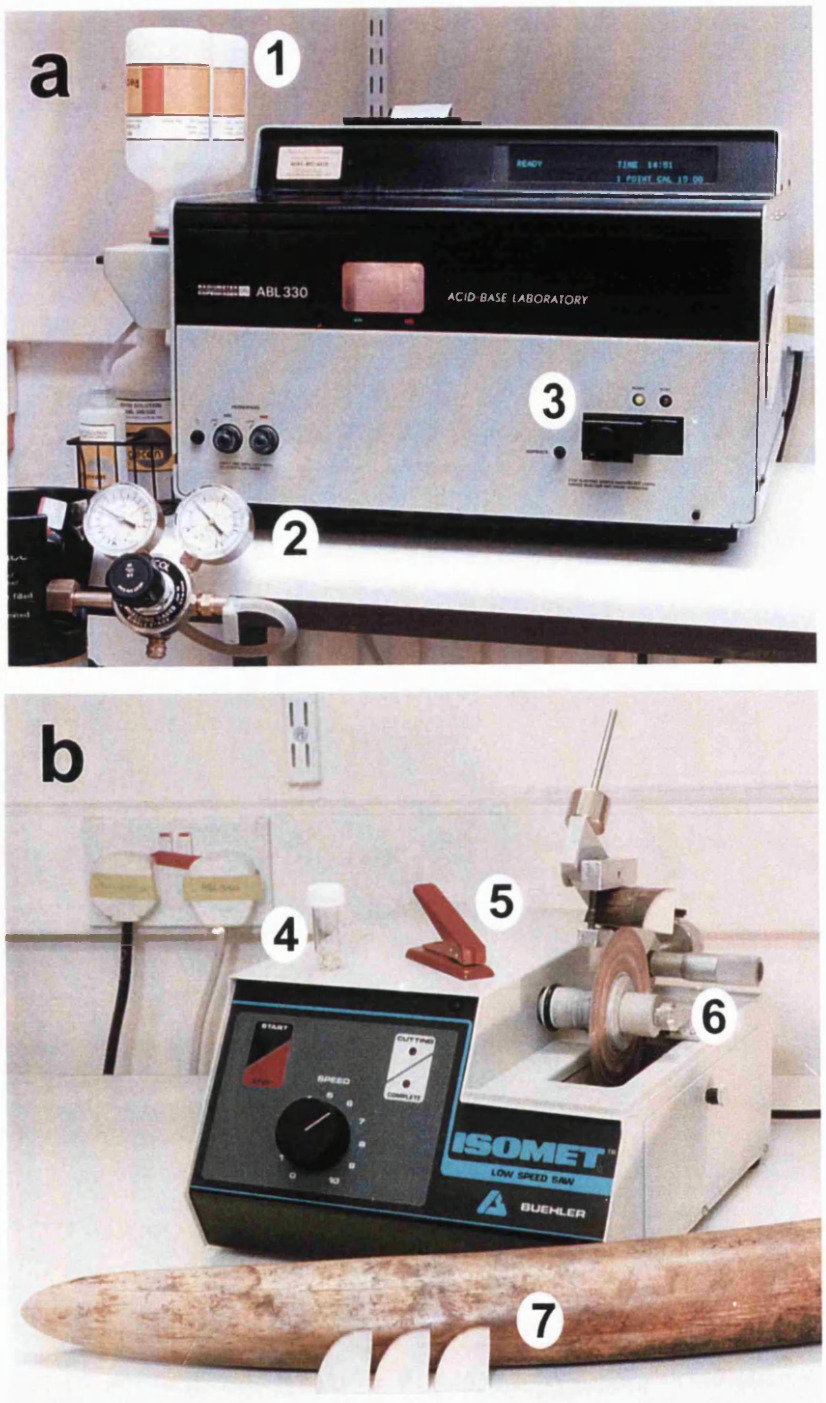


Figure 2.1 Photographic illustration of (a) ABL330 blood gas analyser and (b) Isomet low speed saw with ivory discs shown at different stages of preparation.

Key : 1) High-low pH calibrating solutions;
2) Carbon dioxide supply; 3) Sample injection point;
4) Ivory discs; 5) 5mm hole punch; 6) Low speed blade;
7) Elephant ivory.

Results

Relationship between pH, P_{CO_2} and $[HCO_3^-]$ in tissue culture media

Figure 2.2 demonstrates the relationship between pH, P_{CO_2} and $[HCO_3^-]$ in tissue culture media. Unmodified MEM with Earle's salts and 10% foetal calf serum (FCS) results in an operating pH of about 7.2 in a 5% CO_2 atmosphere. Increasing the concentration of CO_2 to 10% (equivalent to a partial pressure of 85mmHg), while HCO_3^- remains relatively constant, acidifies the medium to give an operating pH close to 7.0. However, for osteoclast culture a reduced operating pH is more conveniently achieved by adding concentrated HCl directly to media containing Earle's salts and 10% FCS. Addition of 41µl of 12 M HCl to 50 ml of culture medium provides 10 mEq/l H^+ / l and results in an operating pH of about 7.0 in a 5% CO_2 atmosphere. Medium containing a very low bicarbonate concentration (Hanks' salts) is intended for use with atmospheric air. Figure 2.2 demonstrates that when used in a tissue culture incubator containing 5% CO_2, this medium becomes strongly acidic.

Modulation of chick osteoclasts by changes in extracellular pH

Embryonic chick osteoclasts exhibit a similar sigmoidal pH response curve to mature rat osteoclasts (Figure 2.3 & Figure 2.4). However, chick osteoclasts differ from rat osteoclasts in that the response curve is shifted about

0.1 pH units upwards, and is slightly less steep (Arnett and Spowage, 1996). Addition of 10 mEq/l H^+ to the culture medium reduced the final pH from 7.164 to 6.984 (PCO_2 = 41.4 mmHg), which increased the number of pits per disc from 74 to 125 ($p<0.01$), addition of 5 mEq/l H^+ reduced the final pH to 7.075, and resulted in an increase in the number of pits per disc to 97. Addition of 5, 10 and 15 mEq/l OH^- (as NaOH) increased the final pH from 7.164 to 7.274, 7.355 and 7.408 respectively. This resulted in a reduction in the number of pits per disc from 74 to 26, 16 and 12 respectively ($p<0.01$). The greatest change in the numbers of resorption pits formed was associated with a pH difference of only about 0.11 unit, between final pH values of 7.274 and 7.164. Over this range, the average number of resorption pits increased nearly 3-fold, and the average number of pits per osteoclast increased about 2.5-fold.

Long term effects of extracellular protons on mature rat osteoclasts

The stimulation of mature rat osteoclast resorption by extracellular protons does not diminish with time in culture (Figure 2.5). There was no indication of "tachyphyllaxis", such that after 7 days a pH decrease of about 0.1 unit caused a 15-fold increase in resorption pit formation (20.6 compared to 308.2; $p<0.01$). The results show clearly that small pH shifts were not associated with any long term changes in osteoclast number. At all time points resorption pit number was significantly greater in acidified medium compared to control (non-acidified) medium ($p<0.01$). PCO_2 was 46.7 ± 1.8 mmHg over the 7 day period.

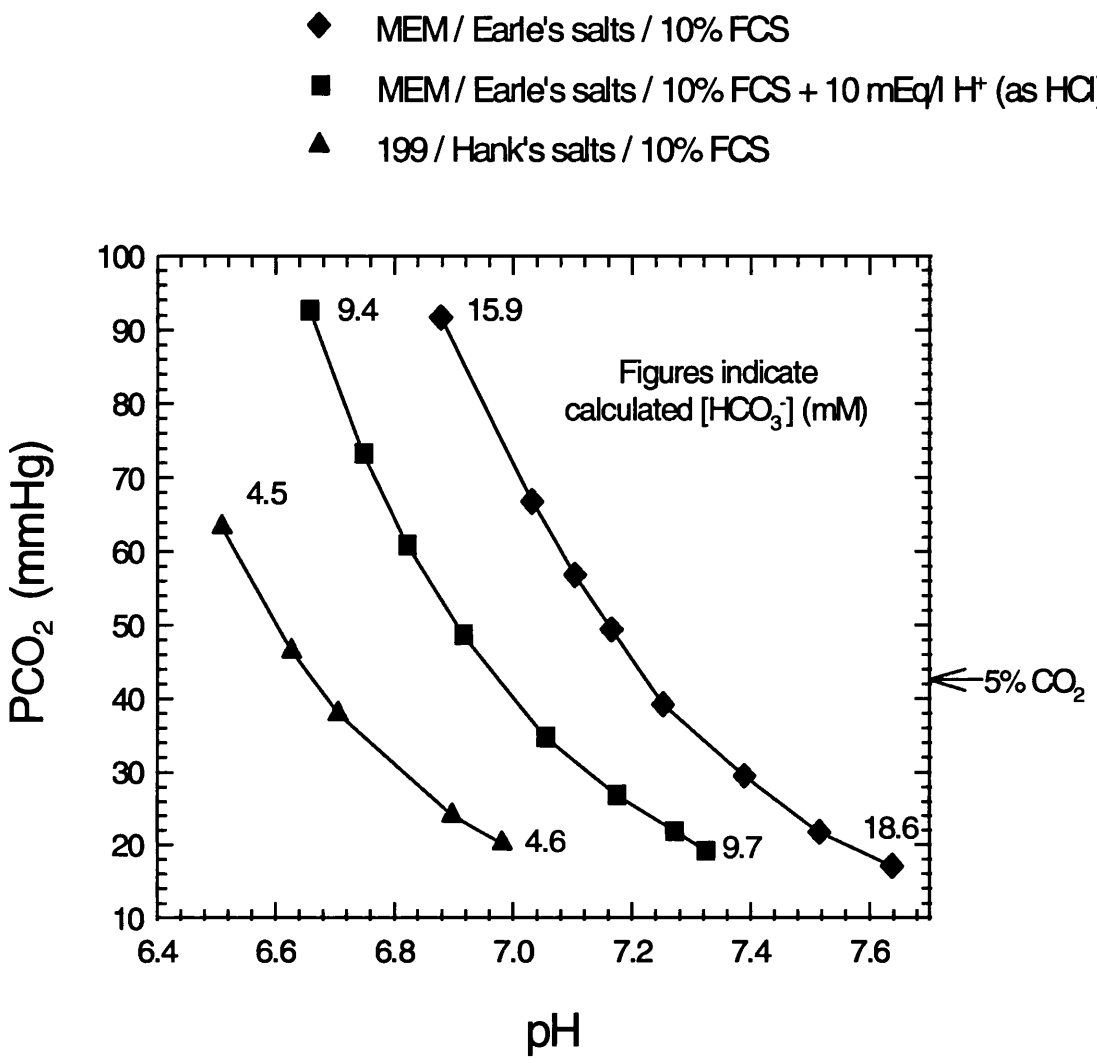


Figure 2.2 Relationship between pH, P_{CO_2} and $[HCO_3^-]$ in tissue culture media.

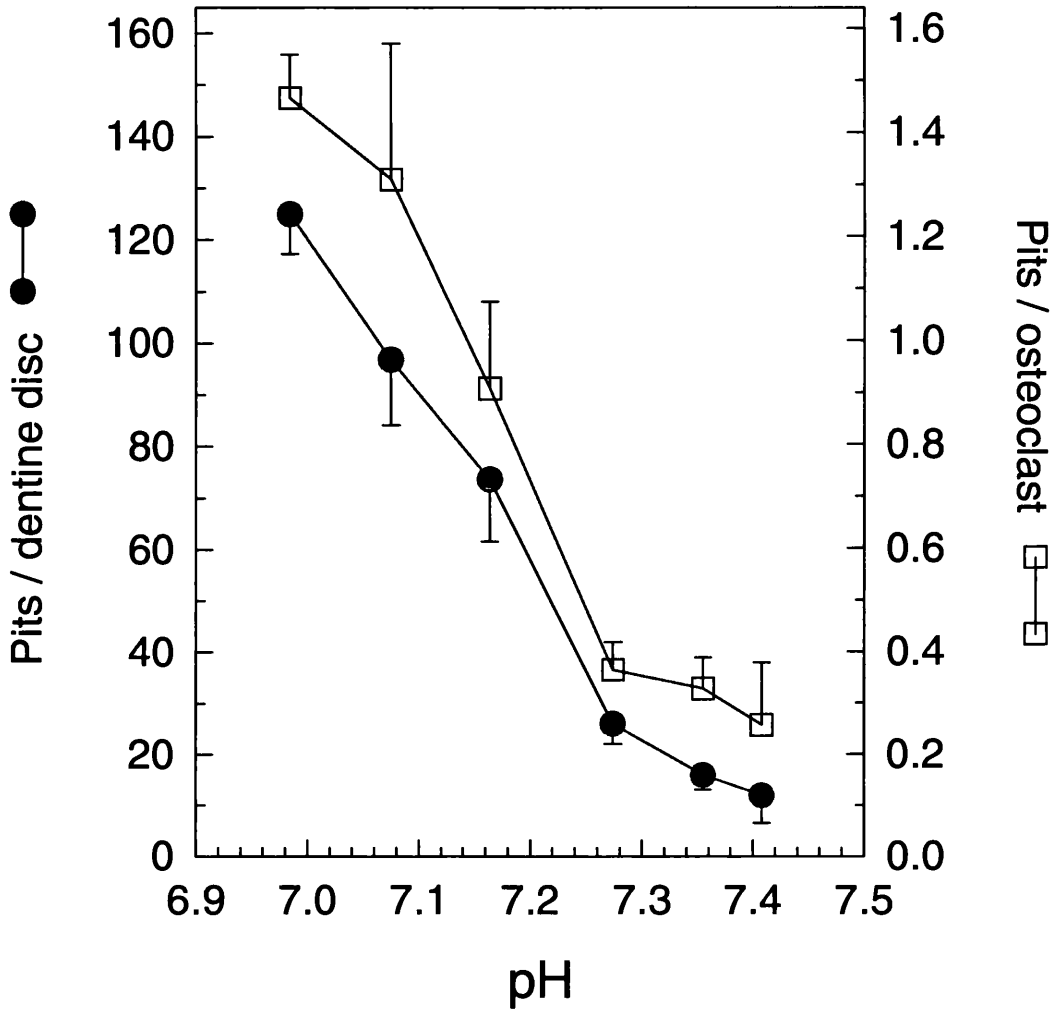


Figure 2.3 Modulation of chick osteoclast resorption pit formation by small changes in extracellular pH. Closed circle plots (left to right): effect of adding 10, 5, 0 mEq/l H^+, and 5, 10 and 15 mEq/l OH^- to culture medium on pit number per dentine disc; open square plots: effect on pits per osteoclast. HCO_3^- concentrations were 9.5, 11.8, 14.3, 19.1, 23.6 and 27.1 mmol/l respectively. Cells were cultured on 5mm dentine discs in for 26h. Values are means ± SEM (n = 5). PCO_2 was 41.4 mmHg. For clarity, symbols denoting statistical significances are omitted.

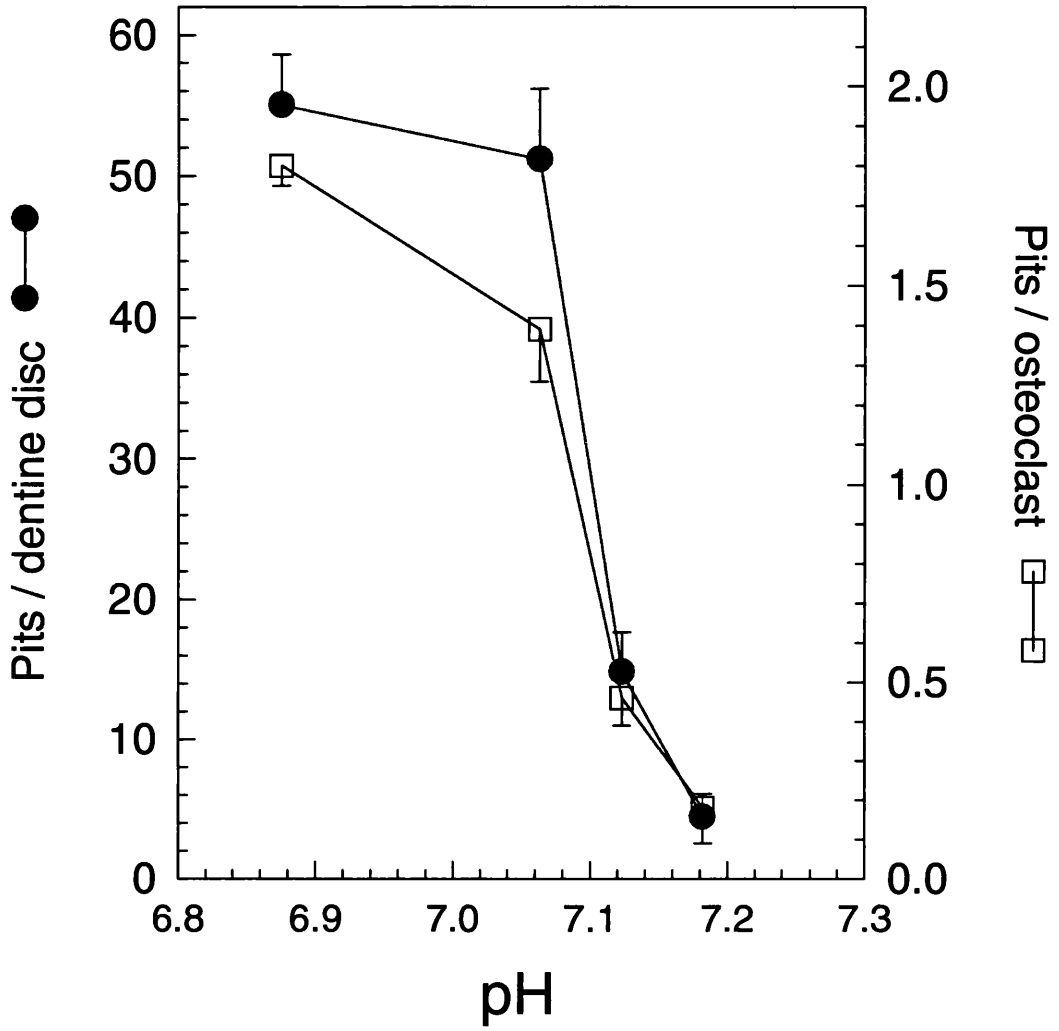


Figure 2.4 "On-off" switching of bone resorption by rat osteoclasts, associated with small extracellular pH differences, in low density 26h cultures, . Closed circle plots (left to right): effect of adding 10, 5, 2.5 and 0 mEq/l H^+ to culture medium. Values are means ± SEM (n = 5). For clarity, symbols denoting statistical significances are omitted. Reproduced with the permission of Dr. T.R. Arnett, UCL.

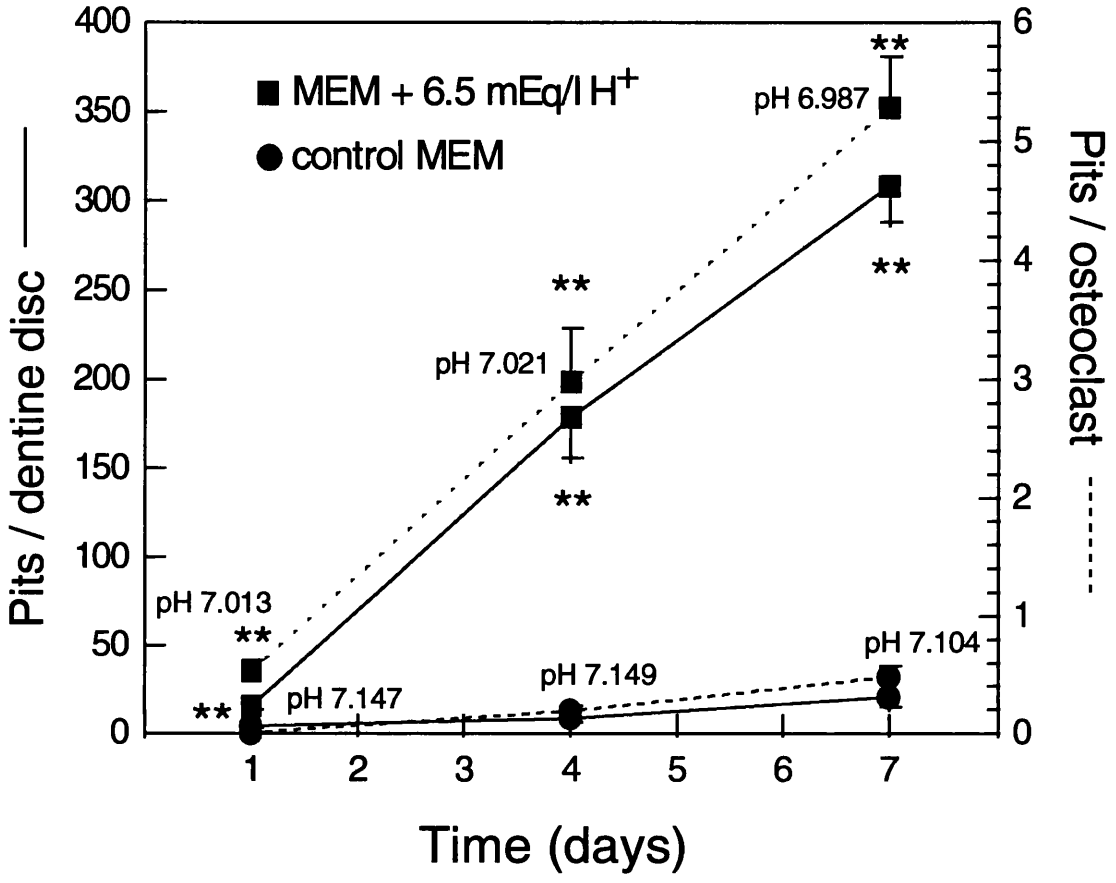


Figure 2.5 Long-term activation by small decreases in extracellular pH of mature rat osteoclasts cultured on 5mm dentine discs . PCO_2 was 46.7 ± 1.8 mmHg over the 7 day period. Cell numbers were not significantly altered over time. Values are means ± SEM (n = 5); ** $p<0.01$ vs. control MEM.

Interaction of extracellular pH with $1,25(OH)_2D_3$

The interaction between the effects of pH and $1,25(OH)_2D_3$ were investigated using rat osteoclast cultures incubated for 26 hours with 10 nM $1,25(OH)_2D_3$ in either control (non-acidified) or acidified MEM (Figure 2.6). In the absence of $1,25(OH)_2D_3$, acidification (pH reduction from 7.215 to 6.922) produced a 4.5-fold stimulation of resorption pit formation ($p<0.01$). In non-acidified medium $1,25(OH)_2D_3$ caused a 2.5-fold stimulation of pit formation. However, in the presence of 10 nM $1,25(OH)_2D_3$ the acid effect was further enhanced, with a 12-fold stimulation compared with control ($p<0.01$). Thus, the stimulatory effect of $1,25(OH)_2D_3$ was much greater in the acid conditions than in non-acidified condition.

The interaction between the effects of extracellular protons and indomethacin, ATP, and $1,25(OH)_2D_3$ were investigated using rat osteoclast cultures incubated for 26 hours with 2 μM indomethacin, 2 μM ATP and 10 nM $1,25(OH)_2D_3$ in acidified MEM (Figure 2.7). Acidification (pH reduction from 7.214 to 6.983) produced a 2-fold stimulation of resorption pit formation compared to non-acidified control ($p<0.01$). With the addition of 2 μM indomethacin, 2 μM ATP or 10 nM $1,25(OH)_2D_3$ to acidified cultures, resorption was further stimulated. Indomethacin, ATP, and $1,25(OH)_2D_3$ gave similar levels of resorption pit formation (29.13 ± 1.47 *vs* acid control 19.6), and had similar ratios of number of pits per osteoclast (1.83 ± 0.03 *vs*. acid control 1.18). Including 2 μM indomethacin, 2 μM ATP, and 10 nM $1,25(OH)_2D_3$ together in

acidified cultures resulted in a 2.4-fold stimulation compared to acid control ($p<0.01$), and an average 1.6-fold stimulation of resorption pit formation compared to indomethacin, ATP, or $1,25(OH)_2D_3$ individually.

Effects of extracellular pH on osteoclast formation

To obtain reliable and consistent levels of osteoclast formation and bone resorption using the murine marrow assay, it has been necessary to alter certain aspects of the original method (Takahashi *et al.*, 1988a). It was found that culturing the marrow cells for the first 24 hours in 96-well plates increased the number of cells adhered to the ivory discs. Cell density appears to be especially important in this assay as osteoclast formation only occurs in areas of high cell concentration. However, due to the small media volume (150µl) and large cell count, it was important to avoid excessive media acidification. This was achieved by transferring the ivory discs to 6-well plates which contained a large volume of media (*e.g.* approximately 1 ml / 5mm disc). Large volumes of media coupled with regular medium changes reduce the amount of cell-mediated acidification, and avoid medium exhaustion. As the murine marrow assay is "serum sensitive", it is also suggested that several batches of serum are screened to determine that which provides the best results. Screening of six batches of serum gave a 5-fold variation in pit area (data not shown). As long bones were fragmented and not simply flushed as mentioned above, it was necessary to ascertain that mature osteoclasts had not been released. Therefore several discs were examined at day 3 of culture . No osteoclasts or resorption pits were ever

observed at this 3 day stage, indicating that osteoclasts and resorption pits observed after 10 to 14 days in culture resulted entirely from the formation of new osteoclasts.

The following results demonstrate the overall inhibitory action of low pH on osteoclast formation in murine marrow cultures. Increasing the final extracellular pH from 7.107 to 7.150 (Figure 2.8) by the addition of 5 mEq/l OH^- (as NaOH) resulted in a 2-fold increase ($p<0.01$) in the area covered by TRAP-positive multinucleate cells, but a 7-fold decrease ($p<0.01$) in resorption pit area (*i.e.* the ratio of pit area : TRAP-positive area decreased from 0.7 to 0.06), over the 10 day culture period (PCO_2 was 45.7 mmHg). Addition of 5 and 10 mEq/l H^+ (as HCl) increased the final pH from 7.107 to 7.000 and 6.893 respectively. This resulted in a reduction in the area covered by TRAP-positive multinucleate cells from 49.5 to 21 and 7.3 $\mu m^2 \times 10^{-5}$ respectively ($p<0.01$ for 10 mEq/l). However, although addition of 5 and 10 mEq/l H^+ resulted in a reduction in the area covered by TRAP-positive multinucleate cells, the ratio of pit area : TRAP-positive area remained relatively unchanged (0.68 ± 0.01).

To determine if the TRAP- positive cells formed in response to added OH^- were indeed functional osteoclasts, mouse marrow cultures were incubated at alkaline pH (7.41) for 10 days, before transferring the cells to an acidified medium (pH 7.03) for a further 4 days (Figure 2.9). Incubation for 14 days at pH 7.41 resulted in abundant TRAP-positive multinucleate cell formation (43.4

μm^2 x 10^{-5}), but almost no resorption (0.29 μm^2 x 10^{-5}). However, in cultures maintained in alkaline medium for 10 days followed by 4 days in acidified medium, formation of TRAP-positive multinucleate cells was similar to 14 days at pH 7.41 (47.7 μm^2 x 10^{-5}), but resorption pit formation was increased to 27.0 μm^2 x 10^{-5}, a 93-fold increase ($p<0.01$). As previously described (Figure 2.8), continuous incubation in acidified media (pH 7.01) for 14 days reduced TRAP-positive multinucleate cell formation (16.1 μm^2 x 10^{-5}; $p<0.01$), but further increased the ratio of pit area : TRAP-positive area to 1.2.

Effects of extracellular pH on calvarial resorption

In the calvarial system, stimulation of calcium release by added protons closely paralleled the effect of extracellular protons on pit formation by isolated mature rat osteoclasts. Increasing the proton concentration from 0 to 15 mEq/l decreased extracellular pH from 7.21 to 6.94 and resulted in an 8.3-fold increase in calcium release from 0.62 to 5.12 mg/l (Figure 2.10). Calcium release measurements from dead bones suggested that a relatively small physicochemical element may be involved at large acid levels. The largest effects observed with proton stimulation (15 mEq/l H^+) were comparable to the maximal calcium release evoked by 10 nM $1,25(OH)_2D_3$, 10 nM 1-34 PTH, or 1 μM PGE_2 (Figure 2.11).

Effects of calcitonin and indomethacin on calcium release from calvaria

The increase in calcium release stimulated with 15 mEq/l H^+ was reduced to control levels in the presence of 10 ng/ml salmon calcitonin (sCT), implying osteoclast-mediated resorption (Figure 2.12). Addition of indomethacin (a cyclooxygenase inhibitor) to acid-stimulated (12 mEq/l H^+) calvaria blocked calcium release (Figure 2.13). 0.01, 0.1 and 1 μM indomethacin resulted in a 1.9, 3, and 3.8-fold reduction in calcium release from acid-stimulated calvaria respectively. 1μM indomethacin inhibited acid-stimulated calcium release to a level equivalent with that seen in non-acidified control cultures.

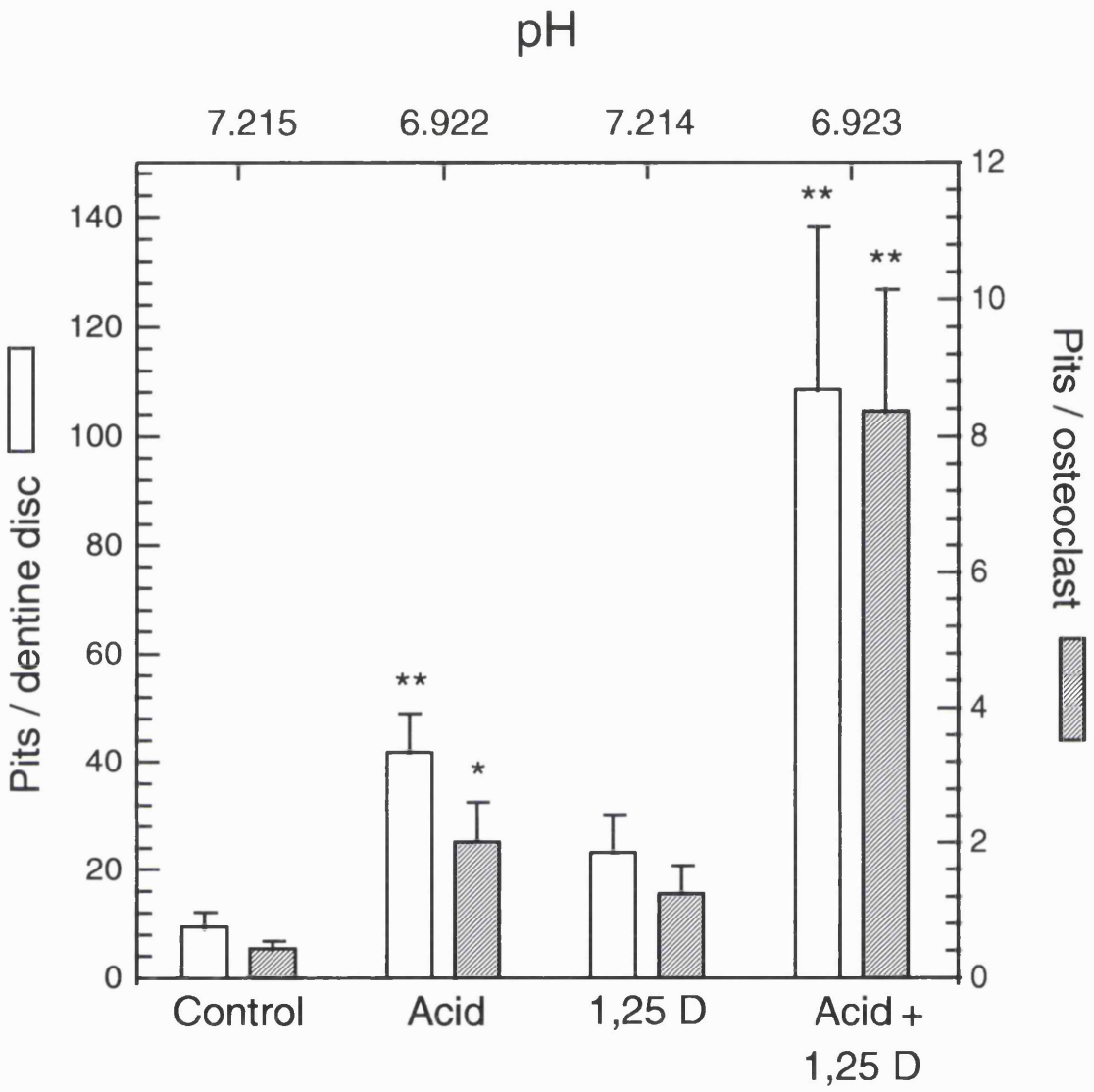


Figure 2.6 Interaction of the effects of $1,25(OH)_2D_3$ (1,25 D; 10 nM) on resorption pit formation by rat osteoclasts cultured in unmodified medium (Control) or in acidified medium (Acid) for 26 h, showing potentiation of $1,25(OH)_2D_3$-stimulated resorption at low pH. PCO_2 was 42.3 mmHg. Values are means ± SEM (n = 5); * $p<0.05$; ** $p<0.01$ with respect to control.

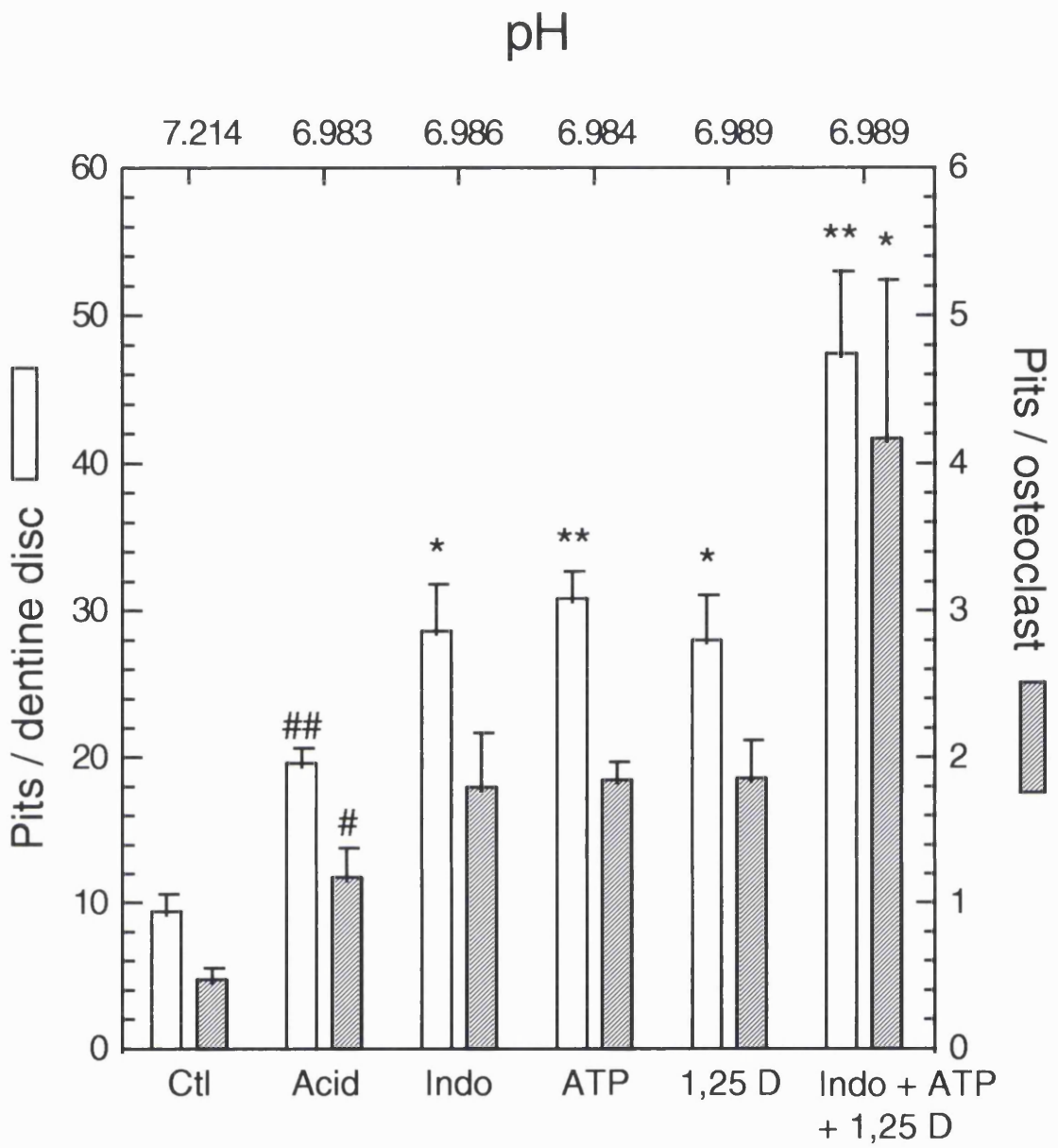


Figure 2.7 Further stimulation of resorption pit formation by rat osteoclasts cultured for 26 h in acidified medium (# $p<0.05$; ## $p<0.01$ with respect to pH 7.214 control value (Ctl)) by indomethacin (Indo; 2μM), ATP (2μM) and $1,25(OH)_2D_3$ (1,25 D; 10 nM). PCO_2 was 36.6 mmHg Values are means ± SEM (n = 5); * $p<0.05$; ** $p<0.01$ with respect to Acid.

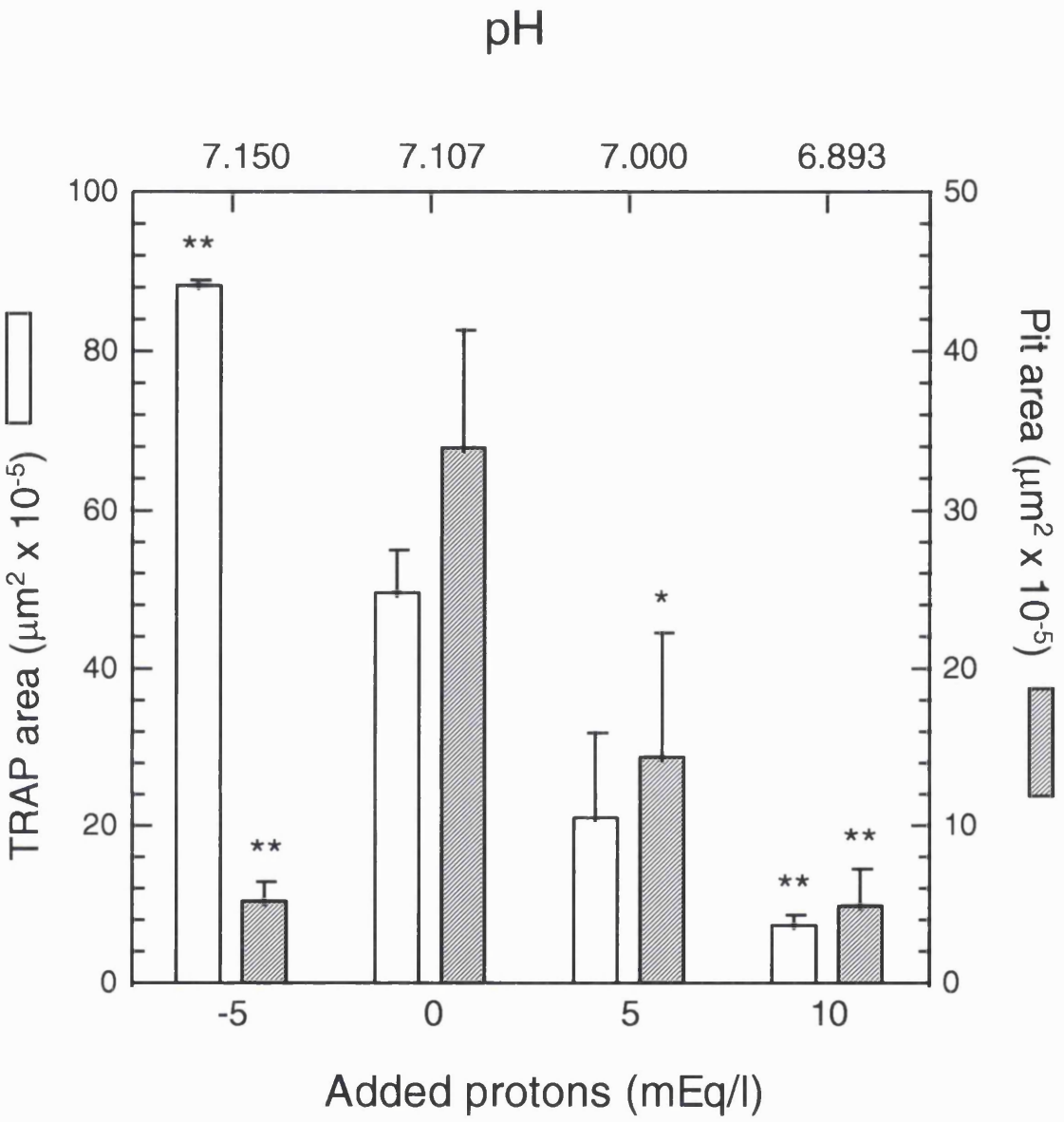


Figure 2.8 Effect of extracellular pH on osteoclast formation and activation in 10 day cultures of mouse marrow. -5 mEq/l H^+ signifies the addition of 5 mEq/l OH^- as NaOH. Stromal cell numbers were unaffected. PCO_2 was 45.7 mmHg. Values are means ± SEM (n = 5); * $p<0.05$ vs control; ** $p<0.01$ vs. Control.

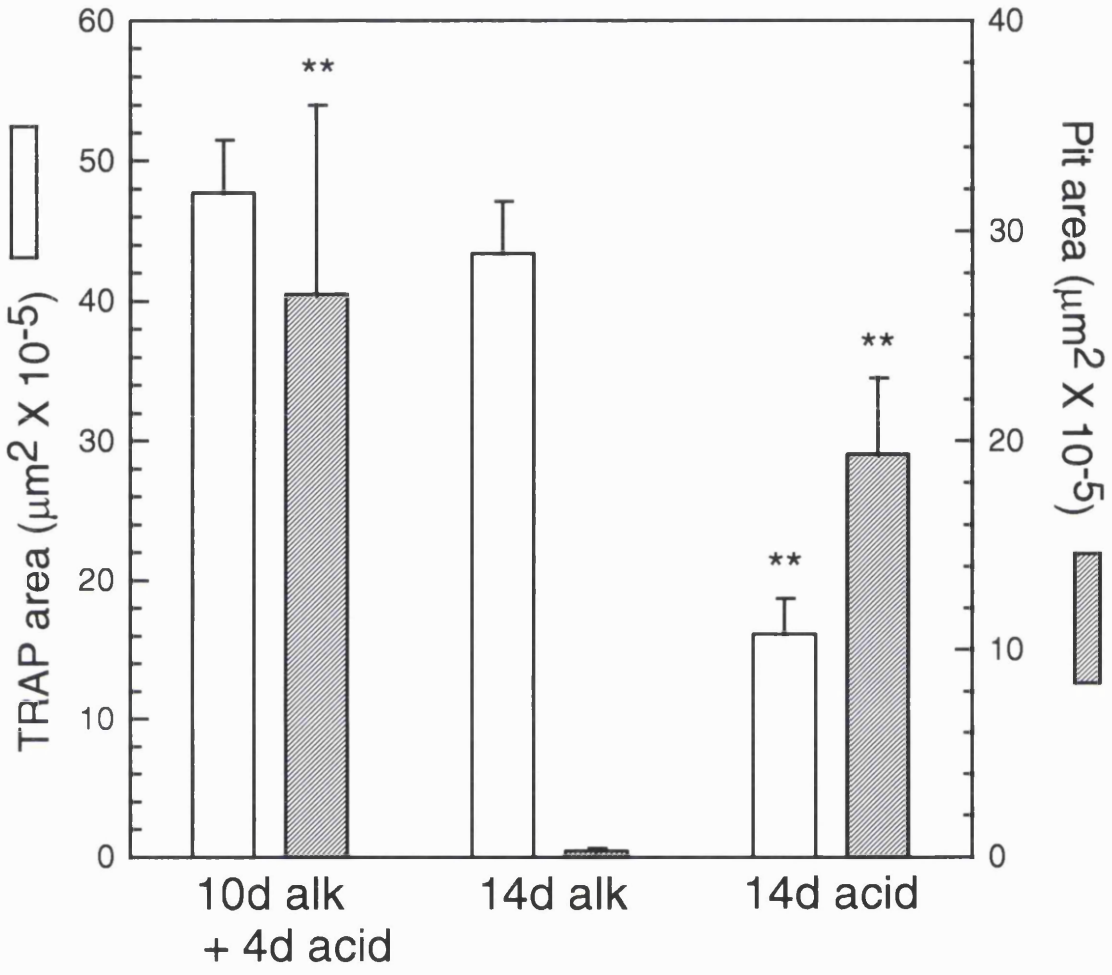


Figure 2.9 Effect of switching from alkaline to acidified media on osteoclast formation in mouse marrow cultures. Cultures were incubated on 5mm dentine discs for (left to right) 10 days in alkaline medium (alk) (pH 7.41) before transfer to acid medium (acid) (pH 7.03) for 4 days; 14 days in alkaline medium (pH 7.41); 14 days in acidified medium (pH 7.01). PCO_2 was 41.6 ± 1.64 mmHg over the 14 day period. Values are means ± SEM (n = 5); ** $p<0.01$ vs 14d alkaline medium.

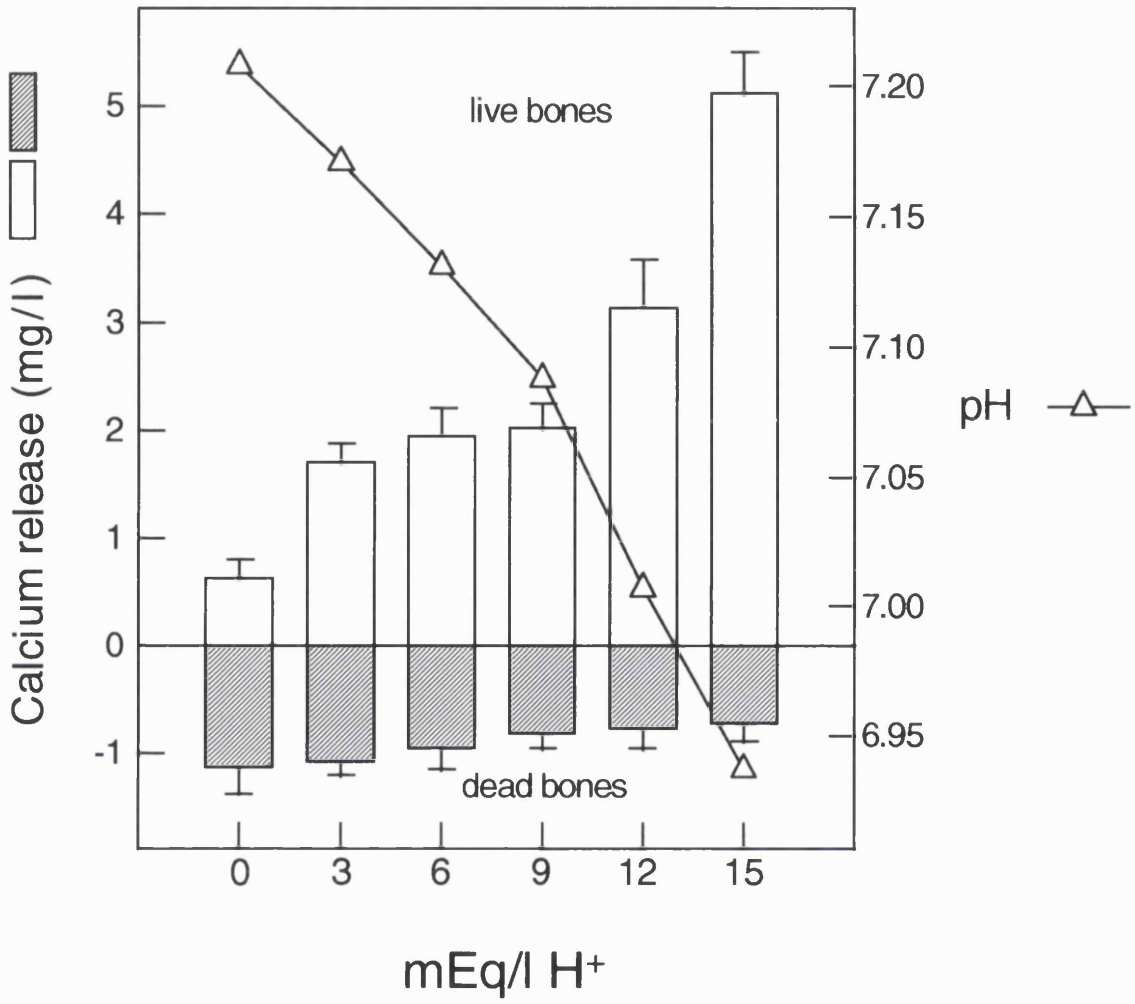


Figure 2.10 Modulation of calcium release from 3 day mouse calvarial cultures by small changes in extracellular pH. Decreasing extracellular pH from control value (7.208) by addition of 3, 6, 9, 12 and 15 mEq/l H^+ to culture medium increased calcium release from 0.62 to 1.71, 1.95, 2.03, 3.14 and 5.12 respectively. HCO_3^- concentrations were 13.2, 12.2, 10.9, 10.4, 8.5 and 7.1 mmol/l respectively. Over the same pH range, devitalised bones (hatched bars) decreased calcium adsorption from 1.13 to 1.08, 0.95, 0.82, 0.77 and 0.72 respectively. PCO_2 was 36.3 mmHg. Values are means ± SEM (n = 5).

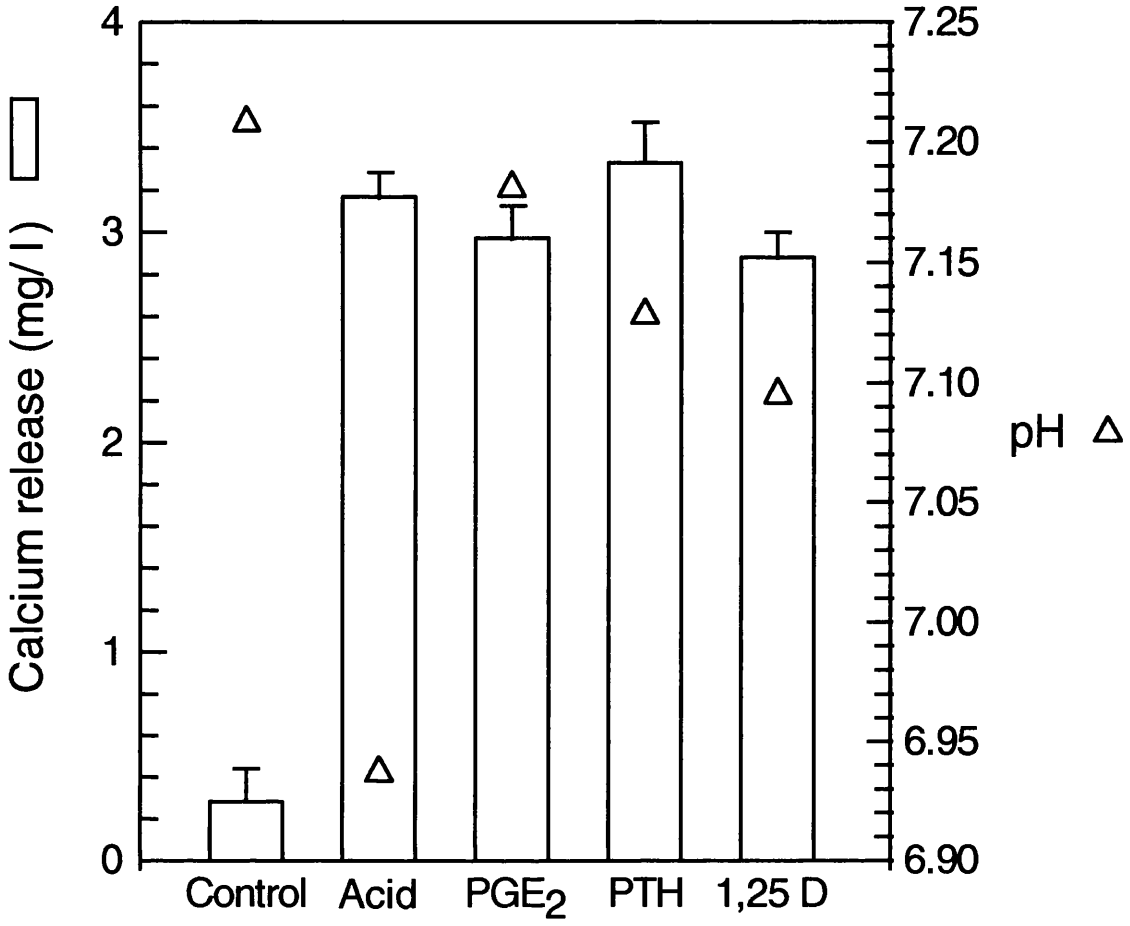


Figure 2.11 Comparison of the effects of HCO_3^- acidosis (acid; 15 mEq/l H^+) with maximal doses of PGE_2 (1μM), parathyroid hormone (PTH; 10 nM), and $1,25(OH)_2D_3$ (1,25 D; 10 nM) on calcium release from 3 day mouse calvarial cultures. PCO_2 was 36.3 mmHg. Values are means ± SEM (n = 5).

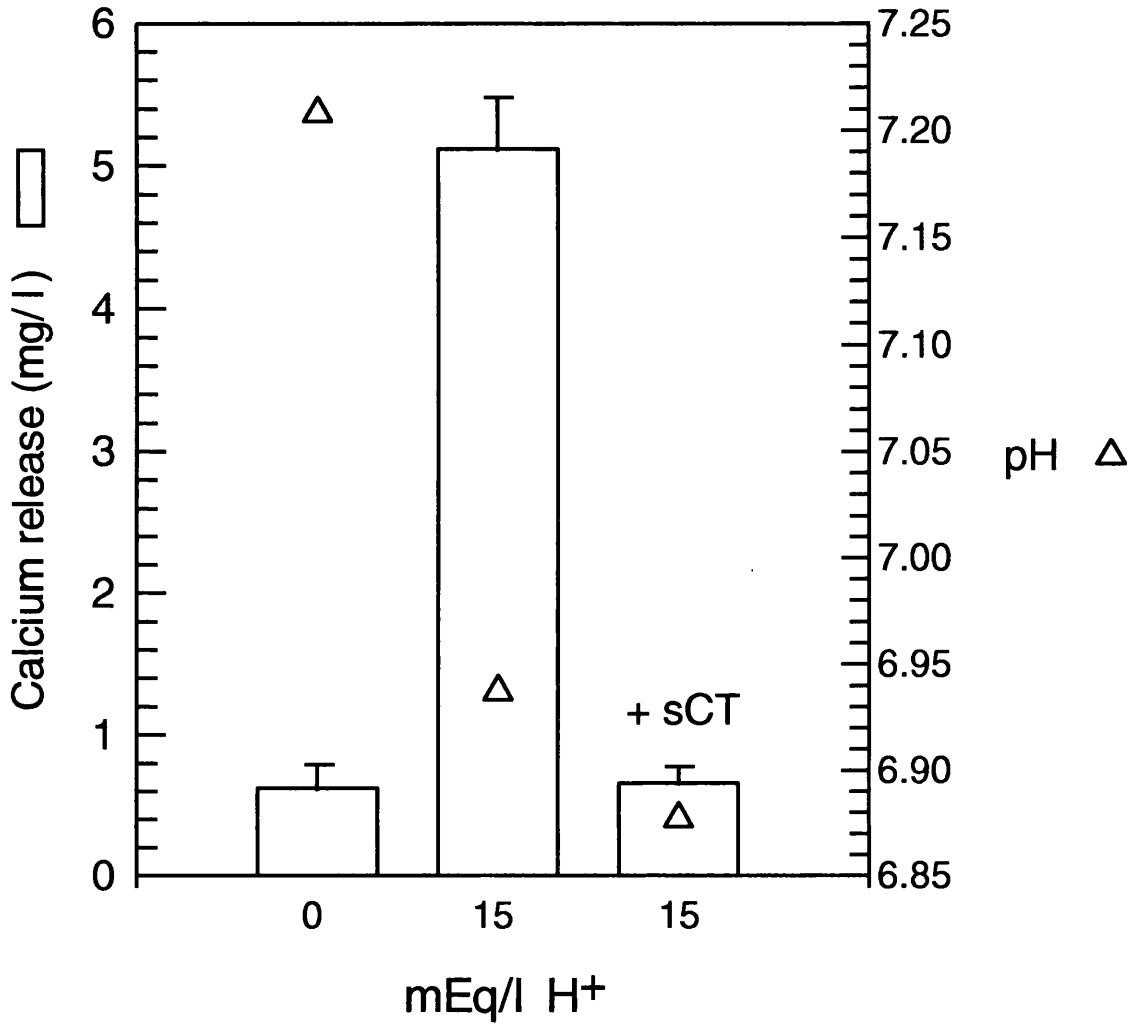


Figure 2.12 Inhibition of HCO_3^--acidosis stimulated calcium release from 3 day mouse calvarial cultures back to control (non-acidified) levels by salmon calcitonin. PCO_2 was 36.3 mmHg. Values are means ± SEM (n = 5).

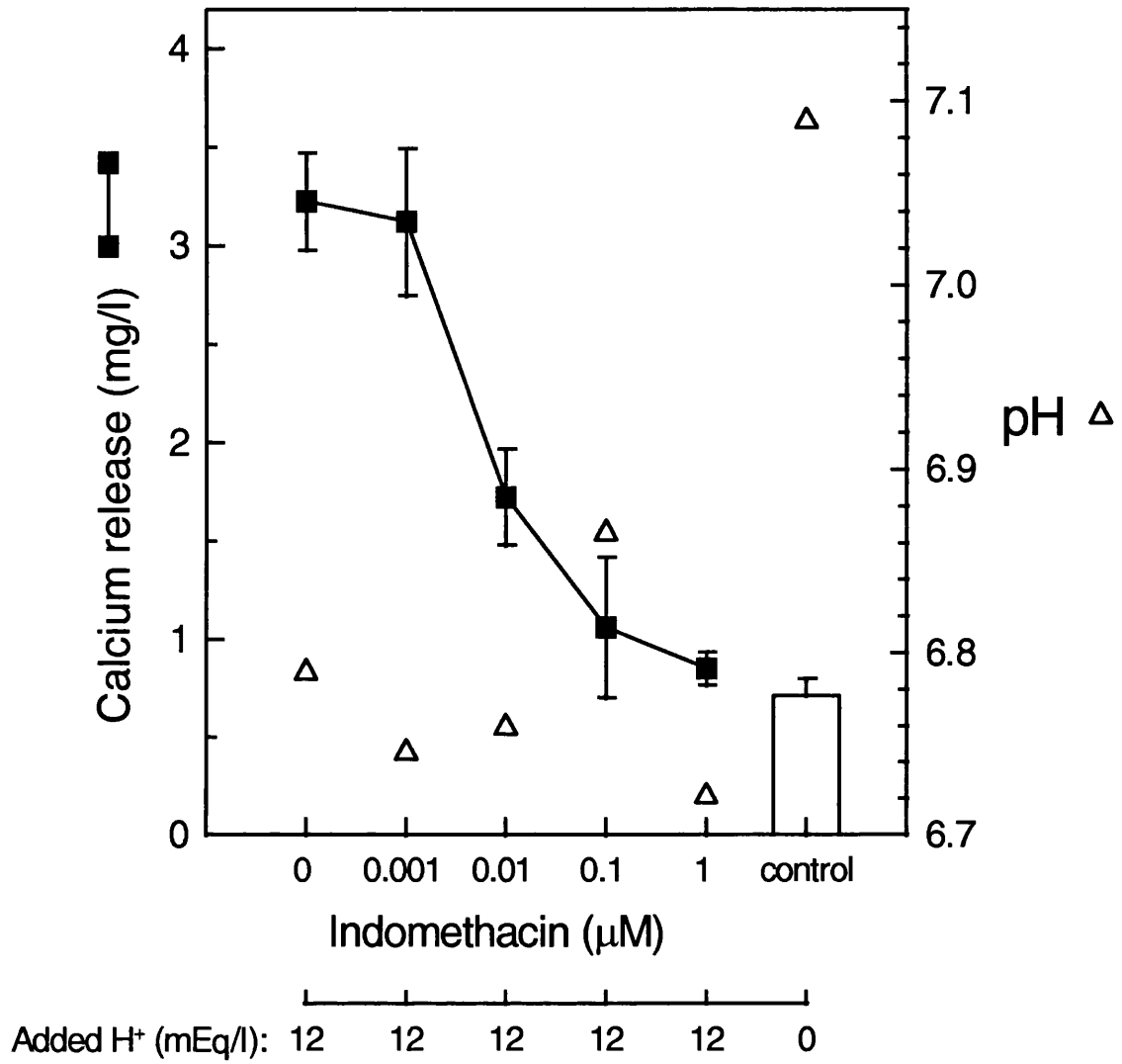


Figure 2.13 Inhibition of proton stimulated (12 mEq/l H^+) calcium release from 3 day mouse calvarial cultures back to control (non-acidified) levels by indomethacin. PCO_2 was 64.0 mmHg. Values are means ± SEM (n = 5).

Effects of respiratory and metabolic acidosis on calcium release from calvaria

To compare the effects of metabolic or respiratory acidosis on calcium release from calvaria (Figure 2.14), culture medium was acidified either by the addition of 4, 8, 12 and 16 mEq/l H^+ as HCl, which reduced the HCO_3^- concentration of the media from 15.8 to 13.5, 11.3, 9.7 and 8.4 mM respectively (a model of metabolic acidosis), or by increasing incubator CO_2 to a nominal 5, 10 and 15% (actual PCO_2 values: 54.5, 87.2 and 108.5 mmHg respectively) to produce a model of respiratory acidosis. Acidification of culture medium by increasing PCO_2 values produced markedly lower calcium releases at comparable pH values to metabolic acidosis. For example, pH 6.85 (achieved by the addition of 16 mEq/l H^+) resulted in a calcium release of 3.17 mg/l, while pH 6.81 (achieved by increasing incubator PCO_2 to 108.5 mmHg) resulted in a calcium release of only 1.49 mg/l, compared to a control value in non-acidified medium of 0.28 mg/l. Devitalised bones adsorbed 0.05 mg/l calcium at pH 6.81.

TRAP-stained half calvaria from cultures treated with 12 to 16 mEq/l H^+, 10nM $1,25(OH)_3D_3$, 10nM 1-34 PTH or 1μM PGE_2 (Figure 2.15) demonstrated large amounts of osteoclastic resorption, with frequent perforations. However, counts of the number of multinucleate cells present on each half calvaria (Figure 2.16) demonstrated that whereas $1,25(OH)_2D_3$ increased cell number compared to control (~400 to ~700; $p<0.01$), PGE_2 had no effect, and 15 mEq/l HCl caused a decrease to ~200 TRAP positive cells ($p<0.01$).

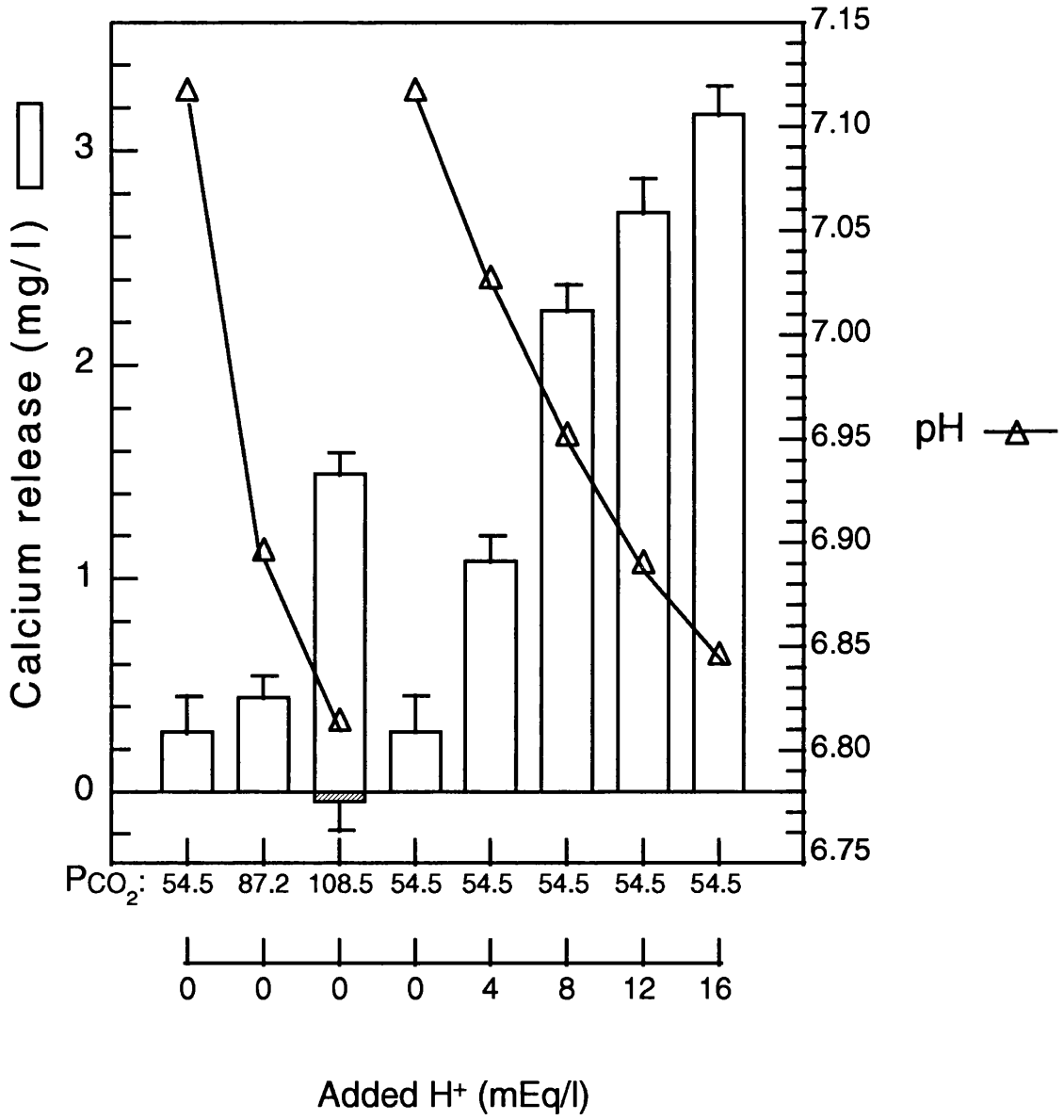


Figure 2.14 Comparison of the effects of metabolic and respiratory acidosis on calcium release from 3 day mouse calvarial cultures. Culture medium was acidified (left to right) by increasing incubator CO_2 to 54.5, 87.2 and 108.5 mmHg to model respiratory acidosis; or by addition of 4, 8, 12 and 16 mEq/l H^+ as HCl to model metabolic acidosis (PCO_2 = 54.5 mmHg). Devitalised bones are shown as hatched bars. Values are means ± SEM (n = 5).

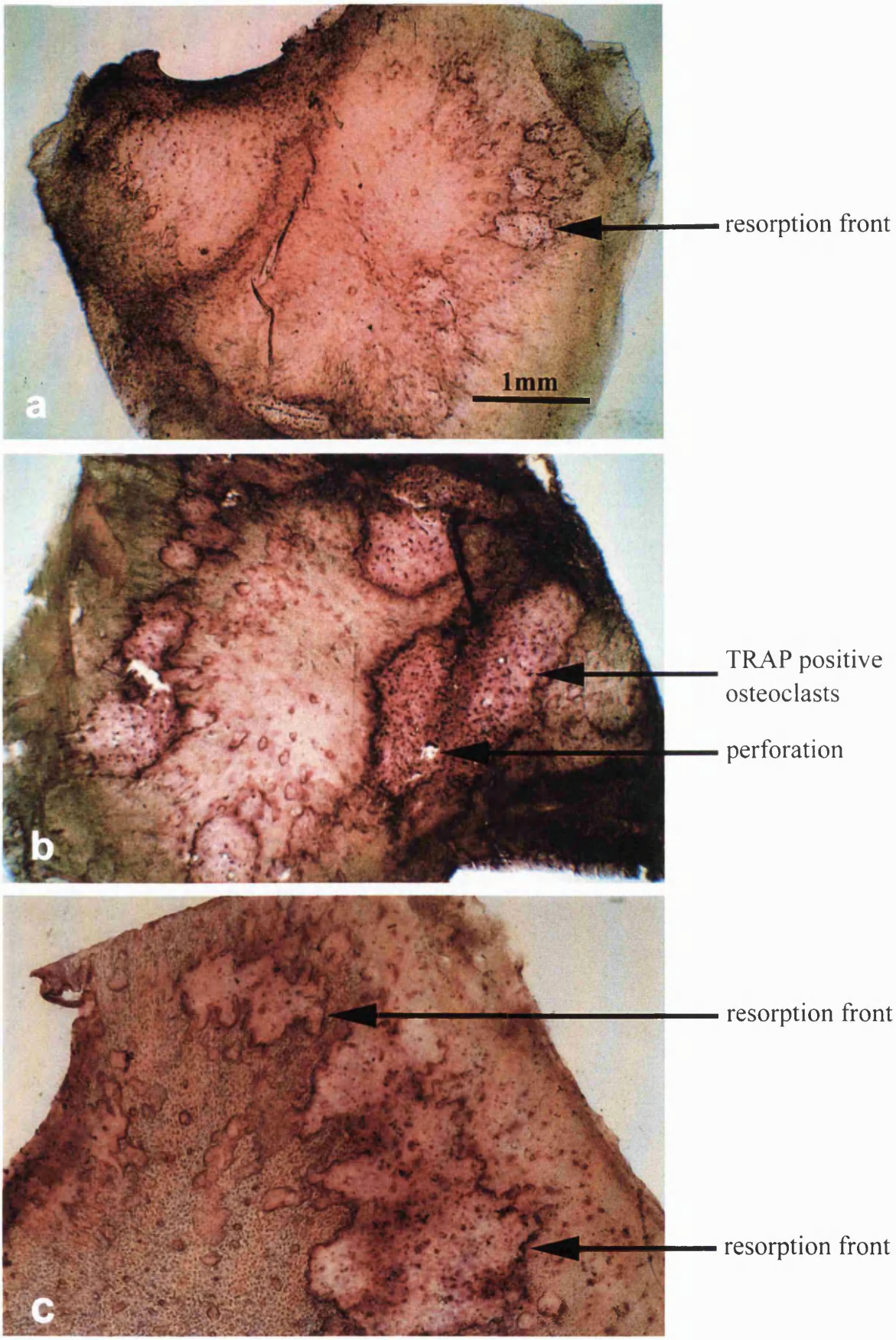


Figure 2.15 Transmitted light photographs of TRAP stained whole- mount half calvaria after 3 days culture. (a) control, pH 7.208 (x20); (b) 1,25(OH)2D3, pH 7.095 (x20); (c) 12 mEq/l H+, pH 7.007 (x20).

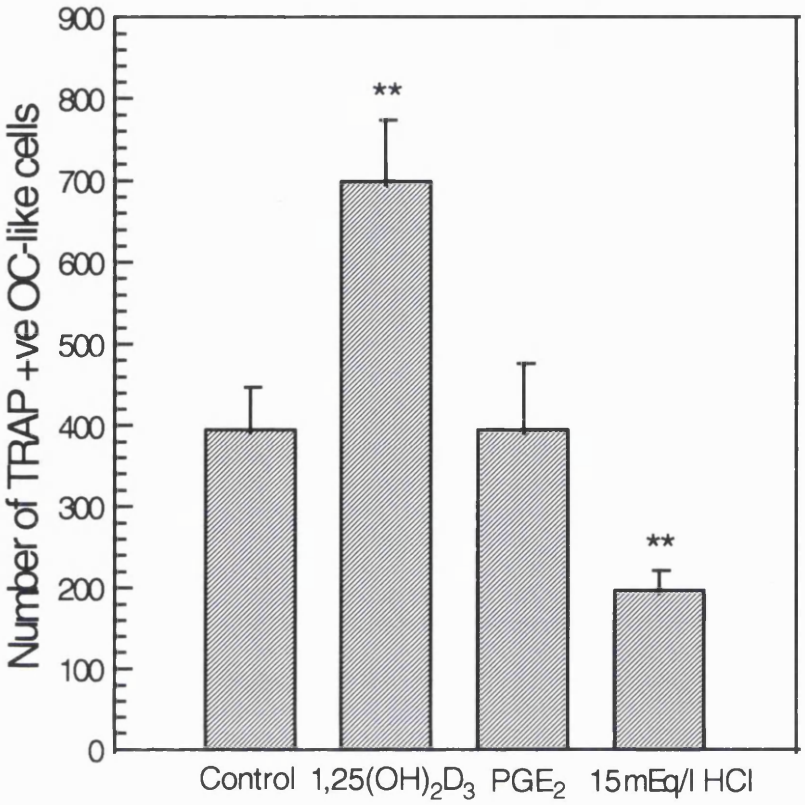


Figure 2.16 Comparison of the effects of $1,25(OH)_2D_3$, PGE_2 and 15 mEq/l H^+ on the number of TRAP-positive multinucleate cells present on half calvaria after 3d culture. Values are means ± SEM (n = 5); ** $p<0.01$ vs. control.

Discussion

Recent work has demonstrated that mature osteoclasts disaggregated from neonatal rat bones are extremely sensitive to small changes in extracellular pH (Arnett and Spowage, 1996), such that a pH difference of as little as 0.10 unit (between pH 7.25 and 7.15) can effectively "switch on" or "switch off" rat osteoclasts. Mechanisms underlying this switching effect are unclear at present, although it has been reported that relatively brief exposure to low pH conditions (6 hours at pH 6.5) is associated with two-fold increases in CA II and TRAP mRNA levels in rabbit osteoclasts (Asotra *et al.*, 1994a, 1994b).

Experiments using the disaggregated osteoclast assay demonstrate that at particular pH levels there is less than one pit per osteoclast. Osteoclasts differ in their area and volume (Piper *et al.*, 1995). There is also a positive correlation between the number of nuclei per osteoclast and the number and volume of resorption pits made (Piper *et al.*, 1992). This suggests that osteoclasts are heterogeneous, and thus will differ in their response to a particular pH level (i.e some osteoclasts will be activated while others are not at the same pH). However, the benefit of studying a population of heterogeneous osteoclasts such as those found in the disaggregated osteoclast assay allows the response to be

seen as an average for all the osteoclasts per slice, and can therefore give values of less than one pit per osteoclast.

Embryonic chick osteoclasts are also extremely pH-sensitive, and are able to form resorption pits in somewhat more alkaline conditions (pH 7.4) than mammalian osteoclasts. My results confirm the work of Arnett and Dempster (1987) and Murrills *et al.*, (1993), who demonstrated that chick osteoclastic bone resorption was consistently greater at low pH; but contradict the findings of Walsh *et al.*, (1990) who demonstrated that avian osteoclast activity was optimal at pH 7.20 - 7.40, and was inhibited below pH 7.10. The difference with the results from the latter laboratory may be due to the fact that osteoclasts were buffered with either HEPES or PIPES, which the authors acknowledge may have adverse effects on osteoclast metabolism. Perhaps because of the rapid remodelling that must accompany the high rates of bone growth seen in developing birds, and the lack of response to calcitonin, chick osteoclasts would appear to be "constitutively activated". Chick osteoclasts have a basal rate of resorption in non acidified conditions which is generally much higher than that of the neonatal rat (Arnett and Dempster, 1987). Slightly larger changes in extracellular pH are needed to "switch on" or "switch off" chick osteoclasts. Osteoclast pH-sensitivity has been conserved in these two species, as well as in mouse, human and rabbit osteoclasts (Shibutani and Heersche, 1993; Tamura *et al.*, 1993; Matayoshi *et al.*, 1996). The phenomenon of osteoclast activation by extracellular protons may have evolved in vertebrates as a last line of defence to

protect the body against the effects of systemic acidosis. Indeed, the fact that avian and mammalian osteoclasts differ in response to calcitonin (Arnett and Dempster, 1987) but respond similarly to low pH suggests that H^+ sensitivity is a more primitive characteristic conserved during evolution of higher vertebrates.

In relatively short-term experiments, the effects of extracellular pH on mammalian osteoclastic resorption seem clear cut. In longer term cultures, there have been conflicting data. Murrills *et al.*, (1993) showed that the relative stimulatory effect of low pH on resorption by rat osteoclasts diminished over several days in culture; although these experiments studied the effects of severely acid media (<pH 6.7), which can reduce osteoclast survival. However, results presented in this chapter show that the powerful stimulatory effect of low pH on pit formation by mature rat osteoclasts does not diminish with time in culture, and may increase somewhat, provided the cytotoxic effects of very low pH (*i.e.* <6.9) are avoided. There was no indication of "tachyphyllaxis" (escape), such that with 7 days in culture a pH decrease of about 0.1 unit caused a 15-fold increase in resorption pit formation. The small pH shifts were not associated with any long term changes in osteoclast number. Long term stimulatory effects of low pH on osteoclastic resorption may be pertinent to *in vivo* situations, for example rheumatoid arthritis, where the local pH can be reduced (Steen *et al.*, 1992) and bone is lost.

The effects of extracellular pH on mature osteoclasts are well documented, but little is known about the effects on osteoclast formation. Shibutani and Heersche (1993) demonstrated that rabbit osteoclast differentiation and proliferation were optimal at pH 7.0 to 7.5, but decreased at pH 6.5 over 48 hours. However, osteoclast proliferation seen in the experiments described by Shibutani and Heersche contrast with results I have obtained, which show that over 7 days of culture (non-acidified or acidified medium) rat osteoclast number remained constant.

Results presented here show that osteoclast formation and activation in 10 day mouse marrow cultures were extremely sensitive to changes in extracellular pH. Although acidification resulted in moderate inhibitions of TRAP-positive multinucleate cell formation, resorption pit formation was subject to large stimulations when the pH was reduced from 7.15 to 7.10 ("off-on switching"), in a manner similar to that described for mature rat osteoclasts (Arnett and Spowage, 1996). It is unclear from the data whether in fact it is low pH that is inducing osteoclast formation and resorption since HCO_3^- concentrations are also decreased in these cultures. It may be that in a similar manner to osteoclast-mediated calcium release from calvaria, decreased medium bicarbonate and not just a fall in pH is necessary to stimulate osteoclast formation. The results additionally indicate that the TRAP-positive multinucleate cells, whose formation was stimulated in alkaline conditions, were indeed functional osteoclasts which were activated simply by reducing pH. It is an interesting paradox that in the

mouse marrow system, extracellular protons inhibit osteoclast formation, but stimulate the activity of these cells once formed. Additionally, osteoclasts are known to be formed by the fusion of circulating mononuclear precursor cells of haematopoietic origin (Fujikawa *et al.*, 1996a). Results from other systems, have shown that the optimal pH for cell fusion is around pH 7.5 (Chang *et al.*, 1989), raising the possibility that extracellular protons could directly influence pre-osteoclast fusion.

Experiments with 3-day cultures of mouse calvaria have shown a similar response to extracellular pH. This was more sensitive than previously thought (Goldhaber and Rabadjija, 1987). Changes in pH of only 0.1 unit were associated with 2 to 3-fold alterations in osteoclast-mediated calcium release. Devitalised (dead) bones took up calcium from the medium over the same pH range which caused cell-mediated calcium release from live bones. This uptake decreased as the amount of protons added increased, suggesting a relatively small physicochemical component may be involved. Proton-mediated calcium release from dead bones appears to be a function of changes in solution equilibria that control bone mineral growth and dissolution (Bushinsky *et al.*, 1983). The calcium efflux seen most likely represents dissolution of either hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), brushite ($CaHPO_4$), or calcium carbonate ($CaCO_3$) from bone (Bushinsky *et al.*, 1986).

From the stoichiometry of hydroxyapatite dissolution the importance of H^+ at physiological pH is obvious (Schlesinger *et al.*, 1994):

$$[Ca_{10}(PO_4)_6(OH)_2]_{solid} + 14\ H^+ = 10\ Ca^{2+} + 6\ H_2PO_4^- + 2\ H_2O$$

Evidence that the addition of protons to the culture medium stimulated cell-mediated bone resorption was obtained by demonstrating that calcitonin (a specific inhibitor of osteoclastic bone resorption (Chambers and Moore, 1983; Arnett and Dempster, 1986, 1987)), was able to inhibit calcium release from calvaria exposed to 15 mEq/l of added protons. A preliminary report has suggested that calcitonin receptor mRNA expression in osteoclasts formed from mouse marrow cells can be modulated by changes in culture medium pH, with an increase in expression with decreasing pH (Weinstein *et al.*, 1998). These results may suggest that as osteoclasts become more active at low pH, calcitonin receptor mRNA expression is increased to allow for regulation of osteoclast function by increased calcitonin sensitivity.

The finding that indomethacin inhibits the bone resorption stimulated by added protons suggests that prostaglandin synthesis is involved in this phenomenon. It is possible that hydrogen ions act on cells in the calvaria, (*e.g* osteoblasts), to produce agents that lead to osteoclastic stimulation (*e.g.* prostaglandins) (Goldhaber and Rabadjija, 1987). Rabadjija and colleagues (1990) further demonstrated that the addition of protons to mouse calvaria

cultures significantly increased the release of PGE_2 and cAMP, and suggested that the bone resorption due to added protons was dependent on their production. Indomethacin (2.8×10^{-7}M) inhibited the release of both PGE_2 and cAMP from H^+ treated bones. Marshall *et al.*, (1996) noted that when mouse calvaria were incubated with 1 μM indomethacin, the number of TRAP-positive osteoclasts counted on the bone surface were reduced, a reduction that did not occur with calcitonin. Seemingly, TRAP-positive osteoclasts become less adherent to bone and more adherent to the endocranial membrane with indomethacin treatment, and thus resorption is decreased (Marshall *et al.*, 1995; 1996). On the other hand, in the isolated mature rat osteoclast assay, prostaglandins and cAMP analogs are inhibitory (Arnett and Dempster, 1987), and addition of indomethacin leads to a stimulation of resorption pit formation (see Chapter 3).

Although extracellular protons can stimulate resorption as powerfully as maximal doses of $1,25(OH)_2D_3$, PTH or PGE_2, acidification is accompanied by reductions in the number of TRAP-positive osteoclasts visible in whole mount preparations of half calvaria. The data suggest that extracellular protons may stimulate bone resorption by "switching on" mature osteoclasts already present in calvarial bones, rather than by stimulating the recruitment of new osteoclasts. These results mirror my findings in 10 day mouse marrow cultures, where reducing extracellular pH results in a decrease in the number of TRAP-positive osteoclasts formed, but still causes a stimulation of resorption pit formation.

Results presented in this chapter confirm the observation that metabolic acidosis (*i.e.* decreasing HCO_3^-) is a more effective stimulator of osteoclastic resorption than respiratory acidosis (*i.e.* increasing PCO_2) in calvaria (Bushinsky, 1989). The results demonstrate that increasing PCO_2 in calvaria cultures increases resorption, however the pH has to be lower to obtain an equivalent amount of calcium release compared to decreasing HCO_3^-. Varying the PCO_2 between 54.5 and 87.2 caused only a small amount of calcium release despite a large drop in pH (7.12 to 6.89). However, increasing the PCO_2 from 87.2 to 108.5 caused a large (3.3-fold) increase in calcium release, although the pH dropped by only 0.08 of a unit. This suggests that the "set point" for osteoclast activation is altered depending on whether the acidosis is related to alterations in PCO_2 or HCO_3^- concentration. This contrasts with the work of Arnett *et al.*, (1994) who demonstrated that isolated rat osteoclasts were more sensitive to stimulation by CO_2 acidosis than HCO_3^- acidosis. However, my own preliminary work (unpublished) with 26 hour disaggregated rat osteoclast cultures showed no significant difference in pit number or size in CO_2 or HCO_3^- acidosis. This suggests that low pH stimulates osteoclasts to resorb, and in calvaria the resorption response is more sensitive HCO_3^-.

The reasons why a reduction in bicarbonate concentration should have a greater effect on osteoclastic bone resorption in calvaria than an increase in the partial pressure of carbon dioxide are unclear. However, a low HCO_3^- concentration (as in metabolic acidosis) might be expected to facilitate the

exchange of intracellular HCO_3^- for Cl^-, leading to enhanced H^+ availability for increased resorption. Increasing CO_2 (as in respiratory acidosis) would not be expected to stimulate the HCO_3^- / Cl^- exchanger. It is also possible that increasing incubator CO_2 levels could promote calcium carbonate accretion and the formation of carbonated apatite (Bushinsky, 1995). This may explain the apparent reduction in calcium efflux seen in calvaria cultures incubated in medium acidified by increased PCO_2. During metabolic acidosis however, the low HCO_3^- concentration would favour the dissolution of bone calcium carbonate and carbonated apatite.

Osteoblast mitogens such as ATP and IL-1 are known to enhance medium acidification, which could conceivably stimulate osteoclasts, along with any other mechanisms that may be involved (Aisa *et al.*, 1995; Kaplan and Dixon, 1996). In the results presented in this chapter, PTH, and in some experiments $1,25(OH)_2D_3$ and PGE_2 were associated with medium acidification which could in itself stimulate resorption to some extent. The data are in agreement with the work of Vaes (1968), who showed that PTH-treated mouse calvaria acidified their culture media at a faster rate and released more citrate and lactate than control bones. Recent work by Barrett *et al.*, (1997) has demonstrated that one important consequence of PTH receptor activation in bone is enhanced local acidification of the extracellular space. Utilising the technique of microphysiometry, Barrett *et al.*, showed that PTH could directly and dose-dependently enhance acid production by human osteoblast-like SaOS-2 cells.

This may represent a mechanism by which PTH acting on osteoblasts stimulates osteoclasts.

Clearly, the effects of pH on osteoclasts can interact with those of other osteolytic agents. For example, resorption pit formation can be additionally stimulated by $1,25(OH)_2D_3$, cyclooxygenase inhibitors (see Chapter 3 for further discussion), or ATP (see Chapter 4 for further discussion). Similar effects have also been demonstrated with metabolic acidosis and PTH in mouse calvaria (Bushinsky and Nilsson, 1995). Anderson *et al.*, (1986) demonstrated using mouse calvaria and acridine orange (a fluorescent weak base) that PTH could increase osteoclast intracellular acidity, and suggested an intimate link between acidification of osteoclasts and bone resorption. It is unclear to what extent the effects of these varying modes of stimulation are additive, or what the upper limit of resorptive efficiency for an osteoclast might be. However, these findings emphasise the critical importance of extracellular protons in modulating osteoclast function and reinforce the need for accurate monitoring of culture medium pH in all bone resorption experiments, a requirement that has been widely overlooked.

The availability of a clinical blood gas analyser has allowed the careful measurement and analysis of medium pH and PCO_2 before, during, and at the end of experiments. Coupled with the use of calibration curves measured for culture medium with differing bicarbonate concentrations, the relationship between, and

the significance of pH, PCO_2, and $[HCO_3^-]$ in tissue culture media is apparent. In view of the extreme sensitivity of osteoclasts to changes in extracellular pH, it is important to note that the equilibrium pH of unmodified MEM can vary between batches (pH 7.2 ± 0.1). The equilibrium pH of unmodified DMEM is slightly higher (pH 7.35-7.4). This may help to explain some of the variability in resorption pit formation in the disaggregated osteoclast assay, since osteoclasts cultured in MEM which has a low equilibrium pH may be activated to resorb without the need for addition of H^+ as HCl. Conversely, osteoclasts cultured in unmodified DMEM will not resorb.

The mechanism underlying the effects of extracellular protons on osteoclastic resorption is unknown. In view of the small changes in extracellular proton concentration involved, it seems unlikely that adding protons simply decreases the gradient against which osteoclasts must pump H^+ to create and maintain an acid microcompartment necessary for resorption (Baron *et al.*, 1985). Work with rat and chick osteoclasts has shown that extracellular acidification leads to an increase in the expression of actin-rich podosomes and formation of actin-rich "clear zones" within the osteoclast, indicating increased adhesion to substrate (Teti *et al.*, 1989b; Murrills *et al.*, 1993). However, along with effects on carbonic anhydrase expression (as mentioned above) acidosis can also induce plasmalemmal vacuolar type H^+ ATPase (V-ATPase) activity. V-ATPases are localised to the ruffled border of osteoclasts and are the primary cellular mechanism responsible for acidification of the resorption lacunae (Blair *et al.*,

1989; Vaananen *et al.*, 1990). Therefore, induction of plasmalemmal V-ATPase activity by chronic acidosis, generated either systemically due to metabolic disease or locally at sites of inflammation, is likely to stimulate osteoclastic bone resorption and thus promote bone loss (Nordstrom *et al.*, 1997). However, another possibility is that osteoclasts possess a specific pH sensing apparatus linked to an intracellular signalling pathway (Arnett and Spowage, 1996).

Additionally, results presented in chapter 4 suggest a link between ATP (a ubiquitous extracellular signalling molecule), and protons in receptor modulation. Although unproven, a tentative theory is that protons and ATP may interact to allow the opening of a cation / proton channel in the osteoclast cell membrane. This could conceivably increase the intracellular concentration of protons, which can then be actively pumped out of the cell via a V-ATPase into the resorption lacunae, and so increase resorption pit formation (Arnett and King, 1997).

Protons may be mediating their effects directly on osteoclasts (Arnett and Dempster, 1986). This is supported by studies from Wang *et al* (1997) using individually micro-isolated osteoclasts cultured on dentine slices has shown that lowering the pH from 7.35 to 6.9 resulted in a 50% increase in the number of actively resorbing micro-isolated osteoclasts. However, to a certain extent it may be irrelevant whether protons are acting directly on osteoclasts or acting on osteoclasts via osteoblasts, since within bone both cells will be in close proximity to each another. Most osteolytic agents act on osteoclasts via osteoblasts. It is

also possible that protons may serve a physiological role as a local osteoblast-osteoclast coupling factor (Teti *et al.*, 1989b; Arnett, 1990). Indeed, it has been shown in mouse calvaria cultures that lowering extracellular pH (or possibly intracellular pH) by addition of protons triggers prostaglandin biosynthesis, leading to activation of bone resorbing cells and bone resorption (Rabadjija *et al.*, 1990).

Although the normal pH range of the extracellular environment in bone is not known, it is possible that the results presented here may have relevance to the modulation of bone turnover *in vivo*. For example, it is known that from childhood to old age, humans eating a typical western diet develop a slight but progressive increase in blood acidity and decrease in plasma bicarbonate concentrations, (low-grade metabolic acidosis) (Barzel, 1995; Frassetto and Sebastian, 1996). Along with this general acidosis, decreased perfusion of bone with ageing may cause an accumulation of lactate and a further decrease in the pH of bone interstitial fluid. Even if this decrease were small, a slightly elevated rate of bone resorption and a slightly diminished rate of osteoblastic collagen synthesis over several years could contribute to the bone loss associated with osteoporosis (Ramp *et al.*, 1994). Recent studies have shown that, in postmenopausal women, administration of potassium bicarbonate to neutralise endogenous acid increased blood pH by 0.02 unit and plasma bicarbonate by 1.8mmol/l, which, it was suggested, led to a reduction in bone resorption and an increased rate of bone formation (Sebastian *et al.*, 1994). The findings presented

could also be pertinent to diseases that feature localised bone destruction, such as arthritis and periodontal disease. For example, Falchuk *et al.*, (1970) demonstrated that the pH in inflammatory synovial fluid is lower than fluid from joints showing no signs of inflammation, suggesting the possibility that the appendicular bone loss commonly found in rheumatoid arthritis could be caused by local acidification enhancing osteoclastic bone resorption.

Low pH modulates both osteoclast formation and activation, and has the ability to interact with and enhance a wide range of other osteolytic agents. The results presented in this chapter demonstrate the important role of extracellular pH in bone physiology, and provide new clues to understanding the pathogenesis in metabolic bone disorders.

Chapter 3

Cyclooxygenase inhibitors stimulate pit formation by mature rat osteoclasts

Introduction

Since the initial discovery that prostaglandin-E (PGE) was involved in bone resorption (Klein and Raisz, 1970), arachidonic acid metabolites, (*e.g.* PGE_2), have been the focus of a large number of experimental bone studies. These studies have shown that prostaglandins are powerful, multifunctional local regulators of both the formation and resorption of bone. Prostaglandins are important in bone metabolism as they are produced in abundance not only by osteoblasts (Feyen *et al.*, 1984), but also by cells adjacent to bone in the marrow and periosteal tissues (Raisz, 1995). Cells involved in the response to injury and inflammation may also have an important influence on skeletal tissues.

Prostaglandin synthesis

Cyclo-oxygenase (COX) is the key enzyme involved in the conversion of arachidonic acid from membrane phospholipids to form prostanoids, while leukotrienes are formed via the lipoxygenase pathway. COX converts arachidonic acid to prostaglandin G_2 (PGG_2) and then reduces PGG_2 to prostaglandin H_2 (PGH_2) in a peroxidase reaction. PGH_2 is then converted by intracellular isomerases and reductases to prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2), prostacyclin (PGI_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and thromboxane A_2 (Smith, 1992). These products either simply diffuse across the cell membrane, or are transported from the cell via an organic anion transporter (Kanai *et al.*, 1995), to exert their effects in an autocrine or paracrine manner.

There are two distinct isoforms of the COX membrane protein (Kurumbail *et al.*, 1996), representing independent biosynthetic pathways. COX-1 is constitutively expressed in most tissues, whereas COX-2 is induced by pro-inflammatory stimuli, such as mitogens and cytokines and is the more highly regulated of the two enzymes (Pilbeam *et al.*, 1993; Kawaguchi *et al.*, 1994, 1995). The two isoforms are approximately 60% identical at amino acid and nucleic acid levels, and both enzymes are about 70kDa in size (Kurumbail *et al.*, 1996). Regulation of prostaglandin production by mechanical forces, hormones (*e.g.* parathyroid hormone), cytokines (*e.g.* interleukin-1), and growth factors (*e.g.* TGF-α & TGF-β) appears to be due largely to the transcriptional regulation of COX-2 (Kawaguchi *et al.*, 1994; Forwood, 1996; Pilbeam *et al.*, 1997; Maciel

et al., 1997). Prostaglandins have also been postulated to act in positive feedback loops amplifying their own production by stimulating cyclic adenosine monophosphate (cAMP) activity in cultured mouse calvaria and murine osteoblastic MC3T3-E1 cells (Kawaguchi *et al.*, 1994; Pilbeam *et al.*, 1995).

Effects of prostaglandins on osteoclast resorption and formation

In 1970, Klein and Raisz demonstrated that prostaglandins stimulated bone resorption in foetal rat long bones in 48 to 96 hour tissue culture, and later showed that PGE_2 was the most potent stimulator of resorption in this system after a transient inhibition (Dietrich *et al.*, 1975). Prostaglandins also have a similar stimulatory effect on osteoclastic resorption in neonatal mouse calvaria (Lerner, 1980). Paradoxically, early work on the effects of prostaglandins on isolated osteoclasts established that *in vitro*, they had a direct inhibitory effect (Chambers and Dunn, 1982; Chambers and Ali, 1983a; Chambers, 1985b; Arnett and Dempster 1987), similar to that observed with calcitonin. Prostaglandins were found to transiently inhibit osteoclast spreading and motility (Chambers and Ali, 1983b; Chambers *et al.*, 1984a) and, like calcitonin, operate by increasing the cyclic AMP levels in osteoclasts. PGE_1 and PGE_2 are known to inhibit bone resorption by isolated osteoclasts for at least 6 hours (Fuller and Chambers, 1989b). A similar transient inhibition of bone resorption has also been observed in mouse calvarial bones *in vitro* (Lerner *et al.*, 1987). Lakkakorpi & Vaananen (1990) reported that PGE_2 rapidly dispersed the specific microfilament structure (*e.g.* F-actin, vinculin, and talin) in isolated resorbing osteoclasts, demonstrating

a direct inhibitory effect. The fact that prostaglandins inhibit isolated osteoclasts, but stimulate resorption in bone organ culture, suggests that prostaglandins are acting on cell types other than mature osteoclasts in organ culture (Collins and Chambers, 1991).

The osteolytic action of several hormones and cytokines is dependent to some degree on endogenous prostaglandin synthesis. For example, interleukin-1 (IL-1), a potent bone-resorbing cytokine, has both prostaglandin dependent and independent effects on bone resorption. Akatsu *et al.*, (1991) and Lader and Flanagan (1998) demonstrated that recombinant human IL-1α increased both bone resorption and osteoclast number, and that this effect was suppressed by indomethacin, suggesting a role for endogenous prostaglandins. However, IL-1 has also been reported to stimulate bone resorption independently of prostaglandin synthesis (Garrett and Mundy, 1989). PGE_2 may also be a potential mediator of extracellular ATP action in osteoblast-like cells. Pre-treatment with indomethacin suppressed both ATP-induced PGE_2 synthesis and DNA synthesis in these cells (Suzuki *et al.*, 1993).

Proton-stimulated resorption in neonatal mouse calvaria may also have a prostaglandin dependent mechanism of action (Rabadjija *et al.*, 1990), since indomethacin abolishes the stimulatory effect (see Chapter 2). Acid stimulated calcium release in mouse calvaria was also shown to be blocked by the 5-lipoxygenase inhibitors, MK 886 and BW 70C (Arnett *et al.*, 1997).

Prostaglandins also have a role in the movement of osteoclasts to bone or periosteum. When PGE_2 is blocked by indomethacin, osteoclasts become less adherent to bone and more adherent to the endocranial membrane (Marshall *et al.*, 1996), therefore resorption is reduced. The β_3 integrin subunit seems to play an important role in the movement of osteoclasts from the periosteum to bone, but not in the reverse process induced by indomethacin (Holt and Marshall, 1998).

In addition to the effects of prostaglandins on bone resorption, leukotrienes, (the products of the lipoxygenase pathway) increase both osteoclast formation and resorption *in vitro* and *in vivo* (Meghji *et al.*, 1988; Gallwitz *et al.*, 1993; Garcia *et al.*, 1996), although other groups have reported that with isolated rat osteoclasts none of the lipoxygenase pathway products had a significant direct effect (Fuller and Chambers, 1989b). Prostaglandins and leukotrienes may therefore play an important role in the localised bone loss associated with inflammatory lesions, such as periodontal disease and rheumatoid arthritis (Kawaguchi *et al.*, 1995).

Recent results using murine bone marrow cultures have indicated that prostaglandins may promote the differentiation of osteoclasts from haematopoietic precursors by a mechanism involving cyclic adenosine 3',5'-monophosphate (cAMP). It was demonstrated that PGE_1 and PGE_2 (10^{-9} to 10^{-5} M) significantly stimulated the formation of osteoclast-like cells (Akatsu *et al.*,

1989; Collins and Chambers, 1991), suggesting that prostaglandins promote osteoclast formation, but inhibit osteoclast activity once formed. The effects of prostaglandins may be to increase the commitment of early precursors to the osteoclastic differentiation pathway, while at the same time acting on osteoblasts to increase their endogenous prostaglandin production. This combination leads to increased osteoclast formation (Suda *et al.*, 1995b). Indeed, Marshall *et al.*, (1995) showed, using bromo-deoxyuridine labelling, that osteoclast formation stimulated by PGE_2 was the result of differentiation and not proliferation of postmitotic precursors. Soekanto *et al.*, (1994) demonstrated that the addition of indomethacin or sodium salicylate to murine marrow cultures dose dependently inhibited the formation of TRAP positive osteoclasts, suggesting that prostaglandins are necessary for osteoclast formation.

In the human bone marrow assay, prostaglandins appear to exert opposite effects on osteoclast formation compared to the murine bone marrow assay. For example, Chenu *et al.*, (1990) reported that prostaglandins inhibited osteoclast-like cell formation and hence resorption, while Roux *et al.*, (1997) recently demonstrated that the inhibitory effect of PGE_2 on human osteoclast differentiation from cord blood monocytes resulted in an increase in the number of macrophages formed. However, neither of these two groups demonstrated resorption pit formation, an unequivocal marker of osteoclast formation *in vitro*.

In contrast to the work mentioned above, Flanagan *et al.*, (1995) showed that PGE_2 increased bone resorption by human osteoclasts formed *in vitro*, an effect which was blocked by indomethacin. Recent work from the same group has demonstrated the critical importance of PGE_2 for both human osteoclast formation and resorption. PGE_2 dose responsively increased osteoclast numbers and bone resorption, while reducing macrophage CD 14-positive cells in culture. Addition of indomethacin, or NS-398 (the COX-2 inhibitor) abolished osteoclast parameters. These results provide strong evidence for PGE_2 exerting a similar effect on osteoclast formation in both the human and murine species (Lader and Flanagan, 1998).

Prostaglandins and bone formation

The first reports on the effects of prostaglandins on osteoblastic collagen synthesis *in vitro* showed both inhibitory and stimulatory effects. Incorporation of proline into collagen was enhanced by PGE_2 and $PGF_{2\alpha}$ in cultures of embryonic chick frontal bone, while high PGE_2 concentrations were inhibitory in cultures of foetal rat calvaria (Blumenkrantz and Sondergaard, 1972; Raisz and Koolemans-Beyen, 1974). More recent studies have demonstrated a biphasic effect of PGE_2 on bone formation in cultured foetal rat calvaria: at low concentrations PGE_2 increased osteoblastic collagen synthesis, whereas at high concentrations the major effect was inhibitory (Raisz and Fall, 1990). A similar dose-related biphasic effect has been reported in cultured human bone cells, with

stimulation at 10^{-9} M and inhibition at 10^{-6} M (Baylink *et al.*, 1995). Prostaglandins (PGE_1 and PGE_2) have also been shown to stimulate the formation of mineralised nodules *in vitro* (Flanagan & Chambers, 1992)

Stimulation of bone formation can also be demonstrated in both humans and experimental animals *in vivo* (Jee *et al.*, 1985; Jorgensen *et al.*, 1988; for review see Jee and Ma, 1997). A role for endogenous prostaglandins in skeletal growth and repair is suggested by mechanical loading experiments, which show that PGE_2 and PGI_2 are involved in the initial transduction of strain information (Pead and Lanyon, 1989), and also increase the release of insulin-like growth factor II (IGF-II) (Zaman *et al.*, 1997).

When prostaglandin production is blocked by indomethacin *in vivo*, mechanically induced bone formation is markedly reduced (Pead and Lanyon, 1989; Chow and Chambers, 1994). In adult rats, short-term indomethacin treatment inhibits fracture healing (Hogevold *et al.*, 1992), while long-term treatment reduces trabecular bone mass and strength (Saino *et al.*, 1997), again suggesting an important role for prostaglandins in bone formation. However, indomethacin is relatively non-specific, and blocks both the constitutive and inducible forms of COX. Recent work using the COX-2 specific inhibitor NS-398, has suggested that bone formation occurring in response to mechanical stimulation *in vivo*, is dependent on the production of inducible COX-2 (Forwood, 1996).

In contrast to the stimulatory effects of PGE_2 on bone formation, 5-lipoxygenase metabolites (*e.g.* leukotrienes) impair the bone forming capacity of osteoblasts in both the nodule formation assay and the calvarial organ culture assay, indicating that metabolites of the 5-lipoxygenase pathway are negative regulators of bone formation (Traianedes *et al.*, 1998).

The duality of response which prostaglandins show in bone probably involves differences in the receptors and signal transduction pathways in osteoblastic cells. There are data supporting the presence of multiple receptors in cells (Coleman *et al.*, 1994), although bone cells and organ cultures appear to only express receptors of the E and F type (Raisz, 1995). Different receptor expression, ligand specificity, or transduction pathways may help to explain the confusing effects of prostaglandins in bone.

Non-steroidal-anti-inflammatory drugs (NSAIDs)

In 1971 Vane discovered that aspirin and similar drugs inhibited the biosynthesis of prostaglandins, and proposed that this explained their mechanism of action. Many other aspirin-like drugs are now available, and are termed non-steroidal-anti-inflammatory drugs (NSAIDs). Indomethacin and ibuprofen belong to this class of drug, a group of structurally unrelated compounds, commonly prescribed to relieve inflammation, pain and fever. They act as relatively non-selective inhibitors of cyclooxygenase activity (*i.e.* COX-1 and COX-2) and thus, prostaglandin production (Mitchell *et al.*, 1994). Recently,

NS-398, a new NSAID with analgesic and antipyretic effects which selectively inhibits COX-2 activity has been described (Futaki *et al.*, 1993). Although NSAIDs have been widely used to study the role of prostaglandins *in vivo*, such studies may be difficult to interpret. For example, it has been shown in cultures of rat calvaria that the effects of NSAIDs are biphasic; low concentrations (10^{-10} M) actually increase rather than decrease prostaglandin production (Raisz *et al.*, 1989).

As mentioned earlier (see Chapter 2), my findings and those of other groups (Goldhaber and Rabadjija, 1987; Rabadjija *et al.*, 1990) demonstrate that indomethacin inhibits acid-stimulated calcium release from cultured calvarial bones, suggesting that prostaglandin synthesis is involved in this phenomenon.

Previous work has shown that resorption pit formation by cultured osteoclasts is strongly enhanced when extracellular pH is reduced (Arnett and Dempster, 1986, 1987; Arnett and Spowage, 1996). It was therefore of interest to determine whether acid-stimulated resorption pit formation in the isolated osteoclast assay was also prostaglandin-mediated, and to ascertain the possible involvement of the COX-1 and COX-2 enzymes.

Methods

Stock solutions of indomethacin, aspirin, ibuprofen, NS-398 and MK-886 were prepared in ethanol and stored for short periods at -20ºC. The final concentration of ethanol in test and control culture medium was constant at 0.2%. Cell culture methods are described in Chapter 2, pages 49 to 58.

Results

Effects of aspirin, indomethacin and ibuprofen on resorption pit formation

5 μM aspirin was associated with a 3-fold stimulation of resorption pit formation by rat osteoclasts in low density 26 hour cultures in unmodified medium (Figure 3.1). However, aspirin caused a slight acidification of the culture media compared to control (pH reduction from 7.209 to 7.191). Indomethacin exerted a reproducible stimulatory effect on resorption pit formation by rat osteoclasts in low density, acid-activated 26 hour cultures (pH 6.91 ± 0.019). Peak effects were seen at 2 μM, with up to a 2.9-fold increase in pit number (Figure 3.2). At higher concentrations, resorption was reduced. Osteoclast numbers were unaffected by indomethacin in the range 0.2 to 20 μM. Ibuprofen also stimulated pit formation by rat osteoclasts in low density, acid-

activated 26 hour cultures (pH 6.94 ± 0.013), but was considerably more potent than indomethacin, with a maximal effect at concentrations as low as 50nM (Figure 3.3).

The selective COX-2 inhibitor, NS-398, elicited a 4 to 5-fold increase in pit formation over the range 0.4 to 10 μM (Figure 3.4), when incubated with rat osteoclasts in low density, acid-activated 26 hour cultures (pH 7.05 ± 0.003). 0.4 μM NS-398 elicited a similar level of resorption to 2 μM indomethacin. The stimulatory effects of indomethacin, ibuprofen and NS-398 were not associated with reductions in culture medium pH, or changes in osteoclast or stromal cell number at any dose level.

Effect of the 5-lipoxygenase inhibitor MK886

2 μM indomethacin elicited a 1.7-fold increase in pit formation. 2 μM NS-398 produced a 2-fold increase compared to control, in acid-activated 26 hour cultures (pH 6.97 ± 0.011). MK886, a specific 5-lipoxygenase inhibitor had no effect on resorption pit formation by rat osteoclasts when cultured alone, with 2 μM indomethacin, or with 2 μM indomethacin and 2 μM NS-398 (Figure 3.5). Culturing 2 μM NS-398 with 2 μM indomethacin did not result in any further significant stimulation compared to 2 μM NS-398 or 2 μM indomethacin alone.

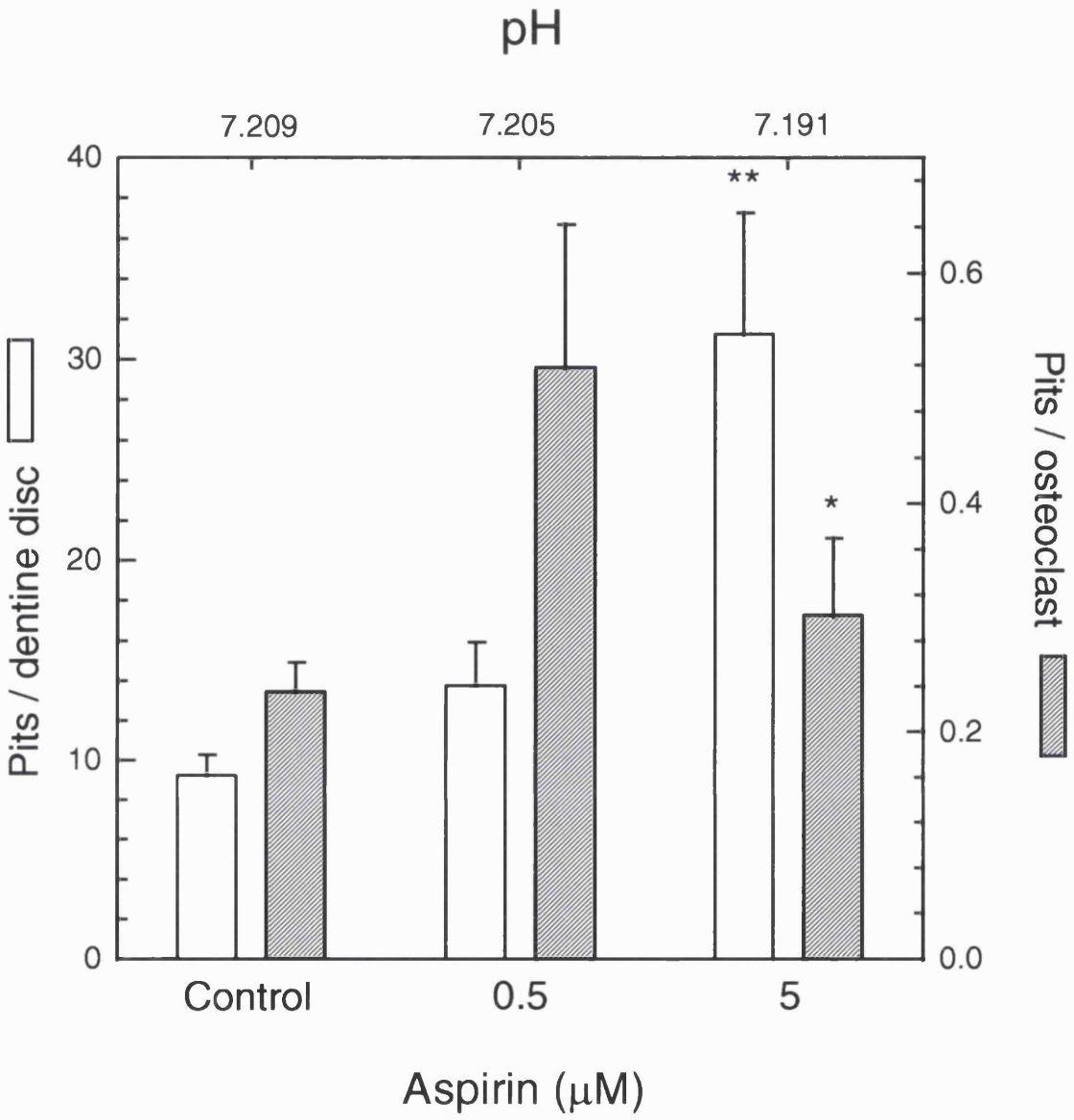


Figure 3.1 Effect of aspirin (acetylsalicylic acid) on resorption pit formation by rat osteoclasts cultured on 5mm dentine discs for 26 hours. Values are means ± SEM (n = 5); * $p<0.05$; ** $p<0.01$ with respect to control.

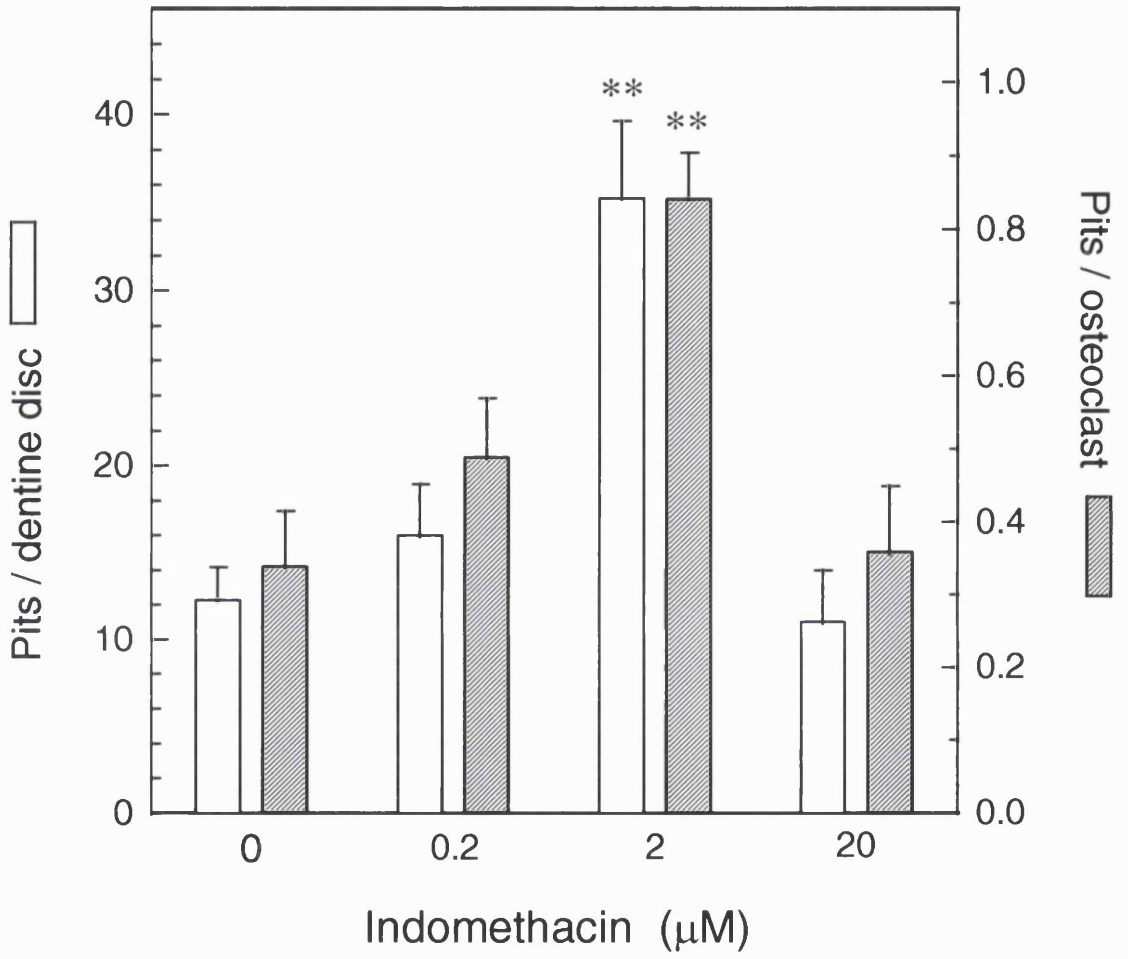


Figure 3.2 Effect of indomethacin on resorption pit formation by rat osteoclasts cultured on 5mm dentine discs for 26 hours. Final culture medium pH values were 6.887, 6.913, 6.923 and 6.933 for 0, 0.2, 2 and 20 μM indomethacin, respectively. Values are means ± SEM (n = 5); ** $p<0.01$ with respect to control.

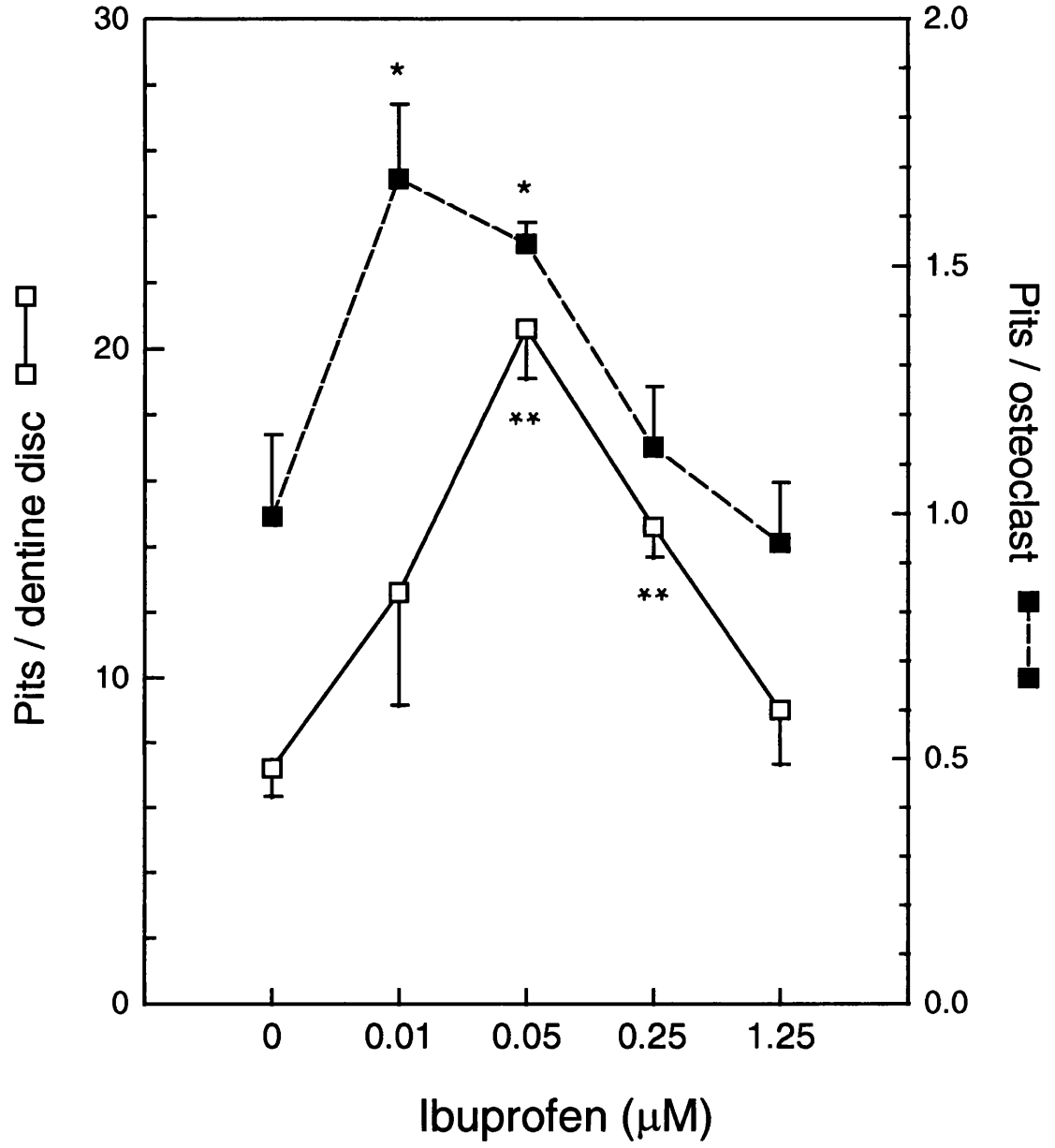


Figure 3.3 Effect of ibuprofen on resorption pit formation by rat osteoclasts cultured on 5mm dentine discs for 26 hours. Final culture medium pH values were 6.920, 6.940, 6.945, 6.921 and 6.948 for 0, 0.01, 0.05, 0.25 and 1.25 μM ibuprofen, respectively. Values are means ± SEM (n = 5); * $p<0.05$; ** $p<0.01$ with respect to control.

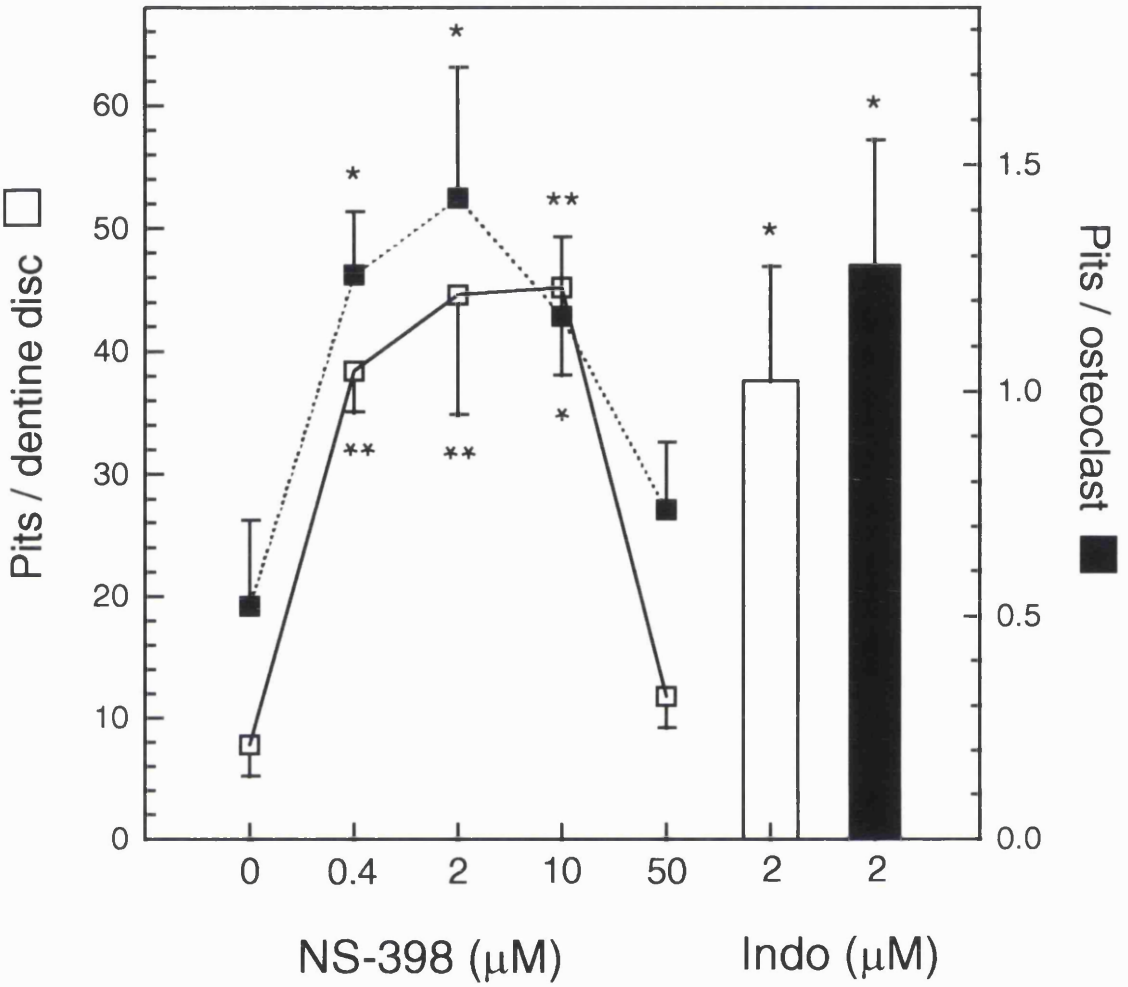


Figure 3.4 Effect of the selective COX-2 inhibitor, NS-398 on resorption pit formation by rat osteoclasts cultured on 5mm dentine discs for 26 hours. Final culture medium pH values were 7.049, 7.043, 7.048, 7.051, 7.050 and 7.049 for 0, 0.4, 2, 10 and 50 μM NS-398, and 2 μM indomethacin (Indo), respectively. Values are means ± SEM (n = 5); * $p<0.05$; ** $p<0.01$ with respect to control.

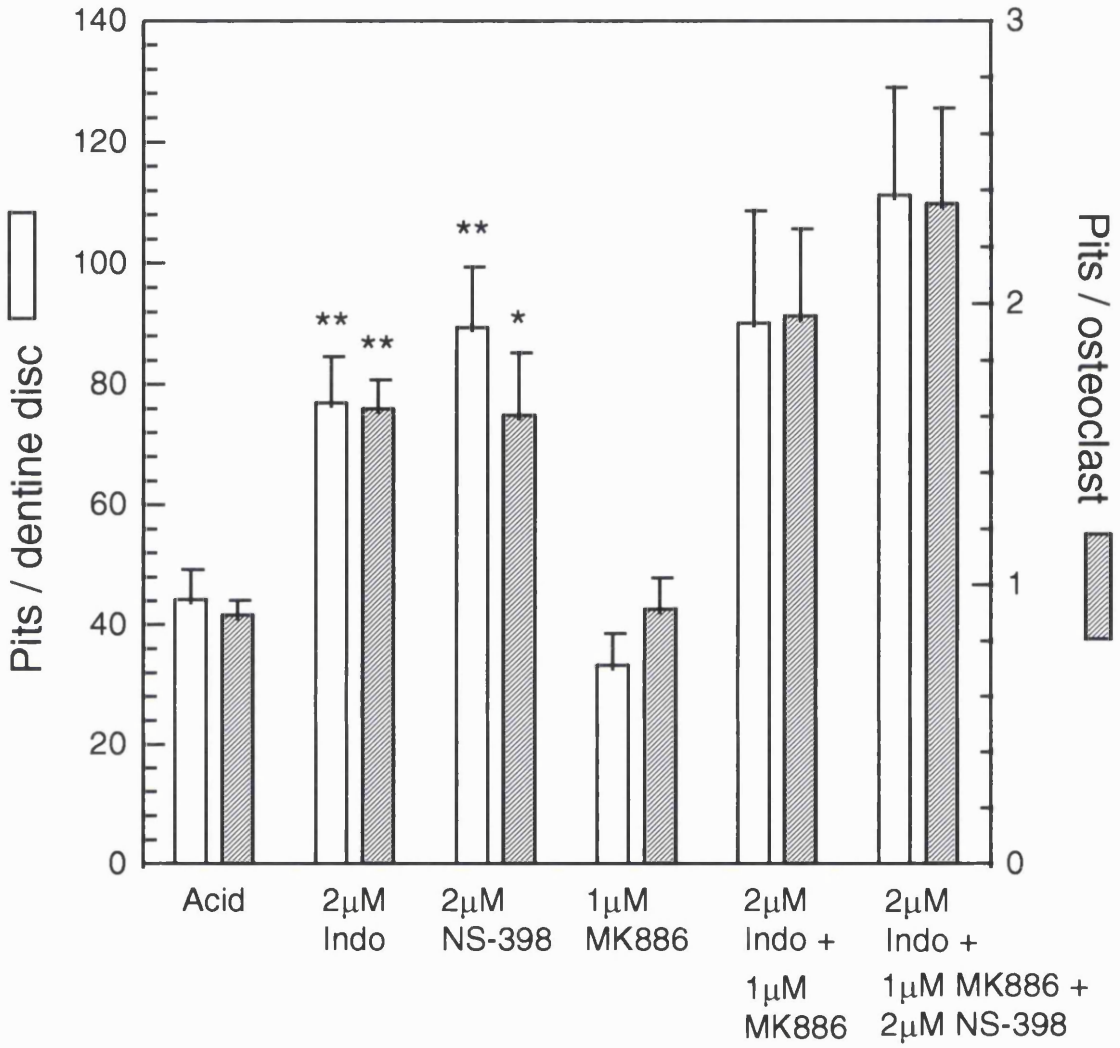


Figure 3.5 Effect of the 5-lipoxygenase inhibitor MK886 on resorption pit formation by rat osteoclasts cultured on 5mm dentine discs for 26 hours, incubated alone, with 2 μM indomethacin (Indo) or 2 μM indo and 2 μM NS-398. Final culture medium pH values were 6.969 ± 0.011. Values are means ± SEM (n = 5); * $p<0.05$; ** $p<0.01$ with respect to control.

Discussion

The results show that COX inhibitors markedly stimulate resorption pit formation by rat osteoclasts, at doses equivalent to those used by investigators in other bone systems (*e.g.* Raisz *et al.*, 1989; Marshall *et al.*, 1995). Inhibition of COX activity in osteoclast cultures leads to a reduction in endogenous prostaglandin production, and prostaglandins are known to inhibit isolated osteoclasts. The COX inhibitors studied vary in their reported selectivity; aspirin, indomethacin, and ibuprofen are respectively about 160, 60, and 15-fold more active on COX-1 than COX-2, whereas NS-398 is more than 1000 times more active on COX-2 than COX-1 (Frolich, 1997). The maximal stimulation of resorption elicited by indomethacin, ibuprofen, and NS-398 on resorption were roughly similar, suggesting that inhibition of the inducible enzyme, COX-2, was mainly responsible for the observed effects. Aspirin appeared to cause a slight acidification of the culture media compared to control (pH reduction from 7.209 to 7.191), which could account for the increase in resorption pit formation.

It is unclear whether COX-2 induction occurred in response to cell isolation and the tissue culture environment, or reflects *in vivo* enzyme activity in growing rat bones. Recent reports suggest that COX-2 induction may play a key role in mediating the responses of bone cells to cytokines, hormones or

mechanical loading (Forwood, 1996; Maciel *et al.*, 1997; Tai *et al.*, 1997). It has also been reported that interleukin 4 (IL-4) and interleukin 13 (IL-13) inhibit bone resorption by suppressing COX-2 dependent prostaglandin synthesis in osteoblasts (Onoe *et al.*, 1996).

It has been reported that NSAIDs increase carbonic anhydrase activity (CA I and CA II) in red blood cells (Puscas *et al.*, 1996). Osteoclasts utilise CA for the generation of protons during resorption (Asotra *et al.*, 1994b), and inhibitors of CA also inhibit bone resorption (Minkin and Jennings, 1972). If NSAIDs produced a similar stimulatory effect on CA in osteoclasts, then they might be expected to facilitate resorption pit formation by increasing the intracellular availability of protons. Prostaglandins and leukotrienes exert opposite effects (inhibition and stimulation respectively) on red cell CA activity (Puscas and Coltau, 1995), this is consistent with the stimulatory action of leukotrienes on osteoclastic bone resorption (Garcia *et al.*, 1996).

It has been suggested that by blocking the COX pathway with indomethacin, arachidonic acid may be shunted toward increased formation of leukotrienes (Vane and Botting, 1987), (*e.g.* leukotriene B_4), which stimulate resorption (Garcia *et al.*, 1996). However, MK886, a specific 5-lipoxygenase inhibitor, had no effect on resorption pit formation by rat osteoclasts when cultured alone, with 2 μM indomethacin, or with 2 μM indomethacin and 2 μM

NS-398, suggesting that the stimulatory effect of NSAIDs on resorption pit formation is not mediated by an increase in leukotriene production.

Disaggregated rat osteoclast resorption assays must be performed in low pH conditions to facilitate pit formation. The finding that indomethacin (and other COX inhibitors) further stimulates resorption in this system contrasts with the observations of Goldhaber and colleagues, (Goldhaber and Rabadjija, 1987; Rabadjija *et al.*, 1990) who showed that proton-stimulated osteoclastic bone resorption in cultured mouse calvaria was completely blocked by indomethacin. The reasons for this discrepancy and for the opposing actions of prostaglandins in these two systems are not clear, but emphasise the complex nature of prostaglandin action in bone cells.

In conclusion, the results demonstrate that acid-activated isolated rat osteoclasts can be further stimulated by COX-2 inhibitors, suggesting that *in vitro*, osteoclasts are subject to a tonic, prostaglandin-mediated inhibition.

Chapter 4

ATP is a potent stimulator of the activation and formation of rodent osteoclasts

Introduction

ATP as an extracellular messenger molecule

The role of intracellular ATP (adenosine 5'-triphosphate) as an energy source has long been recognised. However, the idea that ATP could act as an extracellular signalling molecule was at first highly controversial. The first report of the potent actions of adenine compounds was published by Drury and Szent-Gyorgyi in 1929. The suggestion that ATP might be acting as an extracellular signalling molecule appeared three decades later when ATP was shown to be released from sensory nerves supplying the rabbit ear (Holton, 1959). In the early 1960s several groups proposed the existence of nerves supplying the gastrointestinal tract that were neither adrenergic nor cholinergic (NANC nerves) (Burnstock *et al.*, 1963; Martinson and Muren, 1963). Efforts were then made to identify the transmitter in NANC nerves, which was suggested

to be ATP (Burnstock *et al.*, 1970). In 1972 Burnstock proposed the concept of purinergic neurotransmission (Burnstock, 1972), and 25 years later ATP and other extracellular nucleotides are recognised as being important messenger molecules for cell-cell communication (Burnstock, 1997).

Cell membrane receptors for extracellular ATP (P_2 purinoceptors), which are entirely distinct from the adenosine receptors (P_1 purinoceptors) were first divided pharmacologically into two major classes by Burnstock (Burnstock and Kennedy, 1985), and are known as P2X and P2Y receptors (see Diagram 4.1). The P2Y receptor family couples to G-proteins to stimulate phospholipases (PLA_2, PLCb and PLD), activating a series of intracellular signalling pathways, including IP_3-dependent mobilisation of intracellular Ca^{2+} (Barnard *et al.*, 1994; Suzuki *et al.*, 1995). The P2X receptors are functionally heterogeneous, but resemble acetylcholine and serotonin-gated cation channels (Brake *et al.*, 1994), with permeability to calcium, sodium, potassium and, most likely, hydrogen ions. Seven main subtypes of P2X and eleven main subtypes of P2Y receptors have been identified to date (Burnstock and King, 1996). ATP is released into the extracellular space via synaptic vesicles from nerve cells, as the result of cell damage and also by active excretion via 'ATP binding cassette' transport proteins (ABC proteins), such as P-glycoproteins and sulphonylurea receptors (Burnstock, 1997; Wagstaff *et al.*, 1998).

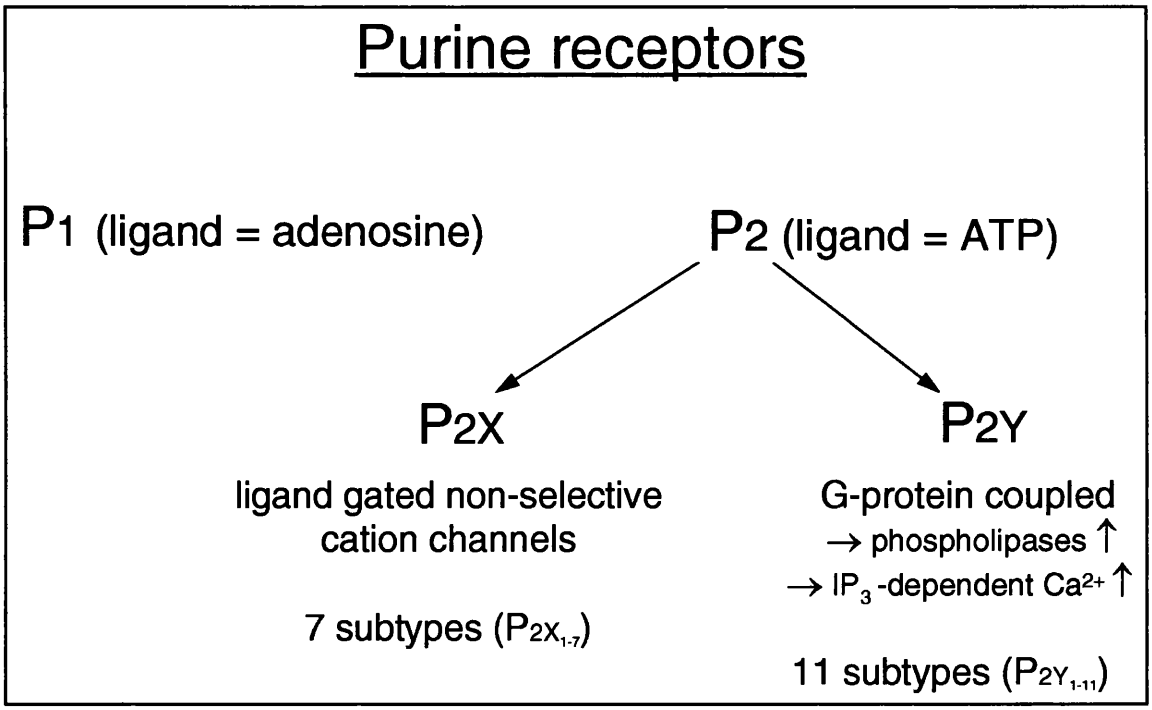


Figure 4.1 Classification of purine receptors.

ATP and bone cells

There is now growing evidence that extracellular ATP may exert functional effects on bone cells. Early work demonstrated that addition of ATP and other purinoceptor agonists to the medium bathing cultured bone cells transiently increased their cytosolic free calcium concentration (Kumagai *et al.*, 1989, 1991; Schofl *et al.*, 1992). Evidence suggests that rat osteoblast-like UMR 106 osteosarcoma cells express mixed populations of purinoceptors (probably P2Y with a proportion of receptor subtypes that varies between individual cells), and respond to extracellular ATP by increasing intracellular Ca^{2+} release (Yu and Ferrier, 1993a). Heterogeneity of receptor expression also exists within populations of human osteoblasts (Dixon *et al.*, 1997a). Recent results have suggested that osteoblast-like cells (human bone-derived cells and SaOS

osteosarcoma cells) not only express multiple P2Y receptor types (Bowler *et al.*, 1998a), but are also able to release ATP under sheer force (Bowler *et al.*, 1998b).

Exogenous ATP (50μM) added to isolated rabbit osteoclasts induces an intracellular Ca^{2+} pulse via two pathways, one involving Ca^{2+} influx, the other involving G protein-coupled internal Ca^{2+} release (Yu and Ferrier, 1993b, 1994). There is also a transient intracellular pH decrease that is Ca^{2+}-independent (Yu and Ferrier, 1995). These findings are consistent with the expression on osteoclasts of both P2Y receptors and P2X receptors. Recent experiments by Weidema *et al.*, (1997; 1998) have provided the first electrophysiological evidence for the co-existence of both P2X and P2Y receptors on bone resorbing cells. Histochemical evidence also exists for the expression of the $P2Y_2$ (P2U) receptor on osteoclasts derived from human giant cell tumours (Bowler *et al.*, 1995), although the same group has also reported that the effects of ATP on resorption pit formation by giant cell tumour osteoclasts are not mediated via the $P2Y_2$ receptor (Bowler *et al.*, 1998c), suggesting that the effects are mediated by a P2 receptor other than the $P2Y_2$ subtype.

Osteoclasts and osteoblastic cells differ in their cytosolic calcium responses to purinergic receptor activation. For example, application of high dose extracellular ATP (50 or 100 μM) inhibited the calcium response to a subsequent application of ATP in UMR 106 cells, but not in rabbit osteoclasts, suggesting that osteoclasts can adapt to the extracellular ATP, whereas UMR 106

cells cannot. This may represent a mechanism for the differential regulation of osteoblasts and osteoclasts in bone (Luo *et al.*, 1997).

It has also been demonstrated that, following purinoceptor activation in SaOS-2 osteosarcoma cells, expression of the immediate early response gene *c-fos* is induced. Induction probably occurs via elevated intracellular calcium interacting with the cAMP response element of *c-fos* (Bowler *et al.*, 1996). Extracellular nucleotides can also synergise with parathyroid hormone (PTH) to further stimulate *c-fos* expression and bone resorption by avian osteoclasts *in vitro*, suggesting that extracellular nucleotides present in the bone microenvironment may be capable of modulating bone cells and controlling the remodelling process by interacting with other factors (Bowler and Gallagher, 1996). Although both ATP and adenosine are able to act as mitogens for cultured bone cells (Shimegi, 1996), extracellular ATP has also been shown to reduce the amount of bone formed by primary rat osteoblasts in an *in vitro* appositional bone formation model (Jones *et al.*, 1997).

Interaction between ATP and extracellular pH

Recent evidence has shown that extracellular acidification is required for $P2X_2$ receptors to show their full sensitivity to extracellular ATP (King *et al.*, 1996). Using recombinant $P2X_2$ receptors expressed in defolliculated *Xenopus* oocytes, Wildman *et al.*, (1997) noted that small acidic and alkaline shifts, (as little as 0.03 pH-units) were able to enhance or diminish the response to ATP,

respectively. It was also found that the response of the recombinant $P2X_2$ receptor to ATP at low pH was potentiated by neurotransmitters (*e.g.* noradrenaline) and related substances (*e.g.* histamine). The pH-activation profile of the recombinant $P2X_2$ receptor is very similar to that for resorption pit formation by rat osteoclasts (Arnett and Spowage, 1996). No other P2 receptors appear to be so sensitive to extracellular pH, although it is known that the action of adenosine on the P1 receptor is potentiated by extracellular acidification (Hiley *et al.*, 1995).

The effects of extracellular ATP on mature osteoclast resorption pit formation and on osteoclast formation have not been investigated thus far. The aim of the present study was to examine the interaction between extracellular ATP and extracellular protons on osteoclast formation and activation.

Methods

Fresh stock solutions of ATP, adenosine, apyrase and suramin were prepared in phosphate buffered solution (PBS) for each experiment. Stock solutions of dexamethasone and $1,25(OH)_2D_3$ were prepared in ethanol and stored for short periods at -20°C. The final concentration of ethanol in test and control culture medium was constant at 0.2%. ATP and adenosine solutions, both of which are acidic, were titrated to pH 7.0 with NaOH immediately before use to avoid unwanted pH effects on osteoclast function (Arnett and Spowage, 1996). Cell culture methods are described in Chapter 2, pages 49 to 58.

Antisense experiments

To investigate the possible involvement of the pH sensitive $P2X_2$ receptor for ATP in the acid activation effect of osteoclasts, a region of the $P2X_2$ mRNA with low sequence identity compared to other P2X subunit mRNAs was selected (selected by Dr A Townsend-Nicholson, Autonomic Neuroscience Institute, London, U.K.) for the design of antisense oligonucleotides. This region encompasses the initiation codon and ranges from nucleotides 31-51 of the rat $P2X_2$ cDNA sequence (Genbank accession number U14414). Phosphorothioate-modified antisense (5'-CAAGCGCCGGACCATGGCCGC-3') and mismatched control (5'-CAAGCCGCCGGCGTAGACGCC-3') oligodeoxynucleotides were synthesised (Genosys Biotechnologies Ltd, Pampisford, Cambridgeshire). As

well as being specific for the mRNA sequence, functional antisense oligonucleotides must be stable to nucleases in order to reach the target. They must be soluble, but still be able to pass through the cell membrane (see Stein *et al.*, 1991 for review). Normal oligonucleotides were found to be extremely sensitive to nucleases, and so modified oligonucleotides such as phosphorothioates (as used here) were devised which have been shown to have a prolonged half-life within the cell (Matsukura *et al.*, 1987).

Fresh antisense solutions were prepared in PBS immediately before each experiment. Previous studies have shown that osteoclasts readily take up oligonucleotides in culture without need for cationic lipophilic agents (Reddy *et al.*, 1994 & Tanaka *et al.*, 1996). Osteoclast cultures were preincubated with the oligonucleotides for 24 hours at pH 7.3 (*i.e.* conditions in which osteoclasts are "switched off" (Arnett and Spowage, 1996)) before being transferred to low pH (around pH 7.0) for a further 24 hours to allow acid activation.

Time lapse video microscopy

Low density mixed cell populations containing osteoclasts were obtained by rapidly mincing the long bones of one two day old rat pup; killed by cervical dislocation, in 1ml MEM, followed by vortexing for 20 seconds. The resulting cell suspension was allowed to sediment for 1 hour in one well of a 6-well plate. During this hour, the culture was incubated in a humidified atmosphere of 5% CO_2 / 95% air. The well was then rinsed twice with PBS to remove non-

adherent cells, before adding 2ml of PBS. The culture was transferred to a 37°C humidified chamber above an Olympus IMT-2 inverted microscope with phase contrast optics (Olympus Optical Company Ltd, Tokyo, Japan). One osteoclast was selected for time lapse recording using a Sony CCD colour video camera (DXC-151A; Sony Corporation, Tokyo, Japan) and a Panasonic time-lapse video cassette recorder (AG-6730; Matsushita Electric Industrial Company Ltd, Osaka, Japan) running at 1/60th of normal speed. Ten minutes after the recording began ATP was added to the culture to give a final concentration of 2mM.

Results

Effect of ATP on mature rat osteoclasts

Extracellular ATP exerted a reproducible, biphasic effect on resorption pit formation by rat osteoclasts in low density, acid-activated 26 hour cultures (pH 6.94 ± 0.016). At low concentrations (0.2 and 2μM) striking stimulations were observed, with up to a 3.5-fold increase in pit number (Figure 4.2). At higher concentrations, resorption was progressively reduced, which was largely due to a selective cytotoxic effect of ATP on osteoclasts. Numbers of mononuclear cells (*i.e.* cells of osteoblastic / fibroblastic morphology) were unaltered by ATP (see Table 4.1 below).

Table 4.1

[ATP] (μM)	Number of mononuclear cells / disc
0	1230 ± 95
0.2	1323 ± 213
2	1200 ± 114
20	1228 ± 167
200	1390 ± 151
2000	1001 ± 143

Lack of effect of ATP on the number of mononuclear cells cultured on 5mm dentine discs in acidified medium (pH 6.937 ± 0.016) for 26h. Values are means ± SEM (n = 5).

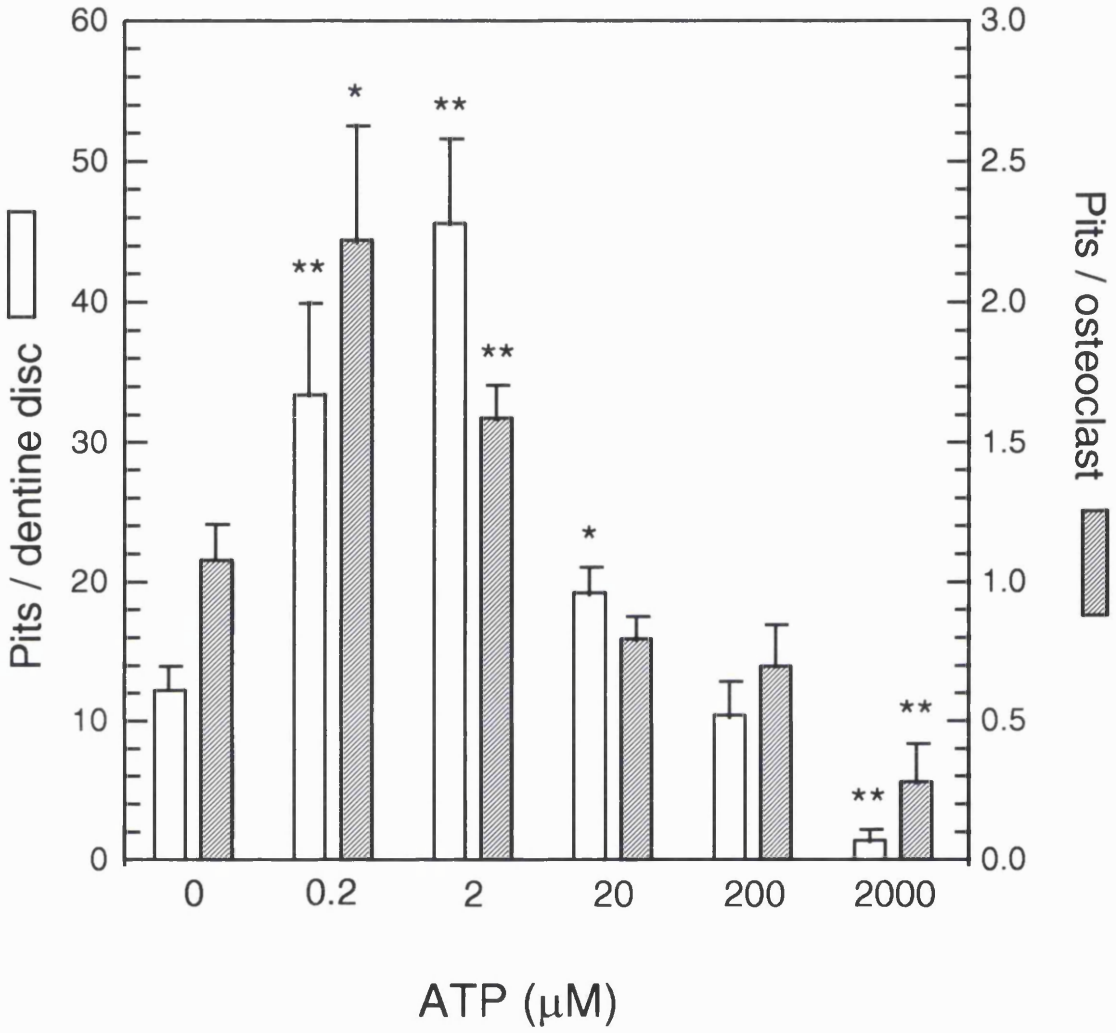


Figure 4.2 Biphasic effect of ATP on resorption pit formation by rat osteoclasts cultured on 5mm dentine discs in acidified medium (pH 6.937 ± 0.016) for 26h, with stimulation effect evident at lower concentrations and a selective cytotoxic effect at the highest concentration. Values are means ± SEM (n = 5); * $p<0.05$; ** $p<0.01$ with respect to control.

Time lapse video microscopy revealed that 60-90 minutes following addition of 2mM ATP to freshly isolated mature rat osteoclasts, the cell abruptly retracted and died in a manner reminiscent of apoptosis (Figure 4.3).

In contrast to ATP, adenosine (the P1 receptor agonist) had no significant effect on either resorption pit formation or osteoclast numbers over a similar dose range, except at the highest dose (2mM), where a selective cytotoxicity was observed (Figure 4.4). Numbers of mononuclear cells were unaltered by adenosine (see Table 4.2 below).

Table 4.2

[Adenosine] (μM)	Number of mononuclear cells / disc
0	873 ± 45
0.2	665 ± 72
2	762 ± 54
20	813 ± 91
200	909 ± 73
2000	583 ± 86

Lack of effect of adenosine on the number of mononuclear cells cultured on 5mm dentine discs in acidified medium (pH 6.973 ± 0.026) for 26h. Values are means ± SEM (n = 5).

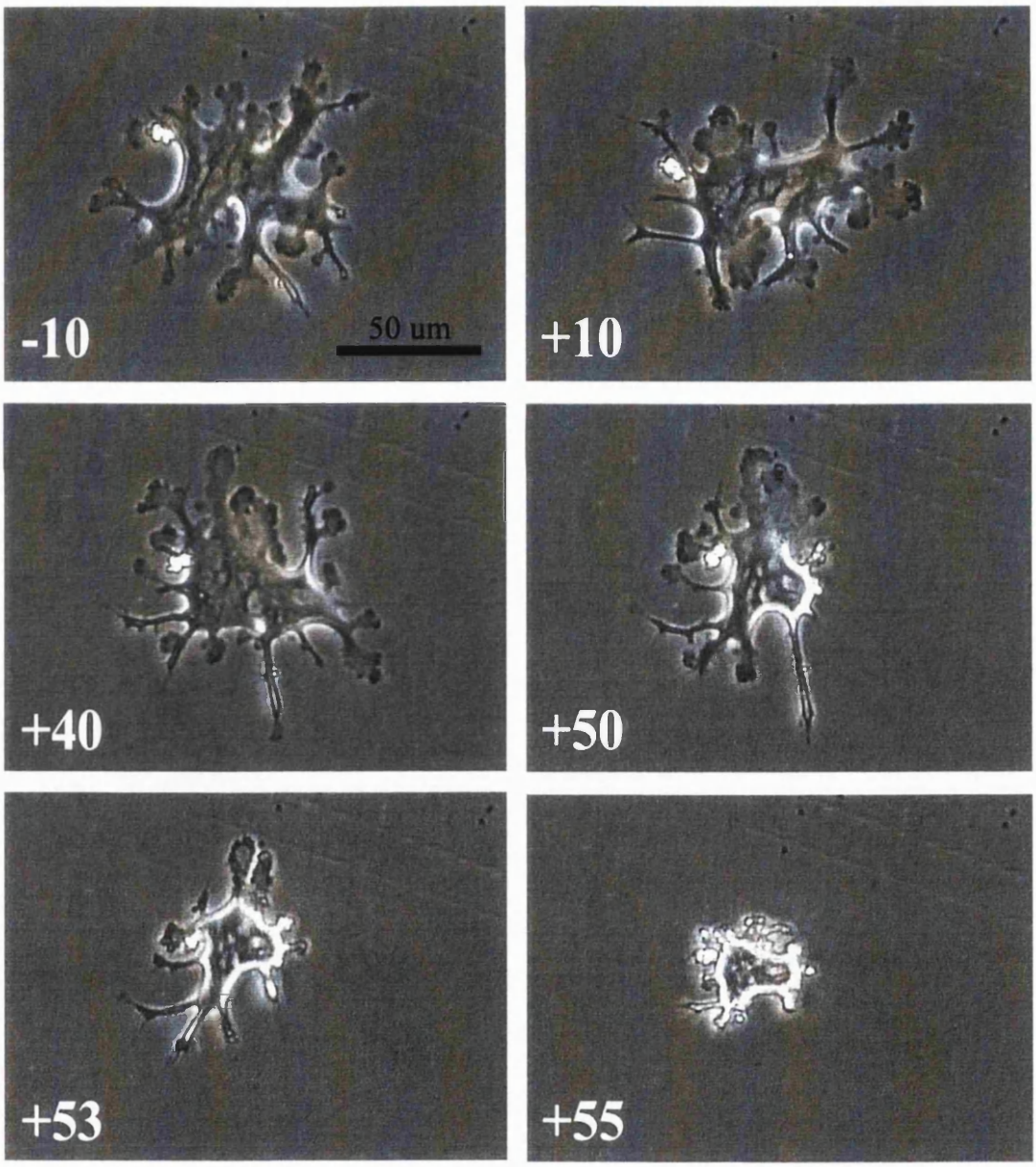


Figure 4.3 Time lapse sequence illustrating relatively rapid cytotoxic effect of 2mM ATP on rat osteoclasts cultured on plastic.

Approximately 1h after addition of ATP, the cell abruptly retracts and dies in a manner reminiscent of apoptosis. Numbers indicate time in minutes before (-) and after (+) addition of ATP.

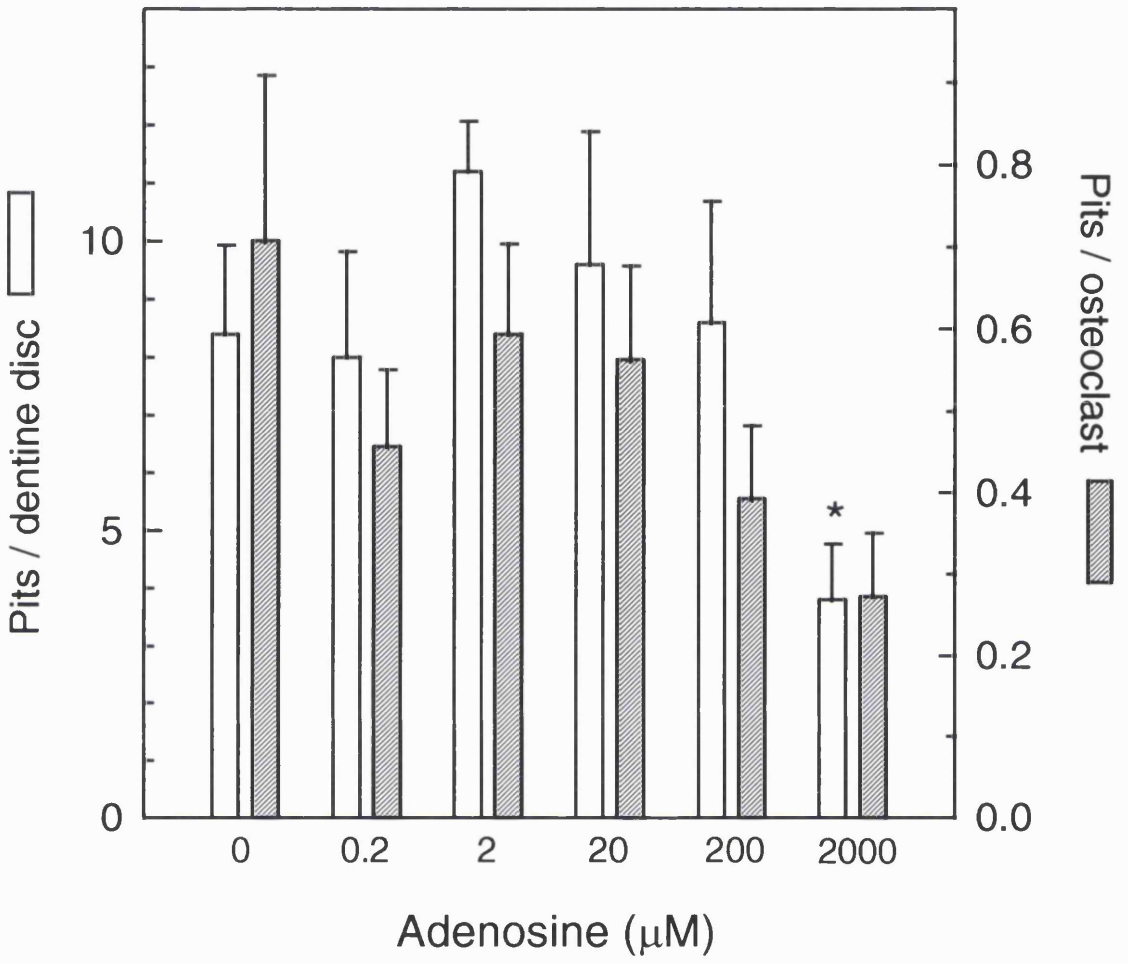


Figure 4.4 Lack of effect of adenosine on resorption pit formation by rat osteoclasts cultured on 5mm dentine discs in acidified medium (pH 6.973 ± 0.026) for 26h, except for a selective cytotoxic effect at the highest concentration. Values are means ± SEM (n = 5); * $p<0.05$; with respect to control.

Interaction of ATP and low pH

The interaction between the effects of low pH and extracellular ATP were investigated using rat osteoclast cultures incubated for 26 hours with or without 2 μM ATP in either control (non-acidified) or acidified MEM (Figure 4.5). In the absence of ATP, acidification (pH reduction from 7.166 to 6.945) produced a modest effect, eliciting a 3-fold stimulation of resorption pit formation; and in non-acidified medium ATP caused a 2.5-fold stimulation of pit formation. However, in the presence of 2 μM ATP, the acid effect was enhanced greatly, with a 17-fold stimulation compared with control. Thus, the stimulatory effect of ATP was much greater in acid conditions than in non-acidified conditions and *vice versa.*

Effect of apyrase, suramin, and antisense oligonucleotides

To investigate whether the acid activation of resorption was dependent on the presence of traces of extracellular ATP, osteoclasts were cultured for 26 hours with apyrase, the ecto-ATPase which hydrolyses extracellular ATP to AMP. In this experiment, acidification resulted in a 3.5-fold stimulation of resorption pit formation. Apyrase inhibited acid-activated resorption, with a near maximal effect at 0.1 units per ml (Figure 4.6). There was no evidence that apyrase affected cell viability or morphology at any dose.

Resorption pit formation by acid-activated osteoclasts was also significantly inhibited by the general P2 receptor antagonist suramin (Figure 4.7). This effect was not due to cytotoxicity.

To investigate the possible involvement of the pH sensitive $P2X_2$ receptor for ATP in the acid-activation effect, osteoclast cultures were pre-incubated for 26 hours with an antisense oligonucleotide corresponding to the rat $P2X_2$ receptor or a mismatched control sequence, before transfer to low pH for a further 26 hours to allow acid-activation. Resorption pit formation by rat osteoclasts activated at pH 7.0 was significantly inhibited by the antisense oligonucleotide, and to a lesser degree by the mismatched control (Figure 4.8).

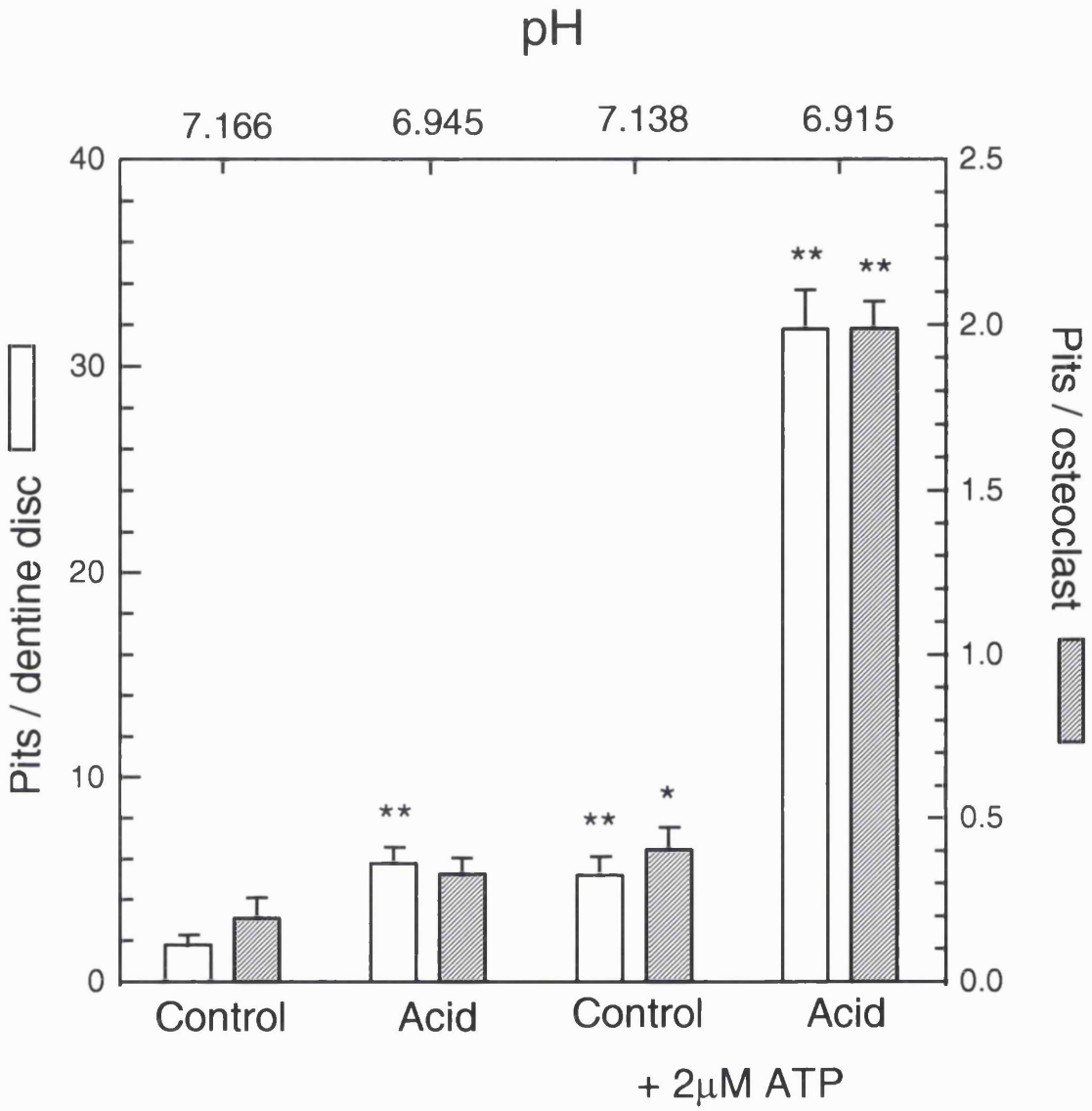


Figure 4.5 Comparison of the effects of ATP on resorption pit formation by rat osteoclasts cultured in unmodified medium (Control) or in acidified medium (Acid) for 26 h, showing potentiation of ATP-stimulated resorption at low pH. Values are means ± SEM (n = 5); * $p<0.05$; ** $p<0.01$ with respect to control.

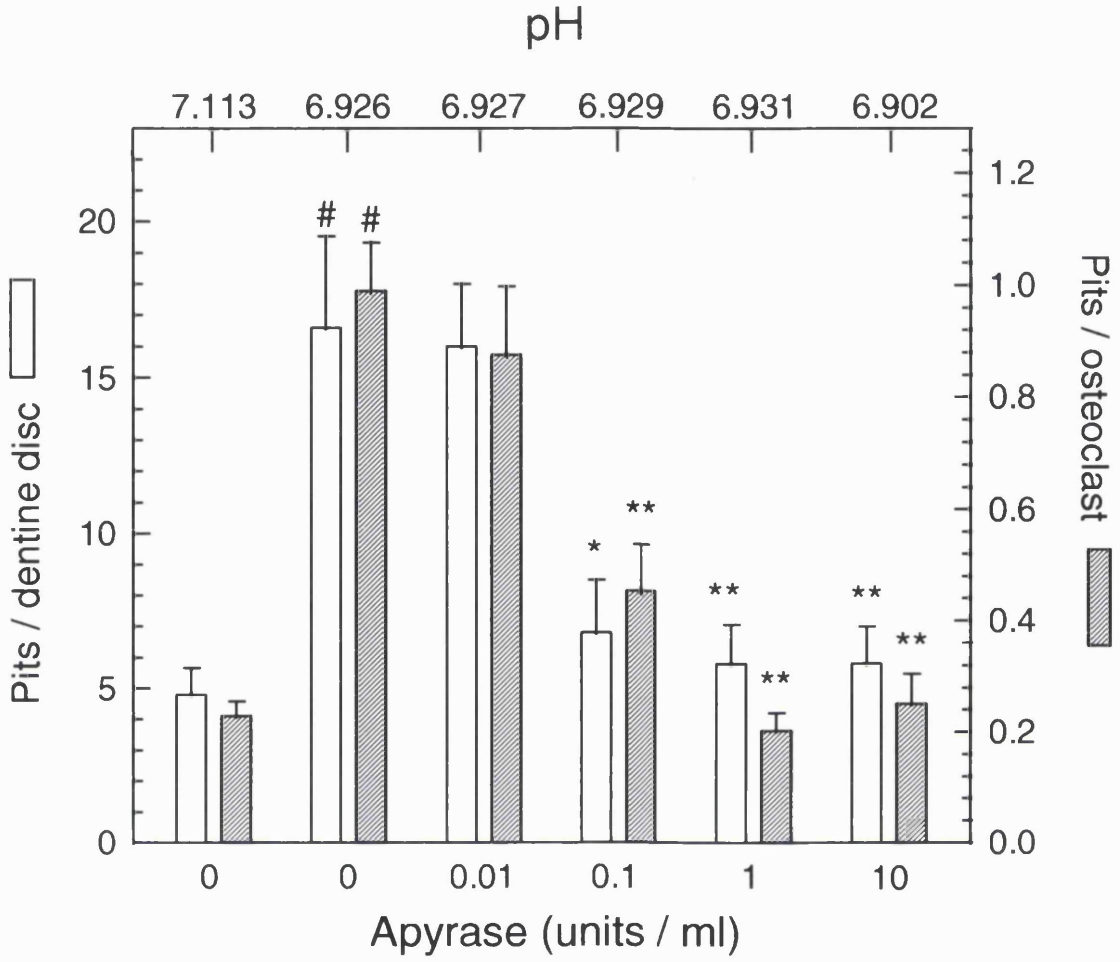


Figure 4.6 Stimulation of resorption pit formation by rat osteoclasts cultured on 5mm dentine discs in acidified medium (#, $p<0.01$ with respect to pH 7.113 control value) and inhibition of acid stimulated pit formation by apyrase (* $p<0.05$; ** $p<0.01$ with respect to pH 6.926 control value). Values are means ± SEM (n = 5).

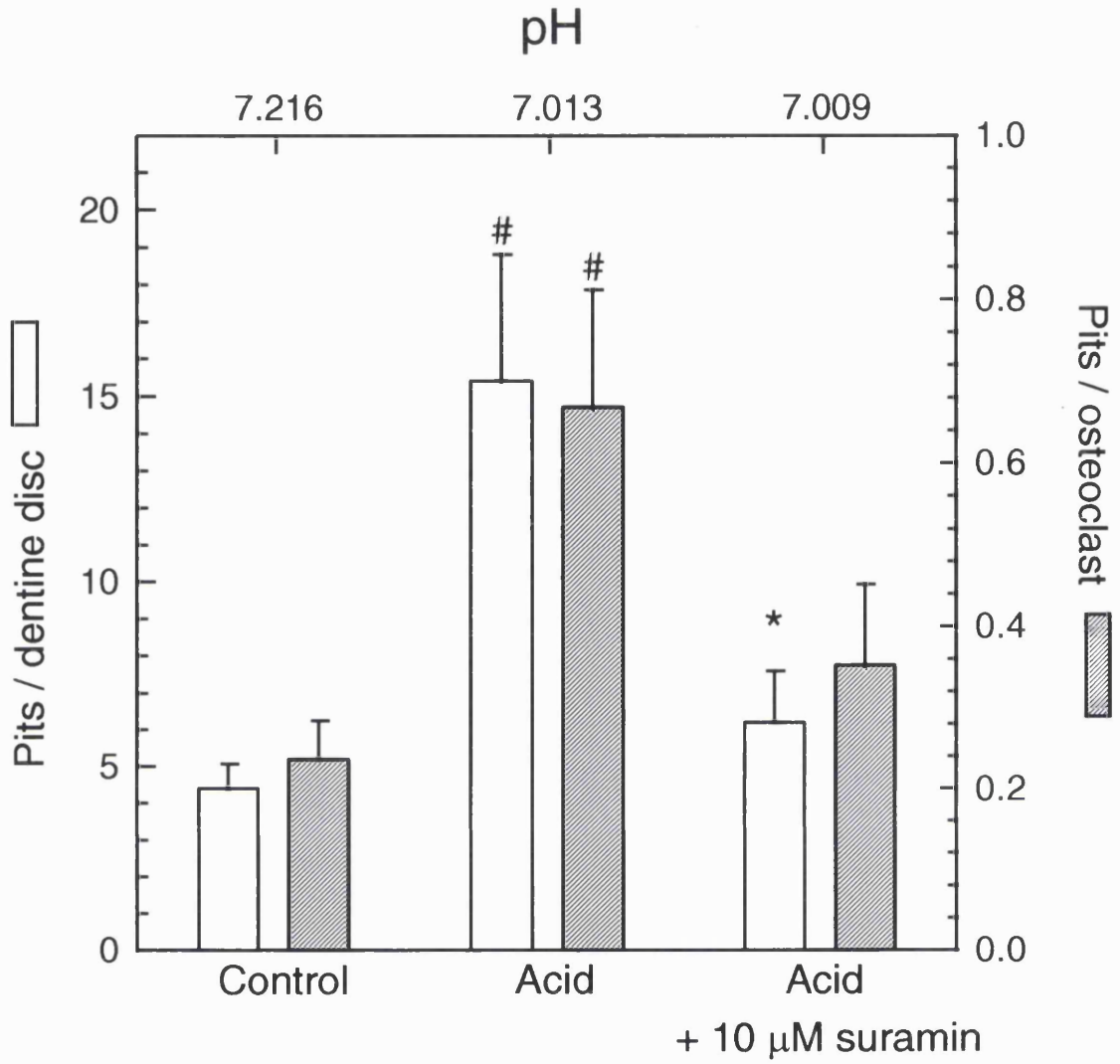


Figure 4.7 Stimulation of resorption pit formation by rat osteoclasts cultured on 5mm dentine discs in acidified medium (#, $p<0.01$ with respect to pH 7.216 control value) and inhibition of acid stimulated pit formation by suramin (* $p<0.05$ with respect to pH 7.013 control value). Values are means ± SEM (n = 5).

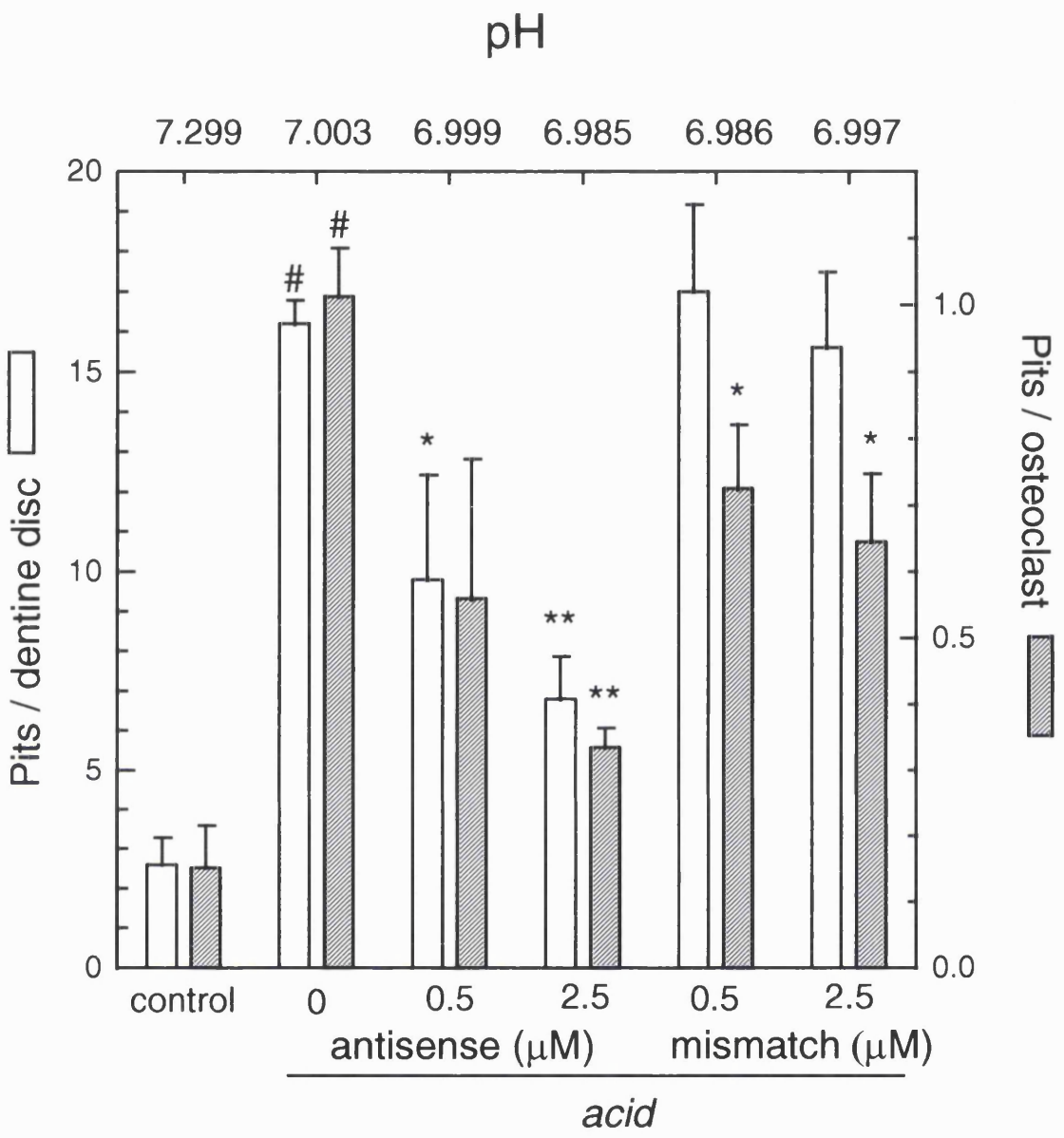


Figure 4.8 Stimulation of resorption pit formation by rat osteoclasts cultured on 5mm dentine discs in acidified medium (#, $p<0.01$ with respect to pH 7.299 control value) and inhibition of acid stimulated pit formation by antisense oligonucleotide corresponding to initiation codon region of rat $P2X_2$ receptor (* $p<0.05$; ** $p<0.01$ with respect to pH 7.003 control value). Values are means ± SEM (n = 5).

Effect of ATP and adenosine on chick osteoclasts

Low concentrations of extracellular ATP (0.1 and 1μM) had no effect on resorption pit formation by chick osteoclasts (Figure 4.9) in acid-activated 26 hour cultures (pH 6.97 ± 0.019). These doses were similar to concentrations which caused a significant stimulation of resorption pit formation in rat osteoclast cultures (Figure 4.2). At higher concentrations (10μM and above), resorption was significantly reduced compared to control due to a selective cytotoxic effect. Mononuclear cell numbers were unaltered by ATP (see Table 4.3 below). In common with ATP, adenosine (the P1 receptor agonist) was without significant effect on either resorption pit formation or osteoclast numbers over a similar dose range, except at the highest dose (500μM), where some cytotoxicity was observed (Figure 4.10).

Table 4.3

[ATP] (μM)	Number of mononuclear cells / disc
0	5887 ± 367
0.1	5825 ± 153
1	5563 ± 380
10	5827 ± 181
100	5837 ± 332
500	5359 ± 200

Lack of effect of ATP on the number of mononuclear cells cultured on 5mm dentine discs in acidified medium (pH 6.972 ± 0.019) for 26h. Values are means ± SEM (n = 5).

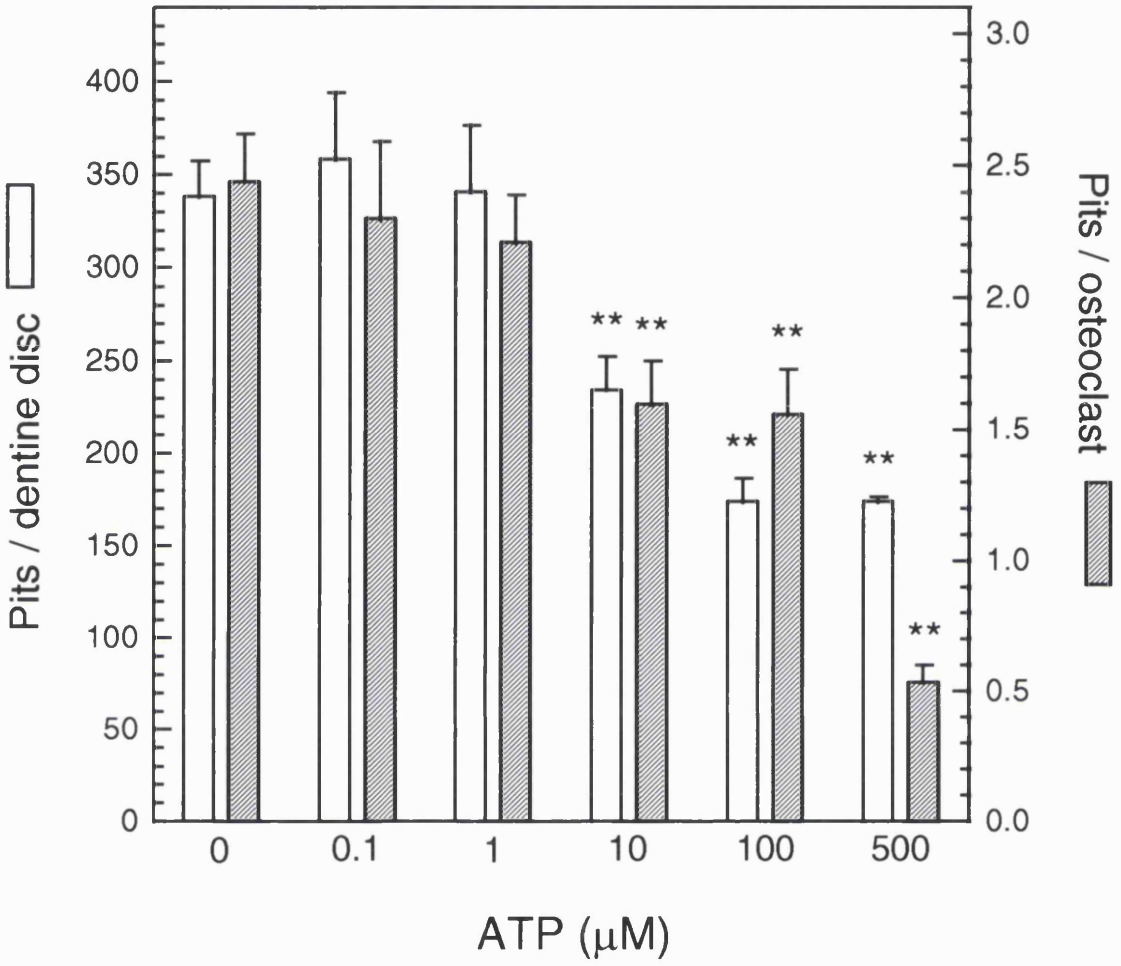


Figure 4.9 Inhibitory effect of ATP on resorption pit formation by chick osteoclasts cultured on 5mm dentine discs in acidified medium (pH 6.972 ± 0.019) for 26h, with a selective cytotoxic effect at the highest concentration. Values are means ± SEM (n = 5); * $p<0.05$; ** $p<0.01$ with respect to control.

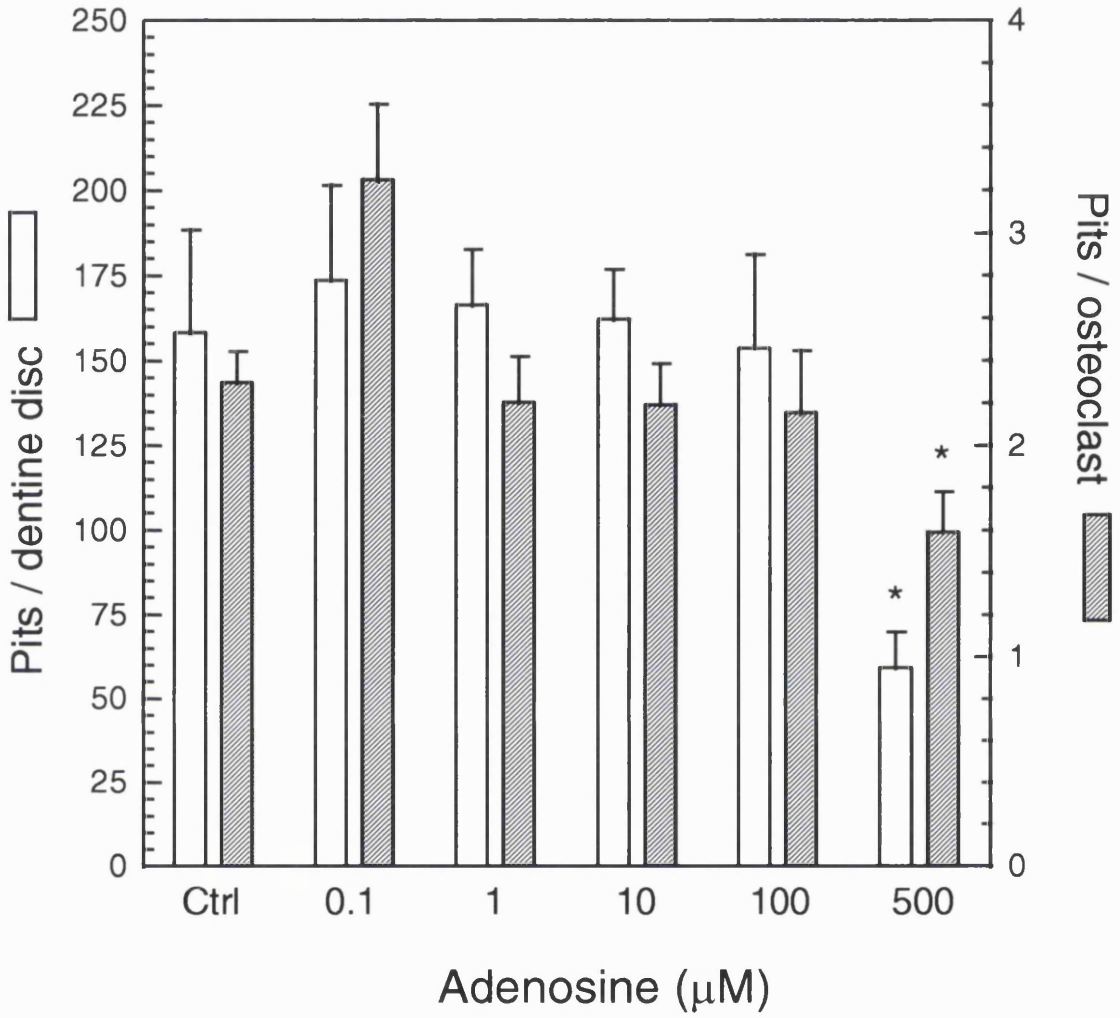


Figure 4.10 Lack of effect of adenosine on resorption pit formation by chick osteoclasts cultured on 5mm dentine discs in acidified medium (pH 6.944 ± 0.007) for 26h, except for a slight cytotoxic effect at the highest concentration. Values are means ± SEM (n = 5); * $p<0.05$; with respect to control.

Effect of ATP on osteoclast formation in murine marrow cultures

In 10 day murine marrow cultures cultured in unmodified MEM at pH 7.2, extracellular ATP at low concentrations stimulated the formation of TRAP-positive osteoclasts and resorption pits. Peak effects were observed over a similar dose range (0.2 to 2 μM) to those seen in the mature rat osteoclast assay. In the presence of 2 μM ATP, osteoclast formation was stimulated 3.3-fold and resorption increased 4-fold compared to control. Higher concentrations either partially (20 μM) or completely inhibited (200 μM) osteoclast formation and resorption (Figure 4.11).

In contrast to ATP, adenosine was without effect on osteoclast recruitment or resorption pit formation over a similar dose range (see Table 4.4 below).

Table 4.4

[Adenosine] (μM)	TRAP positive area ($\mu m^2 \times 10^{-5}$)	Pit area ($\mu m^2 \times 10^{-5}$)
0	83 ± 7.0	43 ± 5.6
0.2	87 ± 7.7	47 ± 2.8
2	87 ± 3.1	41 ± 13.7
20	83 ± 14.1	24 ± 7.5

Effect of adenosine on osteoclast recruitment and resorption pit formation in murine marrow cultures maintained for 10 days on 5mm dentine discs in unmodified medium (pH 7.192 ± 0.016). Values are means ± SEM (n = 5).

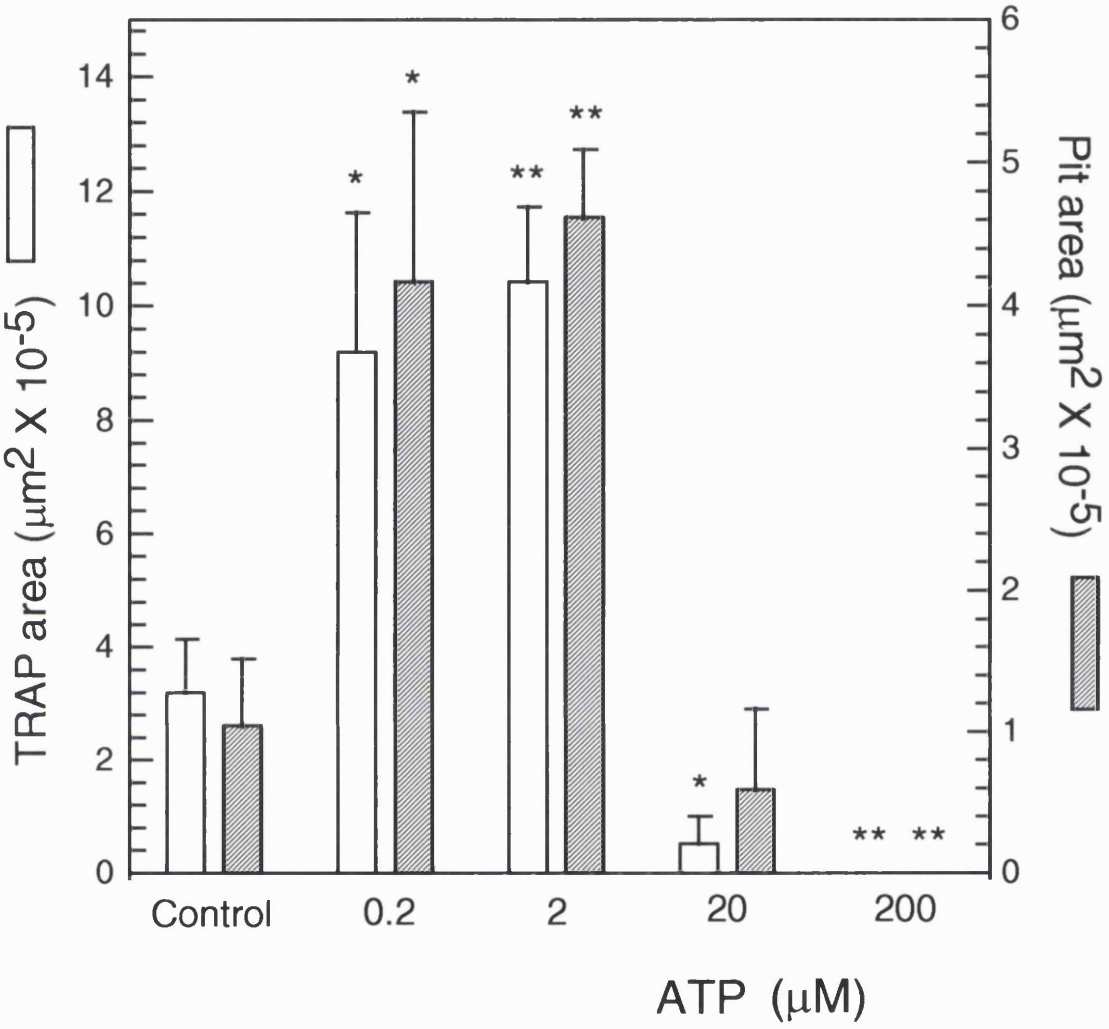


Figure 4.11 Biphasic effect of ATP on osteoclast recruitment and resorption pit formation in mouse marrow cultures maintained for 10 days on 5mm dentine discs in unmodified medium (pH 7.212 ± 0.010). Values are means ± SEM (n = 6); * $p<0.05$; ** $p<0.01$ with respect to control.

Figure 4.12 demonstrates the interaction between ATP and extracellular pH on osteoclast formation over 10 days. Addition of small amounts of hydroxyl ions increased the operating pH by 0.1 of a unit compared with control media (pH increase from 7.168 to 7.277). This resulted in the stimulation of osteoclast formation, but was accompanied by a reduction in resorption pit formation. Addition of 2 μM ATP to the alkalinised cultures stimulated osteoclast formation further (1.6-fold increase), but had little effect on resorption pit formation. Culture of cells for 10 days in acidified conditions (around pH 6.95) resulted in a reduction in osteoclast formation, but a relative increase in the resorptive efficiency. When ATP was added to acidified cultures, osteoclast formation was stimulated as shown in the alkalinised cultures, but this was accompanied by a large increase in resorption pit formation (4.4-fold increase compared to acidified control).

Figure 4.13 demonstrates the typical appearance of a murine marrow assay at day 10 of culture. Note TRAP positive osteoclasts adjacent to areas of resorption.

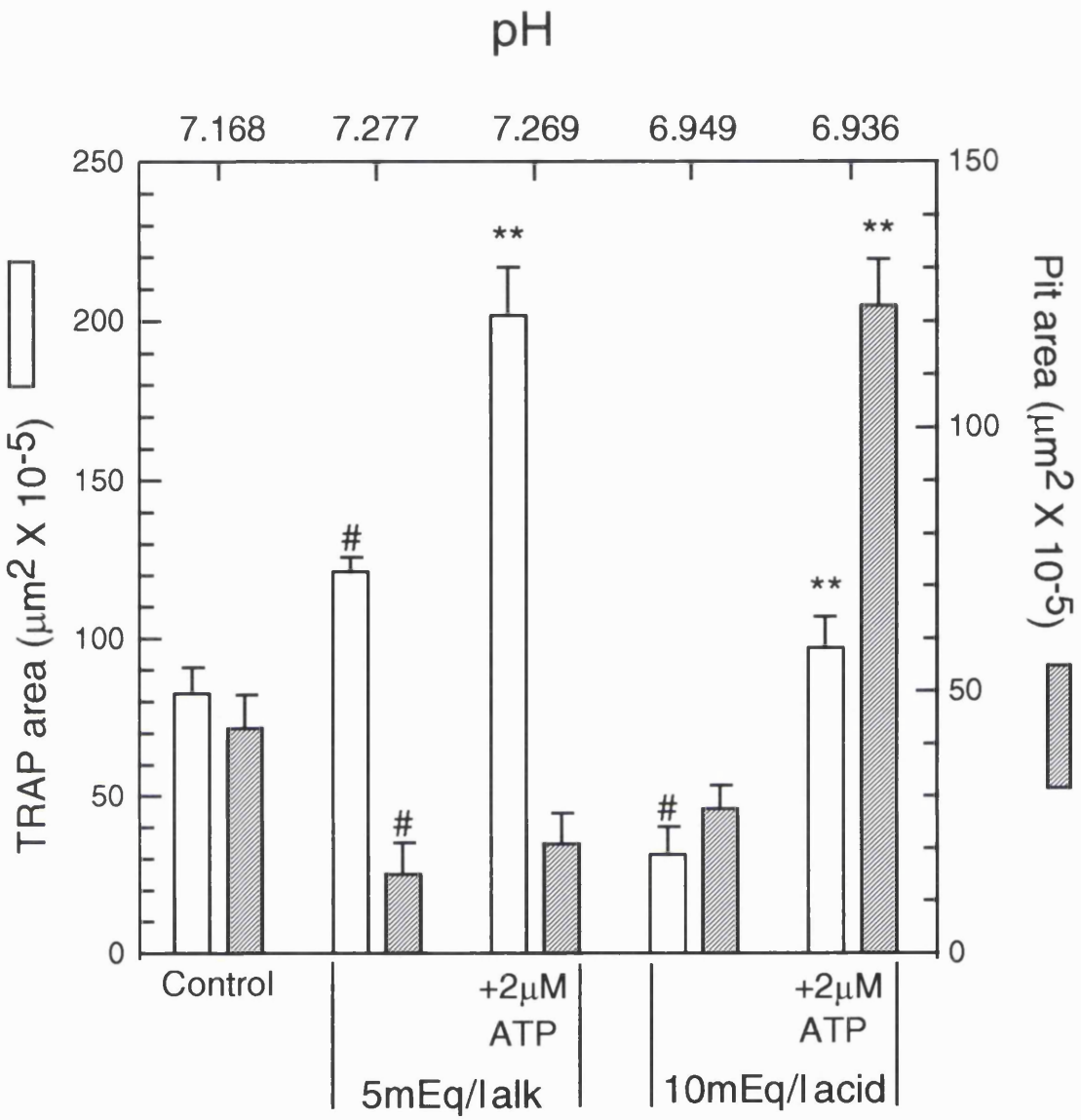
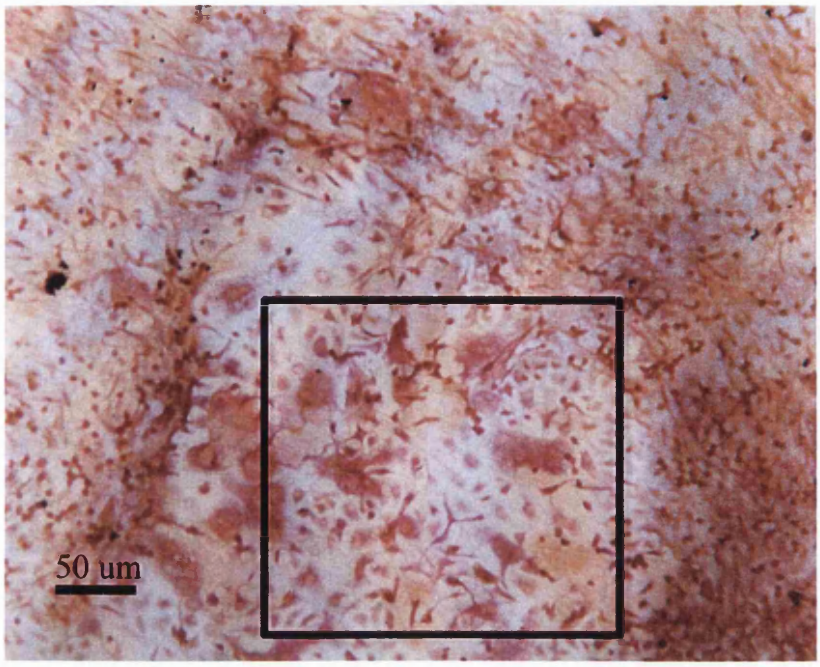


Figure 4.12 Effects of extracellular pH on osteoclast recruitment and resorption pit formation in mouse marrow cultures maintained for 10 days in the absence or presence of 2μM ATP. Values are means ± SEM (n = 5); #, $p<0.01$ with respect to pH 7.168 control value; ** $p<0.01$ with respect to alkaline or acid without ATP.



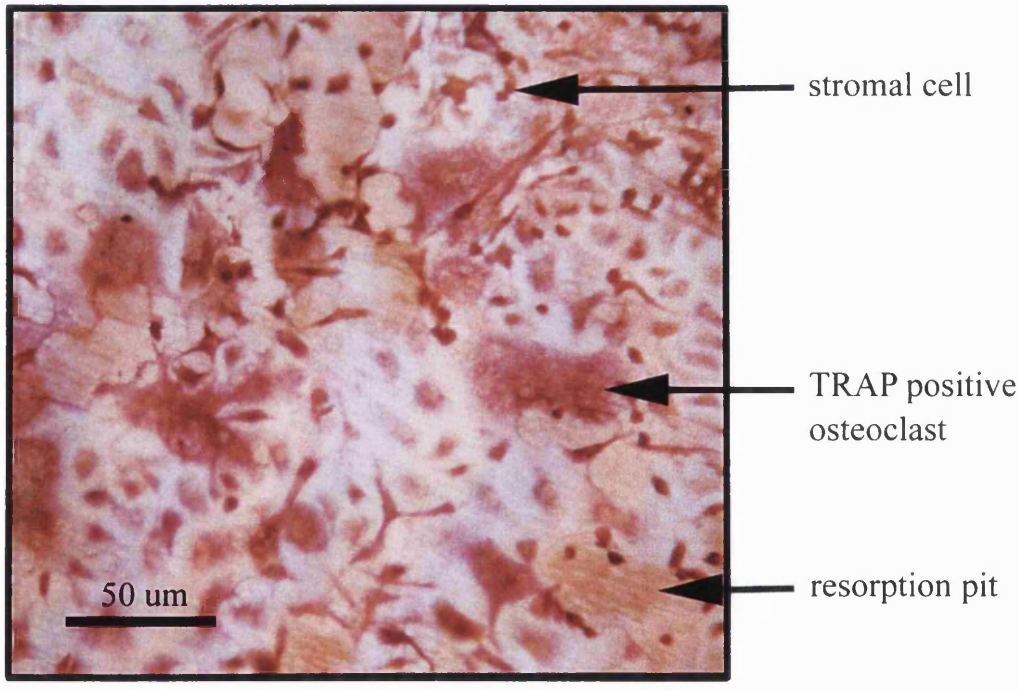


Figure 4.13 Medium (x20 objective) and high power (x40 objective) transmitted light photomicrograph of a 10 day mouse marrow assay showing the typical appearance of abundant TRAP-positive multinucleated osteoclasts adjacent to resorption pits. Note the proximity of the osteoclasts to stromal cell populations.

Discussion

Osteoclast activity is under the control of both systemic hormones and cytokines generated in the bone microenvironment. These factors may act directly on osteoclasts or their precursors, or may act indirectly to control osteoclast formation, differentiation and function (Alsina *et al.*, 1996). The results presented in this chapter indicate that extracellular ATP, which causes striking stimulations of both rat osteoclast resorption pit formation and murine osteoclast recruitment at submicromolar concentrations, must be considered as a potentially important paracrine or autocrine agent for bone.

It is unclear from the present data whether ATP stimulates resorption pit formation via direct effects on mature osteoclasts, indirect effects on cells such as osteoblasts, or both. Electrophysiological evidence (Yu and Ferrier, 1993b, 1994, 1995; Weidema *et al.*, 1997, 1998) and histochemical evidence (Bowler *et al.*, 1995, 1998c) exists for the expression of P2X and P2Y receptors on normal or tumour-derived osteoclasts. P2Y receptors are also present on osteoblast-like cells (Bowler *et al.*, 1995, 1998a). Pure populations of normal mammalian osteoclasts are not routinely available for study and multinucleate osteoclasts constitute only about 1-2% of the cells in the low density rat bone cell cultures used in these experiments. However, whether extracellular ATP is acting

directly or indirectly on osteoclasts may be immaterial, since both cell types are present in the *in vivo* situation.

The rapid, selective, cytotoxic action of ATP at millimolar concentrations on osteoclasts may be mediated by the $P2X_7$ (P2Z) receptor for ATP^{4-}. The $P2X_7$ receptor is a bifunctional molecule through which ATP can open a small cationic channel, and also induce large cytolytic pores permeable to high molecular weight molecules (>600 Da) (Surprenant *et al.*, 1996; Virgino *et al.*, 1997). The results are also in agreement with Nijweide *et al.*, (1995), who found that high concentrations of ATP acting via the $P2X_7$ receptor, (which is found on all cells of haemopoietic origin (*e.g.* osteoclasts) but not on cells of a stromal origin (*e.g.* osteoblasts)) caused cell death. The ability of high concentrations of extracellular ATP to kill cells is well established, with a body of evidence showing the cytotoxicity of extracellular ATP, especially towards cells of the immune system (Di Virgilio *et al.*, 1989, Zanovello *et al.*, 1990 & Zheng *et al.*, 1991). Cell death caused by ATP involves both necrosis and apoptosis, with two P2 purinoceptors implicated: $P2X_7$ (the receptor for ATP^{4-}), and possibly $P2X_1$ which has sequence homology to genes expressed by thymocytes during the onset of apoptosis (Chow *et al.*, 1997).

Adenosine had no effect on resorption pit formation by mature rat osteoclasts, suggesting that P1 receptors are not involved in the modulation of osteoclast function. This result is in line with the findings of Yu and Ferrier

(1993b), who showed that adenosine had virtually no effect on the intracellular calcium concentration in isolated osteoclasts. Adenosine also had no effect on osteoclast formation in murine marrow cultures. Additionally, adenosine has been reported to have no effect on the amount of appositional bone formed in primary rat osteoblast cultures (Jones *et al.*, 1997). Taken together, these results suggest that P2 receptors predominate in the bone microenvironment.

In contrast to the effect of extracellular ATP on mature rat osteoclasts, osteoclasts obtained from embryonic chicks were unresponsive to low dose ATP. However, chick osteoclast resorption pit formation was inhibited by ATP at 10 μM and above. When compared to rat osteoclast resorption pit formation, which was inhibited at 200 μM and above, this suggests that chick osteoclasts are more sensitive to the inhibitory effects of ATP than rat osteoclasts. Chick osteoclasts were also unaffected by adenosine, the P1 receptor agonist, except for a slight cytotoxic effect at 500 μM . Osteoclasts obtained from chicks differ from mammalian osteoclasts in that they are 'constitutively activated' and therefore resorb more bone over time. Furthermore, chick osteoclasts do not show an inhibitory response to calcitonin (Arnett and Dempster, 1987). The results presented here therefore demonstrate additional differences between osteoclasts isolated from mammals and chicks.

As discussed in Chapter 2, an important feature of H^+-stimulated resorption is that that it does not exhibit desensitisation, suggesting that over long

periods of time osteoclasts will remain responsive to pH changes. Embryonic chick osteoclasts, mouse calvarial cultures, and osteoclasts formed from mouse marrow cultures exhibit similar responses to extracellular pH (see Chapter 2). Data presented here show that there is a powerful synergy between the stimulatory effects of low dose ATP and protons on the resorptive activity of mature rat osteoclasts, such that the full sensitivity of osteoclasts to activation by ATP is only evident at low pH (~6.9-7.0) and *vice versa.* In the case of the osteoclast formation experiments, ATP appeared to enhance resorptive efficiency only slightly; this was probably a consequence of the non-acidified medium (pH ~7.2) used to maximise osteoclast recruitment in this assay (see chapter 2).

Apyrase, (an ecto-ATPase) incubated with acid activated rat osteoclasts, inhibited resorption pit formation without affecting cell viability or morphology, suggesting that the pH response of mature rat osteoclasts may be dependent on trace levels of free or bound extracellular ATP. The concentrations of ATP in question may be quite low, given the powerful stimulatory effect seen in the presence of 200 nM ATP.

There are two main possibilities for the origin of extracellular ATP in osteoclast-containing bone cell cultures. First, ATP could be released from cells damaged or stressed during the isolation process. In most cells cytoplasmic ATP is >5mM in concentration, and a significant proportion can be released without loss of cell viability (Gordon, 1986). The high intracellular concentration of

ATP suggests that one ruptured cell could release enough ATP to influence resorption in a region corresponding to 25,000 times its own volume. Second, ATP can be exported from intact cells via transport proteins. Recent results from Wagstaff *et al.*, (1998) have shown that osteoclasts express a novel member of the ATP binding cassette (ABC) superfamily. ABC proteins are a family of ATP-dependent transport proteins, capable of transporting a variety of species, such as small ions, sugars, and ATP itself across the cell plasma membrane. It has also been reported that the constitutive release of ATP from osteoblasts can be modulated by fluid shear forces, suggesting that ATP may have a role in bone mechanotransduction (Bowler *et al.*, 1998b). Interestingly, McSheehy and Chambers, (1986b) reported that osteoblasts released an unknown soluble factor of relatively low molecular weight that stimulated osteoclasts. Given that osteoclastic resorption pit formation and osteoclast recruitment are stimulated by extracellular ATP in the sub-micromolar range, only nanomolar concentrations of ATP need to be present to exert a powerful stimulatory effect. This may represent an important variable in osteoclast assays.

Suramin (a polyanionic general antagonist of ATP action at $P2X_{1\text{-}3}$ & $_5$ receptors (Alexander and Peters, 1998)) was also able to inhibit acid-activated resorption pit formation back to control levels, without affecting cell viability. This result again suggests that ATP and extracellular pH are interacting to stimulate osteoclastic bone resorption. However, care should be taken when interpreting data using suramin, as it is known to suppress bone resorption

regardless of whether it is stimulated by prostaglandins, PTH, $1,25(OH)_2D_3$, or epidermal growth factor (Farsoudi *et al.*, 1993), suggesting a broad and non-specific mechanism of action. The lack of specific P2X receptor antagonists is a recognised problem (Burnstock, 1996), and is an important area of future work.

As stated above, experiments have shown that extracellular acidification is required for the $P2X_2$ receptor subtype to show its full sensitivity to extracellular ATP. An unusual feature of the $P2X_2$ receptor is the presence of 8 histidyl residues in the extracellular loop domain (Brake *et al.*, 1994); histidine is alone among amino acids in having a side group pK value (6.5) close to neutrality. Extracellular pH shifts in the physiological range would be expected to cause changes in the protonation of histidyl residues, with possible alterations in conformation of this domain. No other ATP receptors of the P2X or P2Y families are known to exhibit such pH sensitivity. Although the data presented do not distinguish categorically between P2 receptor types which may be mediating the stimulatory effects of low concentrations of ATP on pit formation by mature rat osteoclasts or osteoclast formation from mouse marrow, suramin antagonism and acid-activation point to the possible involvement of the $P2X_2$ receptor subtype. Therefore, to investigate the possible involvement of the $P2X_2$ receptor in the acid-activation effect, mature rat osteoclasts were incubated with antisense and sense oligonucleotides corresponding to the initiation codon region of the rat $P2X_2$ receptor.

Resorption pit formation by rat osteoclasts activated at pH 7.0 was significantly inhibited by the antisense oligonucleotide corresponding to the rat $P2X_2$ receptor, but not by the mismatched control. This suggests that the $P2X_2$ receptor may be involved in the acid-activation effect. However, there are several problems with the use of antisense oligonucleotides, which should be taken into account when interpreting these results (Wagner, 1994). Oligonucleotides can exhibit non-specific effects. For example, some antisense oligonucleotides may be antiproliferative while others can stimulate cellular activity, or have pronounced cytotoxic effects (Stein and Krieg, 1994). It is also important to demonstrate a decrease in target protein (if possible) if an antisense mechanism is proposed. Quantitative analysis of the target protein by binding or immunoblot assays needs to be performed to assess the success of the knockout, while the specificity of the oligonucleotide can be measured by investigating the levels of related, or associated proteins. While analysing target protein levels, the turnover rate also needs to be taken into consideration as, although there may be a 100% block to new protein formation, protein formed before the application of the oligonucleotide may still remain (Stein and Krieg, 1994). It is also important to demonstrate uptake of the antisense oligonucleotide into the osteoclast, for example via fluorescence microscopy, if an antisense method is proposed (Reddy *et al.*, 1994). Thus, although the antisense oligonucleotide corresponding to the rat $P2X_2$ receptor inhibited acid stimulated pit formation, it may not have occurred via an antisense effect on $P2X_2$ receptor expression.

How are extracellular protons and ATP interacting with the osteoclast to stimulate resorption? A simple hypothesis may be that when the $P2X_2$ receptor is occupied by ATP, protons modulate the receptor to allow the opening of a non-selective cation / proton channel in the osteoclast (dorsal / basolateral) cell membrane. This could act to increase the intracellular concentration of protons when extracellular pH is reduced. These protons could then be actively pumped out of the cell via a vacuolar H^+ ATPase into the resorption lacunae, so increasing resorption pit formation (Arnett and King, 1997). Such a process would enhance the intracellular proton supply formed via the cytoplasmic enzyme II in osteoclasts (Minkin and Jennings, 1972). However, an alternative possibility is that ATP and protons are operating at distinct receptor sites on the osteoclast cell membrane, or indeed on other cells (e.g. osteoblasts) which then act to stimulate osteoclastic bone resorption, possibly in an analogous manner to PTH (McSheehy and Chambers, 1986a).

In osteoclast formation experiments, low doses of ATP were able to stimulate the number of osteoclasts recruited irrespective of the extracellular pH. However, when medium was acidified (pH ~6.9), there was a large stimulation of resorption pit formation, which is in line with previous observations for mature rat osteoclasts (Arnett and Spowage, 1996). This again demonstrates the dramatic interaction between the stimulatory effects of low dose ATP and low pH on the resorptive activity of rat and mouse osteoclasts. Neary and Burnstock (1996) demonstrated that extracellular nucleotides, such as ATP, can have potent

long term trophic roles in cell proliferation and growth. However, the results do not distinguish the mode of action of ATP in increasing osteoclast formation in 10 day murine marrow cultures. ATP could be acting either directly on osteoclast precursor cells, as well as indirectly on the stromal component of marrow cultures; it is also possible that the stimulatory effects of ATP in these long term cultures were due, in some degree, to enhanced osteoclast survival.

TRAP was used to identify osteoclasts in the osteoclast formation experiments, and is widely used as a specific histochemical marker of osteoclasts in bone tissue (Minkin, 1982; Asotra *et al.*, 1994a). However, data on the reliability of TRAP positivity as a marker for osteoclast generation from haemopoietic tissue demonstrated that TRAP did not correlate with the differentiation of bone-resorbing cells in culture; macrophages also form in bone marrow cultures and become TRAP-positive and multinucleate, but remain nonresorptive (Hattersley and Chambers, 1989a). Therefore TRAP staining may not be an absolute marker for functional osteoclasts in these assays, and other identification techniques such as calcitonin receptor staining may have been more appropriate and specific.

Recent work has suggested that mouse and human macrophages express the $P2X_7$ (pore forming) receptor. This receptor has been implicated in the formation of macrophage multinucleated giant cells (MGCs), and may also be involved in intercellular communication, bridging between cytoplasms in a gap

junction-like fashion, and contributing to the formation of MGCs (Chiozzi *et al.*, 1997). Macrophage giant cells and osteoclasts both originate from a common myeloid progenitor cell, demonstrated by recent results from Tondravi *et al.*, (1997) who have shown that when the haematopoietic transcription factor PU.1 is 'knocked out' in mice there is a lack of both macrophages and osteoclasts. As osteoclasts and macrophage giant cells both originate from a common myeloid progenitor cell, the formation of osteoclasts may prove to be comparable to the fusion of macrophage giant cells.

In addition to increasing resorption, ATP may exert negative effects on bone formation. Recent experiments have shown that ATP (albeit at concentrations somewhat higher than those optimal for bone resorption), inhibits appositional bone formation by cultured primary osteoblasts (Jones *et al.*, 1997). ATP also induces cartilage resorption *in vitro* (Leong *et al.*, 1994).

As mentioned earlier, tissue injury and inflammation often result in localised acidosis along with the release of adenosine 5' triphosphate (ATP) and other substances (Steen *et al.*, 1992). In areas of inflammation, local ATP release from damaged cells, mast cells or platelets (Gordon, 1986) could result in concentrations as high as 20μM (Bowler *et al.*, 1995). It is conceivable that ATP could also play a role in tumour osteolysis, given the propensity of transformed cells to release ATP (Burnstock, 1997) and cause local acidification. Additionally, ATP is also known to stimulate proliferation in MCF-7 breast

cancer cells via P2 receptors, suggesting a role in tumour proliferation or progression (Dixon *et al.*, 1997b).

In conclusion, the present findings have shown that sub-micromolar concentrations of ATP coupled with low pH are more than sufficient to stimulate both osteoclastic bone resorption and osteoclast formation, and also suggest a new potential mechanism by which localised bone destruction could occur when ATP is released in acidified tissue.

Chapter 5

General discussion

The present work has focused mainly on the actions of two potent stimulators of bone resorption and osteoclast function: extracellular pH and ATP.

The extreme sensitivity of mature osteoclasts to small changes in extracellular pH, demonstrated by "on-off" switching, suggests that extracellular protons must play an important regulatory role in bone (patho)physiology. Coupled with this is the observation that the powerful stimulatory effect of extracellular protons on mature rat osteoclasts does not diminish with time, and indeed may increase somewhat. Additionally, pre-exposure to low pH does not activate rat osteoclasts to continue resorbing after transfer to non-acidified culture medium, and total resorption is closely related to time spent in low pH conditions, indicating that extracellular protons modulate osteoclastic activity in a continuous and reversible manner (Spowage and Arnett, 1995).

Paradoxically however, I discovered that extracellular acidification decreases osteoclast formation in murine marrow cultures, but stimulates the

activity of these cells once formed (Morrison and Arnett, 1998). Thus, although osteoclasts may be activated to resorb at sites where pH is low, such conditions will inhibit osteoclast formation. However, over the critical range (pH ~7.0-7.25), a small drop in pH dramatically increases osteoclast resorptive activity, but causes only a small decrease in osteoclast formation. These results may be relevant to diseases such as rheumatoid arthritis, where the local pH can be reduced (Steen *et al.*, 1992).

The results demonstrate that a slight reduction in extracellular pH (to ~pH 7.0) is the key step in osteoclast activation. In the isolated osteoclast assay, without this slight acidification of the medium, other osteolytic factors (*e.g.* $1,25(OH)_2D_3$ and ATP) have little effect. However, the mechanism of action of protons on osteoclast formation and activation is unknown at present. In view of the small changes in extracellular proton concentration involved, it seems unlikely that the "on-off" switching phenomenon is due simply to alteration of the gradient against which osteoclasts must effectively pump protons in order to resorb bone (Arnett and Spowage, 1996). However, several other possibilities have been suggested. For example, it is known that the distribution of mRNAs for several V-ATPase subunits in osteoclasts is dependent on the stage of resorption, whereas mRNA for carbonic anhydrase II (CA II) is found diffusely located throughout the osteoclast during the whole resorption cycle (Laitala-Leinonen *et al.*, 1996). Brief exposure to low pH conditions has been reported to cause a small increase in CA II mRNA levels in rabbit osteoclasts (Asotra *et*

al., 1994b), suggesting that if this increase in mRNA levels results in an increase in enzyme expression, greater proton production and hence extrusion could cause the increase in resorption seen. Acidosis is also known to induce an 8-fold increase in V-ATPase activity in osteoclasts (Nordstrom *et al.*, 1997), which may act in conjunction with the increased proton output from CA II to increase resorption.

It is of interest to note that in common with calcitonin and PGE_2 treatment, increasing extracellular pH induces the rapid disappearance of actin rings in osteoclasts (Lakkakorpi and Vaananen, 1990; Murrills *et al.*, 1993; Vaananen, 1996). Since the correct cytoskeletal organisation is obligatory for polarisation and resorption, and actin rings are generally only seen in resorbing osteoclasts (Lakkakorpi and Vaananen, 1996), the interaction with extracellular pH may represent an important point of control.

The steepness of the response of isolated rat and chick osteoclasts to extracellular pH is suggestive of "classical" co-operative activation curves in which several effector molecules must bind simultaneously to a target macromolecule for activation to occur. Therefore, it may be possible that osteoclasts possess a specific pH sensing apparatus linked to an intracellular pathway (Arnett and Spowage, 1996). Indeed, recent work has indicated that both protein kinase A (PKA) and protein kinase C (PKC) signal transduction pathways are involved in osteoclast mediated calcium release from bone (Krieger

et al., 1997). Low pH is also reported to increase calcitonin receptor mRNA expression, suggesting a mechanism whereby pH can modulate osteoclast function by an increased sensitivity to calcitonin (Weinstein *et al.*, 1998).

As mentioned earlier, one theory suggests that the acid-activation of osteoclastic resorption may involve protons and ATP interacting at the $P2X_2$ receptor to open a cation / proton channel in the osteoclast cell membrane. This would critically increase the intracellular availability of protons, which could be pumped out of the cell across the ruffled border, and so facilitate resorption pit formation (Arnett and King, 1997). However, although osteoclasts are known to express P2X and P2Y receptors (Yu and Ferrier, 1995; Bowler *et al.*, 1995; Weidema *et al.*, 1997, 1998; Bowler *et al.*, 1998c), there is no evidence at this present time for $P2X_2$ receptor expression on osteoclasts.

Along with the pathological states producing either systemic or local acidosis, osteoblast mitogens such as ATP and IL-1 are known to enhance medium acidification, which could further stimulate osteoclasts, along with any other mechanism involved (Aisa *et al.*, 1995; Kaplan and Dixon, 1996; Barrett *et al.*, 1997; Pollington and Arnett, 1998, unpublished). Indeed, results presented in chapter 2 illustrate the medium acidification caused by PTH, $1,25(OH)_2D_3$, and PGE_2, which could in itself stimulate some of the resorption seen.

Embryonic chick osteoclasts (Morrison and Arnett, 1997a), neonatal rat osteoclasts (Arnett and Spowage, 1996), mouse calvarial cultures (Meghji *et al.*, 1996, 1997), and osteoclasts formed from mouse marrow cultures (Morrison and Arnett, 1998) exhibited similar responses to extracellular pH. However, in contrast to the effect of extracellular ATP on mature rat osteoclasts (Morrison and Arnett, 1997b), osteoclasts obtained from embryonic chicks were unresponsive to low dose ATP. Indeed, the results suggest that chick osteoclasts are more sensitive to the inhibitory effects of ATP than rat osteoclasts. As mentioned previously, osteoclasts obtained from chicks differ from mammalian osteoclasts in several ways: they are 'constitutively activated' and therefore resorb more bone over time; and do not show an inhibitory response to calcitonin (Arnett and Dempster, 1987). Indeed, the fact that avian and mammalian osteoclasts differ in response to calcitonin but respond similarly to low pH suggests that proton sensitivity is a more primitive characteristic conserved during evolution.

The finding that osteoclasts in calvaria respond to low pH resulting from reduced culture medium HCO_3^- concentrations in a similar manner to isolated osteoclasts adds further weight to the physiological significance of acid stimulated resorption. My work confirms the observation made by Bushinsky (1989) that metabolic acidosis (*i.e.* decreasing HCO_3^-) is a more potent stimulator of osteoclastic resorption than respiratory acidosis (*i.e.* increasing PCO_2) in calvaria, but contrasts with the work of Arnett *et al.*, (1994) who demonstrated

that isolated rat osteoclasts were more sensitive to stimulation by CO_2 acidosis than HCO_3^- acidosis. As discussed earlier, the reasons why a reduction in bicarbonate concentration should have a greater effect on osteoclastic bone resorption in calvaria than an increase in the partial pressure of carbon dioxide are unclear.

In the calvarial assay system, acid-stimulated resorption appears to be dependent on the production of prostaglandins, since results presented in chapter 2 and from other groups demonstrate that indomethacin inhibits resorption (Rabadjija *et al.*, 1990). Addition of indomethacin to calvarial cultures causes TRAP-positive osteoclasts to become less adherent to bone and more adherent to the endocranial membrane (Marshall *et al.*, 1996), therefore the effects of indomethacin in the calvarial cultures may simply represent a lack of osteoclasts present capable of resorption rather than an actual inhibition of resorption *per se*. However, results such as these highlight the disparity between different *in vitro* bone assays. For example, the present work demonstrates that acid-activated isolated rat osteoclasts are further stimulated by indomethacin and other NSAIDs (Morrison and Arnett, 1996a, 1996b, 1997c).

Clearly, the effects of extracellular pH on osteoclasts can interact with other osteolytic agents. The additive or even synergistic effect of low pH with $1,25(OH)_2D_3$ and ATP suggests that although these two factors produce their effects via different receptors and pathways, extracellular pH can modulate

osteoclast resorption to produce a similar response. However, it is unclear how extracellular protons interact with these osteolytic agents.

The interaction between extracellular ATP and low pH is further highlighted by the observation that apyrase (an enzyme which hydrolyses extracellular ATP to AMP), and suramin (a general ATP antagonist) are able to inhibit acid-activated resorption pit formation. This suggests that the pH response of mature rat osteoclasts may be dependent on traces of extracellular ATP, since removal or inhibition reduces resorption (Morrison *et al.*, 1998). Since several bone diseases, such as osteoporosis and rheumatoid arthritis may result, in part, from systemic or local acidosis; the mechanism of proton stimulated bone resorption, and the possible interactions with inflammatory and other osteolytic factors will be an important area for future work.

The work of Suzuki *et al.*, (1993) has shown that PGE_2 is a potential mediator of extracellular ATP action in osteoblast-like MC3T3 cells. Pretreating cells with indomethacin suppressed both ATP-induced PGE_2 synthesis and DNA synthesis. ATP is unlikely to be acting via a prostaglandin-mediated mechanism in isolated osteoclast cultures, since prostaglandins are known to be inhibitory in this system (Arnett and Dempster, 1987). Furthermore, results in chapter 2 demonstrate that when both ATP and indomethacin are added to isolated osteoclast cultures, resorption is further stimulated, suggesting that ATP is not acting via a prostaglandin-mediated mechanism in this assay system. However,

in a similar manner to proton stimulation, ATP stimulation in calvarial cultures has been shown to be inhibited by indomethacin, suggesting that, in this assay at least, prostaglandins are acting as mediators of extracellular ATP action (Meghji *et al.*, 1998).

The large number of actions and interactions of all the known or suspected osteotropic agents may seem an impossible task to begin to understand. However, it is worth remembering that the majority of these factors may simply be acting as permissive rather than true regulatory agents of bone resorption *in vivo*. Thus, a significant problem of the *in vitro* study of the factors involved in bone resorption lies in the fact that many of these complex *in vivo* actions and interactions are disrupted.

During my Ph.D. studies I have chosen to look at the physiological function of osteoclasts, and have described potentially important new functional effects of extracellular pH and ATP on osteoclasts. The key discoveries are as follows: first, the effect of extracellular pH on osteoclast recruitment; second, the lack of tachyphyllaxis demonstrated by isolated osteoclasts when stimulated by low pH; third, the remarkable similarity between isolated osteoclasts and calvarial cultures in response to low pH; and finally, the action of extracellular ATP on osteoclast recruitment and activity. At the present time, it seems likely that extracellular protons are the only factor able to directly activate mature osteoclasts; whether ATP falls into this category remains to be determined.

With such small changes in extracellular pH (often of the order of a few hundredths of one unit) modulating osteoclast activation and formation, the use of a blood gas analyser for measuring medium pH and PCO_2 would appear to be essential for many resorption studies. The results show that proper control and measurement of extracellular pH in culture medium is a very important part of cell culture, one which is however often overlooked.

Future work

The work I have presented in this thesis leads on to a number of questions, which could form the basis of future work. For example, my results demonstrate the key importance of extracellular pH in modulating both the formation and resorptive activity of osteoclasts. However, the actual mechanism of action of protons on osteoclast activity is as yet unknown. It would be of interest therefore to establish whether a pH sensing receptor is present on osteoclasts, and the intracellular signalling pathway to which it may be linked. My work has also demonstrated that ATP is a potent stimulator of the activation and formation of rodent osteoclasts. However, it still remains unclear as to what P2 receptor subtypes are actually present on bone cells. It would be of interest therefore to use the newly developed technique of mico-isolated osteoclasts to ascertain whether ATP has a direct effect on osteoclast function. It should also be possible to employ immunocytochemistry and *in situ* hybridisation techniques to identify P2 receptors more fully. Given time it would also have been of interest to identify specific sequences using the technique of reverse transcriptase

polymerase chain reaction (RT-PCR). The RT-PCR method is extremely sensitive, being able to detect trace amounts of RNA in a sample. However, although the RT-PCR technique demonstrates the expression of a particular mRNA sequence, this is not necessarily indicative of protein expression. It is also important to obtain a pure population of cells, since the origin of the cDNA would be unknown. Analysis of the degree of P2 receptor expression at the protein level would be possible using Western blotting techniques with the antibodies available. The use of enzyme-linked immunosorbent assays (ELISA) could also be very useful in determining the amount of receptor present in the cell population.

Considering the *in vitro* stimulation of osteoclast formation and activity by small changes in extracellular pH, and the fact that from childhood to old age humans eating a typical western diet develop a slight but progressive increase in blood acidity and decrease in plasma bicarbonate concentrations, (low-grade metabolic acidosis) (Barzel, 1995; Frassetto and Sebastian, 1996), it may be that future therapy for osteoporosis will involve correcting this slight systemic metabolic acidosis. Indeed, evidence already suggests that, in postmenopausal women, administration of potassium bicarbonate to neutralise endogenous acid leads to a reduction in bone resorption and an increased rate of bone formation (Sebastian *et al.*, 1994). It is widely accepted that premenopausal women should have a total daily calcium intake of 800 mg. For postmenopausal women, whose calcium absorption is poorer this should be increased to 1500 mg per day

(Kaufman, 1995). In line with these recommendations, calcium carbonate (or a number of other calcium salts) is frequently prescribed to bring the total calcium ingested to that recommended level. However, it may be that it is not the cation, calcium, but rather the anion, carbonate, which is beneficial, buffering the excess acid present in a normal diet.

REFERENCES

Aisa, M. C., Rahman, S., Senin, U., Oyajobi, O. B., Maggio, D., and Russell, R. G. G. (1995). IL-1β-stimulated acid secretion by human osteoblast-like cells and human osteosarcoma cells MG-63. *J Bone Miner Res* **10**, S421 (abstract).

Akatsu, T., Takahashi, N., Debari, K., Morita, I., Murota, S., Nagata, N., Takatani, O., and Suda, T. (1989). Prostaglandins promote osteoclastlike cell formation by a mechanism involving cyclic adenosine 3',5'-monophosphate in mouse bone marrow cell cultures. *J Bone Miner Res* **4**, 29-35.

Akatsu, T., Takahashi, N., Udagawa, N., Imamura, K., Yamaguchi, A., Sato, K., Nagata, N., and Suda, T. (1991). Role of prostaglandins in interleukin-1-induced bone resorption in mice *in vitro*. *J Bone Miner Res* **6**, 183-189.

Alexander, S. P. H., and Peters, J. A. (1998). 1998 receptor and ion channel nomenclature supplement. *TiPs*, 60-62.

Allen, T. D., Testa, N. G., Suda, T., Schor, S. L., Onions, D., Jarrett, O., and Boyde, A. (1981). The production of putative osteoclasts in tissue culture - ultrastructure, formation and behaviour. *Scan Electron Microsc* **3**, 347-54.

Alsina, M., Guise, T. A., and Roodman, G. D. (1996). Cytokine regulation of bone cell differentiation. *Vitam Horm* **52**, 63-98.

Anderson, R. E., Woodbury, D. M., and Jee, W. S. (1986). Humoral and ionic regulation of osteoclast acidity. *Calcif Tissue Int* **39**, 252-258.

Apone, S., Lee, M. Y., and Eyre, D. R. (1997). Osteoclasts generate cross-linked collagen N-telopeptides (NTx) but not free pyridinolines when cultured on human bone. *Bone* **21**, 129-136.

Arkett, S. A., Dixon, S. J., and Sims, S. M. (1994). Effects of extracellular calcium and protons on osteoclast potassium currents. *J Membr Biol* **140**, 163-171.

Arnett, T. R., and Dempster, D. W. (1986). Effect of pH on bone resorption by rat osteoclasts *in vitro*. *Endocrinology* **119**, 119-124.

Arnett, T. R., and Dempster, D. W. (1987). A comparative study of disaggregated chick and rat osteoclasts *in vitro*: effects of calcitonin and prostaglandins. *Endocrinology* **120**, 602-608.

Arnett, T. R., and Dempster, D. W. (1990). Protons and osteoclasts. *J Bone Miner Res* **5**, 1099-1103.

Arnett, T. R. (1990). Update on bone cell biology. *Eur J Orthod* **12**, 81-90.

Arnett, T. R., Boyde, A., Jones, S. J., and Taylor, M. L. (1994). Effects of medium acidification by alteration of carbon dioxide or bicarbonate concentrations on the resorptive activity of rat osteoclasts. *J Bone Miner Res* **9**, 375-379.

Arnett, T. R., and Spowage, M. (1996). Modulation of the resorptive activity of rat osteoclasts by small changes in extracellular pH near the physiological range. *Bone* **18**, 277-279.

Arnett, T. R., Lindsay, R., Kilb, J. M., Moonga, B. S., Spowage, M., and Dempster, D. W. (1996). Selective toxic effects of tamoxifen on osteoclasts: comparison with the effects of oestrogen. *J Endocrinol* **149**, 503-508.

Arnett, T. R., and King, B. F. (1997). ATP as an osteoclast regulator? *J Physiol* **503**, 236.

Arnett, T. R., Morrison, M. S., Henderson, B., and Meghji, S. (1997). 5-Lipoxygenase inhibitors block calvarial resorption stimulated by HCO3-acidosis. *Bone* **20**, 13S (abstract).

Asotra, S., Shibata, Y., Song, S., Anderson, G., and Heersche, J. N. M. (1994a). The regulation of tartrate resistant acid phosphatase (trap) mRNA expression in rabbit osteoclasts as a marker of osteoclastic activity. *J Bone Miner Res* **9**, S372 (abstract).

Asotra, S., Gupta, A. K., Sodek, J., Aubin, J. E., and Heersche, J. N. (1994b). Carbonic anhydrase II mRNA expression in individual osteoclasts under "resorbing" and "nonresorbing" conditions. *J Bone Miner Res* **9**, 1115-1122.

Athanasou, N. A., Heryet, A., Quinn, J., Gatter, K. C., Mason, D. Y., and McGee, J. O. (1986). Osteoclasts contain macrophage and megakaryocyte antigens. *J Pathol* **150**, 239-246.

Aubin, J. E., Turksen, K., and Heersche, J. N. M. (1993). Osteoblastic cell lineage. *In* "Cellular and Molecular Biology of Bone" (D. Evered and S. Harnett, Eds.), pp. 78-85. John Wiley & Sons, Chichester.

Barnard, E. A., Burnstock, G., and Webb, T. E. (1994). G protein-coupled receptors for ATP and other nucleotides: a new receptor family. *TiPS* **15**, 67-70.

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

Baron, R., Neff, L., Tran Van, P., Nefussi, J. R., and Vignery, A. (1986). Kinetic and cytochemical identification of osteoclast precursors and their differentiation into multinucleated osteoclasts. *Am J Pathol* **122**, 363-378.

Baron, R. (1989). Polarity and membrane transport in osteoclasts. *Connect Tissue Res* **20**, 109-120.

Barrett, M. G., Belinsky, G. S., and Tashjian Jr, A. H. (1997). A new action of parathyroid hormone. Receptor-mediated stimulation of extracellular acidification in human osteoblast-like SaOS cells. *J Biol Chem* **272**, 26346-26353.

Barzel, U. S., and Jowsey, J. (1969). The effects of chronic acid and alkali administration on bone turnover in adult rats. *Clin Sci* **36**, 517-524.

Barzel, U. S. (1978). Osteoporosis II : An overview. *In* "Second International Symposium on Osteoporosis" (U. S. Barzel, Ed.), pp. 1-4. Grun & Stratton, Miami Beach, Florida.

Barzel, U. S. (1995). The skeleton as an ion exchange system: implications for the role of acid-base imbalance in the genesis of osteoporosis. *J Bone Miner Res* **10,** 1431-1436.

Baylink, T. M., Mohan, S., Fitzsimmons, R. J., and Baylink, D. J. (1995). Evidence that the mitogenic effect of prostaglandin E_2 on human bone cells involves protein kinase C and calcium pathways. *J Bone Miner Res* **9,** B23 (abstract).

Blair, H. C., Kahn, A. J., Crouch, E. C., Jeffrey, J. J., and Teitelbaum, S. L. (1986). Isolated osteoclasts resorb the organic and inorganic components of bone. *J Cell Biol* **102**, 1164-1172.

Blair, H. C., Teitelbaum, S. L., Schimke, P. A., Konsek, J. D., Koziol, C. M., and Schlesinger, P. H. (1988). Receptor-mediated uptake of a mannose-6-phosphate bearing glycoprotein by isolated chicken osteoclasts. *J Cell Physiol* **137,** 476-482.

Blair, H. C., Teitelbaum, S. L., Chiselli, R., and Gluck, S. (1989). Osteoclastic bone resorption by a polarised vacuolar pump. *Science* **245**, 855-857.

Blavier, L., and Delaissé, J. M. (1995). Matrix metalloproteinases are obligatory for the migration of preosteoclasts to the developing marrow cavity of primitive long bones. *J Cell Sci* **108**, 3649-3659.

Blumenkrantz, N., and Sondergaard, J. (1972). Effect of prostaglandin E1 and F2α on biosynthesis of collagen. *Nature New Biol* **239,** 246-251.

Bowler, W. B., Birch, M. A., Gallagher, J. A., and Bilbe, G. (1995). Identification and cloning of human P2U purinoceptor present in osteoclastoma, bone, and osteoblasts. *J Bone Miner Res* **10**, 1137-1145.

Bowler, W. B., Dixon, C. J., Halleux, C., Hipskind, R. A., Fraser, W. D., Bilbe, G., and Gallagher, J. A. (1996). Extracellular ATP induces c-fos expression through elevation of $[Ca^{2+}]_i$ independently of protein kinase C activation. *J Bone Miner Res* **11**, p9 (abstract).

Bowler, W. B., and Gallagher, J. A. (1996). Extracellular nucleotides enhance parathyroid hormone-stimulated bone resorption. *J Bone Miner Res* **11**, p22 (abstract).

Bowler, W. B., Maier, R., Bilbe, G., and Gallagher, J. A. (1998a). Osteoblasts express multiple G-protein coupled P2Y receptor subtypes. *Bone* **22**, 2S (abstract).

Bowler, W. B., Tattersall, J. A., Hussein, R., Dixon, C. J., Cobbold, P. H., and Gallagher, J. A. (1998b). Release of ATP by osteoblasts: modulation by fluid shear forces. *Bone* **22**, 3S (abstract).

Bowler, W. B., Littlewood-Evans, A., Bilbe, G., Gallagher, J. A., and Dixon, C. J. (1998c). $P2Y_2$ receptors are expressed by human osteoclasts of giant cell tumours but do not mediate ATP-induced bone resorption. *Bone* **22**, 195-200.

Boyce, B. F., Yoneda, T., Lowe, C., Soriano, P., and Mundy, G. R. (1992). Requirement of pp60c-src expression for osteoclasts to form ruffled borders and resorb bone in mice. *J Clin Invest* **90**, 1622-1627.

Boyde, A., Ali, N. N., and Jones, S. J. (1984). Resorption of dentine by isolated osteoclasts *in vitro*. *Br Dent J* **156**, 216-20.

Boyde, A., and Jones, S. J. (1991). Pitfalls in pit measurement [editorial]. *Calcif Tissue Int* **49**, 65-70.

Brake, A. J., Wagenbach, M. J., and Julius, D. (1994). New structural motif for ligand-gated ion channels defined by ionotropic ATP receptor. *Nature* **371**, 519-523.

Burger, E. H., van der Meer, J. W., and Nijweide, P. J. (1984). Osteoclast formation from mononuclear phagocytes: role of bone-forming cells. *J Cell Biol* **99**, 1901-1906.

Burnstock, G., Campbell, G., Bennett, M., and Holman, M. E. (1963). Inhibition of smooth muscle of the taenia coli. *Nature* **200,** 581-582.

Burnstock, G., Campbell, G., Satchell, D., and Smythe, A. (1970). Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. *Br J Pharmac* **40,** 668-688.

Burnstock, G. (1972). Purinergic nerves. *Pharmacol Rev* **24**, 509-581.

Burnstock, G., and Kennedy, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen Pharmac* **16**, 433-440.

Burnstock, G. (1996). *Ciba Foundation Symposium* **198**, 322-323.

Burnstock, G., and King, B. F. (1996). Numbering of cloned P2 purinoceptors. *Drug Dev Res* **38**, 67-71.

Burnstock, G. (1997). The past, present and future of purine nucleotides as signalling molecules. *Neuropharm* **36**, 1127-1139.

Bushinsky, D. A., Krieger, N. S., Geisser, D. I., Grossman, E. B., and Coe, F. L. (1983). Effects of pH on bone calcium and proton fluxes *in vitro*. *Am J Physiol* **245**, F204-209.

Bushinsky, D. A., Goldring, J. M., and Coe, F. L. (1985). Cellular contribution to pH-mediated calcium flux in neonatal mouse calvariae. *Am J Physiol* **248**, F785-789.

Bushinsky, D. A., Levi Setti, R., and Coe, F. L. (1986). Ion microprobe determination of bone surface elements: effects of reduced medium pH. *Am J Physiol* **250,** F1090-1097.

Bushinsky, D. A., and Lechleider, R. J. (1987). Mechanism of proton-induced bone calcium release: calcium carbonate-dissolution. *Am J Physiol* **253**, F998-1005.

Bushinsky, D. A. (1989). Net calcium efflux from live bone during chronic metabolic, but not respiratory, acidosis. *Am J Physiol* **256**, F836-842.

Bushinsky, D. A. (1994). Acidosis and bone. *Miner Electrolyte Metab* **20**, 40-52.

Bushinsky, D. A. (1995). Stimulated osteoclastic and suppressed osteoblastic activity in metabolic but not respiratory acidosis. *Am J Physiol* **268**, C80-88.

Bushinsky, D. A., and Nilsson, E. L. (1995). Additive effects of acidosis and parathyroid hormone on mouse osteoblastic and osteoclastic function. *Am J Physiol* **269**, C1364-1370.

Bushinsky, D. A., Gavrilov, K., Stathopoulos, V. M., Krieger, N. S., Chabala, J. M., and Levi Setti, R. (1996). Effects of osteoclastic resorption on bone surface ion composition. *Am J Physiol* **271**, C1025-1031.

Calvo, M. S., Eyre, D. R., and Gundberg, C. M. (1996). Molecular basis and clinical application of biological markers of bone turnover. *Endocrine Reviews* **17**, 333-368.

Chambers, T. J., and Magnus, C. J. (1982). Calcitonin alters behaviour of isolated osteoclasts. *J Pathol* **136**, 27-39.

Chambers, T. J., and Dunn, C. J. (1982). The effect of parathyroid-hormone, 1,25-dihydroxycholecalciferol and prostaglandins on the cytoplasmic activity of isolated osteoclasts. *J Pathol* **137**, 193-203.

Chambers, T. J., and Dunn, C. J. (1983). Pharmacological control of osteoclastic motility. *Calcif TissueInt* **35**, 566-570.

Chambers, T. J., and Moore, A. (1983). The sensitivity of isolated osteoclasts to morphological transformation by calcitonin. *J Clin Endocrinol Metab* **57**, 819-824.

Chambers, T. J., and Ali, N. N. (1983a). Inhibition of osteoclastic motility by prostaglandins I_2, E_1, E_2 and 6-oxo-E1. *J Pathol* **139**, 383-397.

Chambers, T. J., and Ali, N. N. (1983b). Prostaglandin-E_1, prostaglandin-E_2, prostaglandin-I_2 and prostaglandin-6-oxo-E_1 inhibit the motility of isolated osteoclasts. *Metab Bone Dis Res* **4**, 365 (abstract).

Chambers, T. J., Fuller, K., and Athanasou, N. A. (1984a). The effect of prostaglandins I_2, E_1, E_2 and dibutyryl cyclic AMP on the cytoplasmic spreading of rat osteoclasts. *Br J Exp Pathol* **65**, 557-566.

Chambers, T. J., Revell, P. A., Fuller, K., and Athanasou, N. A. (1984b). Resorption of bone by isolated rabbit osteoclasts. *J Cell Sci* **66**, 383-399.

Chambers, T. J., Darby, J. A., and Fuller, K. (1985a). Mammalian collagenase predisposes bone surfaces to osteoclastic resorption. *Cell Tissue Res* **241**, 671-675.

Chambers, T. J., McSheehy, P. M., Thomson, B. M., and Fuller, K. (1985b). The effect of calcium-regulating hormones and prostaglandins on bone resorption by osteoclasts disaggregated from neonatal rabbit bones. *Endocrinology* **116**, 234-239.

Chambers, T. J., Fuller, K., and Darby, J. A. (1987). Hormonal regulation of acid phosphatase release by osteoclasts disaggregated from neonatal rat bone. *J Cell Physiol* **132**, 90-96.

Chambers, T. J., Owens, J. M., Hattersley, G., Jat, P. S., and Noble, M. D. (1993). Generation of osteoclast-inductive and osteoclastogenic cell lines from the H-2KbtsA58 transgenic mouse. *Proc Natl Acad Sci USA* **90,** 5578-5582.

Chang, D. C., Hunt, J. R., and Gao, P. Q. (1989). Effects of pH on cell fusion induced by electric fields. *Cell Biophys* **14**, 231-243.

Chenu, C., Kurihara, N., Mundy, G. R., and Roodman, G. D. (1990). Prostaglandin E_2 inhibits formation of osteoclastlike cells in long-term human marrow cultures but is not a mediator of the inhibitory effects of transforming growth factor beta. *J Bone Miner Res* **5**, 677-681.

Chiozzi, P., Sanz, J. M., Ferrari, D., Falzoni, S., Aleotti, A., Buell, G. N., Collo, G., and Di Virgilio, F. (1997). Spontaneous cell fusion in macrophage cultures expressing high levels of the P2Z / $P2Z_7$ receptor. *J Cell Biol* **138**, 697-706.

Chow, J. W. M., and Chambers, T. J. (1994). Indomethacin has distinct early and late actions on bone- formation induced by mechanical stimulation. *Am J Physiol* **267**, E287-E292.

Chow, S. C., Kass, G. E. N., and Orrenius, S. (1997). Purines and their roles in apoptosis. *Neuropharm* **36**, 1149-1156.

Coleman, R. A., Smith, W. L., and Narumiya, S. (1994). International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol Rev* **46**, 205-229.

Collins, D. A., and Chambers, T. J. (1991). Effect of prostaglandins E_1, E_2, and F_2 alpha on osteoclast formation in mouse bone marrow cultures. *J Bone Miner Res* **6**, 157-164.

Collins, D. A., and Chambers, T. J. (1992). Prostaglandin E2 promotes osteoclast formation in murine hematopoietic cultures through an action on hematopoietic cells. *J Bone Miner Res* **7,** 555-561.

Colston, K. W., King, R. J., Hayward, J., Fraser, D. I., Horton, M. A., Stevenson, J. C., and Arnett, T. R. (1989). Estrogen receptors and human bone cells: immunocytochemical studies. *J Bone Miner Res* **4,** 625-631.

Davies, J., Warwick, J., Totty, N., Philip, R., Helfrich, M., and Horton, M. (1989). The osteoclast functional antigen, implicated in the regulation of bone resorption, is biochemically related to the vitronectin receptor. *J Cell Biol* **109,** 1817-1826.

Di Virgilio, F., Bronte, V., Collavo, D., and Zanovello, P. (1989). Responses of mouse lymphocytes to extracellular adenosine 5' triphosphate (ATP). Lymphocytes with cytotoxic activity are resistant to the permeabilising effects of ATP. *J Immunol* **143,** 1955-1960.

Dietrich, J. W., Goodson, J. M., and Raisz, L. G. (1975). Stimulation of bone resorption by various prostaglandins in organ culture. *Prostaglandins* **10**, 231-240.

Dixon, C. J., Bowler, W. B., Walsh, C. A., and Gallagher, J. A. (1997a). Effects of extracellular nucleotides on single cells and populations of human osteoblasts: contribution of cell heterogeneity to relative potencies. *Br J Pharmacol* **120**, 777-780.

Dixon, C. J., Bowler, W. B., Fleetwood, P., Ginty, A. F., Gallagher, J. A., and Carron, J. A. (1997b). Extracellular nucleotides stimulate proliferation in MCF-7 breast cancer cells via P_2-purinoceptors. *Br J Cancer* **75**, 34-39.

Dodds, R. A., Connor, J. R., Drake, F., Feild, J., and Gowen, M. (1998). Cathepsin K mRNA detection is restricted to osteoclasts during fetal mouse development. *J Bone Miner Res* **13**, 673-682.

Dominguez, J. H., and Raisz, L. G. (1979). Effects of changing hydrogen ion, carbonic acid, and bicarbonate concentrations on bone resorption *in vitro*. *Calcif Tissue Int* **29**, 7-13.

Drake, F. H., Dodds, R. A., James, I. E., Connor, J. R., Debouck, C., Richardson, S., Lee Rykaczewski, E., Coleman, L., Rieman, D., Barthlow, R., Hastings, G., and Gowen, M. (1996). Cathepsin K, but not cathepsins B, L, or S, is abundantly expressed in human osteoclasts. *J Biol Chem* **271**, 12511-12516.

Drury, A. N., and Szent-Gyorgyi, A. (1929). The physiological activity of adenine compounds with special reference to their action upon the mammalian heart. *J Physiol* **68**, 213-237.

Edwards, M., Sarma, U., and Flanagan, A. M. (1998). Macrophage colony-stimulating factor increases bone resorption by osteoclasts disaggregated from human fetal long bones. *Bone* **22,** 325-329.

Ek-Rylander, B., Flores, M., Wendel, M., Heinegard, D., and Andersson, G. (1994). Dephosphorylation of osteopontin and bone sialoprotein by osteoclastic tartrate-resistant acid phosphatase. Modulation of osteoclast adhesion *in vitro*. *J Biol Chem* **269**, 14853-14856.

Engleman, V. W., Nickols, G. A., Ross, F. P., Horton, M. A., Griggs, D. W., Settle, S. L., Ruminski, P. G., and Teitelbaum, S. L. (1997). A peptidomimetic antagonist of the αvβ3 integrin inhibits bone resorption *in vitro* and prevents osteoporosis *in vivo*. *J Clin Invest* **99**, 2284-2292.

Evans, D. M., and Ralston, S. H. (1996). Nitric oxide and bone. *J Bone Miner Res* **11,** 300-305.

Falchuk, K. H., Goetzl, E. J., and Kulka, J. P. (1970). Respiratory gases of synovial fluids. An approach to synovial tissue circulatory-metabolic imbalance in rheumatoid arthritis. *Am J Med* **49,** 223-231.

Farsoudi, K. H., Pietschmann, P., Cross, H. S., and Peterlik, M. (1993). Suramin is a potent inhibitor of calcemic hormone- and growth factor-induced bone resorption *in vitro*. *J Pharmacol Exp Ther* **264**, 579-583.

Felix, R., Cecchini, M. G., and Fleisch, H. (1990). Macrophage colony stimulating factor restores *in vivo* bone resorption in the op/op osteopetrotic mouse. *Endocrinology* **127,** 2592-2594.

Feuerbach, D., and Feyen, J. H. (1997). Expression of the cell-adhesion molecule VCAM-1 by stromal cells is necessary for osteoclastogenesis. *FEBS Lett* **402**, 21-24.

Feyen, J. H., van der Wilt, G., Moonen, P., Di Bon, A., and Nijweide, P. J. (1984). Stimulation of arachidonic acid metabolism in primary cultures of osteoblast-like cells by hormones and drugs. *Prostaglandins* **28**, 769-781.

Flanagan, A. M., Nui, B., Tinkler, S. M., Horton, M. A., Williams, D. M., and Chambers, T. J. (1988). The multinucleate cells in giant cell granulomas of the jaw are osteoclasts. *Cancer* **62,** 1139-1145.

Flanagan, A. M., and Chambers, T. J. (1989). Osteoclasts are present in the giant cell variant of malignant fibrous histiocytoma. *J Pathol* **159,** 53-57.

Flanagan, A. M., Horton, M. A., Dorey, E. L., Collins, D. A., Evely, R. S., Moseley, J. M., Firkin, F. C., Chambers, T. J., Helfrich, M. H., and Martin, T. J. (1992). An assessment of the ability of human bone marrow cultures to generate osteoclasts. *Int J Exp Pathol* **73**, 387-401.

Flanagan, A. M., and Chambers, T. J. (1992). Stimulation of bone nodule formation *in vitro* by prostaglandins E_1 and E_2. *Endocrinology* **130**, 443-448.

Flanagan, A. M., Stow, M. D., Kendall, N., and Brace, W. (1995). The role of 1,25-dihydroxycholecalciferol and prostaglandin E_2 in the regulation of human osteoclastic bone resorption *in vitro*. *Int J Exp Pathol* **76**, 37-42.

Flanagan, A. M. and Lader, C. (1997). Effect of prostaglandin E2, interleukin 1α, interleukin 6, tumour necrosis factorα and nitric oxide on human osteoclast formation and bone resorption. *J Bone Miner Res* **12**, O15 (abstract).

Forwood, M. R. (1996). Inducible cyclo-oxygenase (COX-2) mediates the induction of bone formation by mechanical loading *in vivo*. *J Bone Miner Res* **11**, 1688-1693.

Frassetto, L., and Sebastian, A. (1996). Age and systemic acid-base equilibrium: analysis of published data. *J Gerontol* **51A**, B91-B99.

Frick, K. K., Jiang, L., and Bushinsky, D. A. (1997). Acute metabolic acidosis inhibits the induction of osteoblastic egr-1 and type 1 collagen. *Am J Physiol* **272**, C1450-1456.

Frolich, J. C. (1997). A classification of NSAIDs according to the relative inhibition of cyclooxygenase isoenzymes. *TiPS* **18**, 30-34.

Fujikawa, Y., Quinn, J. M. W., Sabokbar, A., McGee, J. O. D., and Athanasou, N. A. (1996a). The human osteoclast precursor circulates in the monocyte fraction. *Endocrinology* **137**, 4058-4060.

Fujikawa, Y., Sabokbar, A., Neale, S., and Athanasou, N. A. (1996b). Human osteoclast formation and bone resorption by monocytes and synovial macrophages in rheumatoid arthritis. *Ann Rheum Dis* **55**, 816-822.

Fuller, K., and Chambers, T. J. (1989a). Bone matrix stimulates osteoclastic differentiation in cultures of rabbit bone marrow cells. *J Bone Miner Res* **4**, 179-183.

Fuller, K., and Chambers, T. J. (1989b). Effect of arachidonic acid metabolites on bone resorption by isolated rat osteoclasts. *J Bone Miner Res* **4**, 209-215.

Fuller, K., Owens, J. M., Jaguar, C. J., Wilson, A., Moss, R., and Chambers, T. J. (1993). Macrophage colony-stimulating factor stimulates survival and chemotactic behaviour in isolated osteoclasts. *J Exp Med* **178**, 1733-1744.

Futaki, N., Yoshikawa, K., Hamasaka, Y., Arai, I., Higuchi, S., Iizuka, H., and Otomo, S. (1993). NS-398, a novel non-steroidal anti-inflammatory drug with potent analgesic and antipyretic effects, which causes minimal stomach lesions. *Gen Pharmacol* **24**, 105-110.

Gallwitz, W. E., Mundy, G. R., Lee, C. H., Qiao, M., Roodman, G. D., Raftery, M., Gaskell, S. J., and Bonewald, L. F. (1993). 5-Lipoxygenase metabolites of arachidonic acid stimulate isolated osteoclasts to resorb calcified matrices. *J Biol Chem* **268**, 10087-10094.

Garcia, C., Boyce, B. F., Gilles, J., Dallas, M., Qiao, M., Mundy, G. R., and Bonewald, L. F. (1996). Leukotriene B4 stimulates osteoclastic bone resorption both *in vitro* and *in vivo*. *J Bone Miner Res* **11**, 1619-1627.

Garrett, I. R., and Mundy, G. R. (1989). Relationship between interleukin-1 and prostaglandins in resorbing neonatal calvaria. *J Bone Miner Res* **4**, 789-794.

Goldhaber, P., and Rabadjija, L. (1987). H^+ stimulation of cell-mediated bone resorption in tissue culture. *Am J Physiol* **253**, E90-98.

Gordon, J. L. (1986). Extracellular ATP: effects, sources and fate. *Biochem J* **233**, 309-319.

Gothlin, G., and Ericsson, J. L. E. (1976). The osteoclast: review of ultrastructure, origin, and structure-function relationship. *Clin Orthop* **120**, 201-231.

Goto, K. (1918). Mineral metabolism in experimental acidosis. *J Biol Chem* **36**, 355-376.

Grigoriadis, A. E., Wang, Z. Q., Cecchini, M. G., Hofstetter, W., Felix, R., Fleisch, H. A., and Wagner, E. F. (1994). *c-Fos*: a key regulator of osteoclast-macrophage lineage determination and bone remodelling. *Science* **266**, 443-448.

Gupta, A., Edvards, J. C., and Hruska, K. A. (1996). Cellular distribution and regulation of NHE-1 isoform of the Na^+ / H^+ exchanger in the avian osteoclast. *Bone* **18**, 87-95.

Hall, T. J., and Chambers, T. J. (1989). Optimal bone-resorption by isolated rat osteoclasts requires chloride bicarbonate exchange. *Calcif Tissue Int* **45**, 378-380.

Hattersley, G., and Chambers, T. J. (1989a). Generation of osteoclastic function in mouse bone marrow cultures: multinuclearity and tartrate-resistant acid phosphatase are unreliable markers for osteoclastic differentiation. *Endocrinology* **124**, 1689-1696.

Hattersley, G., and Chambers, T. J. (1989b). Calcitonin receptors as markers for osteoclastic differentiation: correlation between generation of bone-resorptive cells and cells that express calcitonin receptors in mouse bone marrow cultures. *Endocrinology* **125**, 1606-1612.

Hattersley, G., Owens, J., Flanagan, A. M., and Chambers, T. J. (1991). Macrophage colony stimulating factor (M-CSF) is essential for osteoclast formation *in vitro*. *Biochem Biophys Res Commun* **177**, 526-531.

Hayman, A. R., and Cox, T. M. (1994). Purple acid phosphatase of the human macrophage and osteoclast. Characterisation, molecular properties, and crystallisation of the recombinant di-iron-oxo protein secreted by baculovirus-infected insect cells. *J Biol Chem* **269**, 1294-1300.

Hayman, A. R., Jones, S. J., Boyde, A., Foster, D., Colledge, W. H., Carlton, M. B., Evans, M. J., and Cox, T. M. (1996). Mice lacking tartrate-resistant acid phosphatase (Acp 5) have disrupted endochondral ossification and mild osteopetrosis. *Development* **122**, 3151-3162.

Helfrich, M. H., Nesbitt, S. A., Lakkakorpi, P. T., Barnes, M. J., Bodary, S. C., Shankar, G., Mason, W. T., Mendrick, D. L., Vaananen, H. K., and Horton, M. A. (1996). Beta 1 integrins and osteoclast function: involvement in collagen recognition and bone resorption. *Bone* **19**, 317-328.

Hiley, C. R., Bottrill, F. E., Warnock, J., and Richardson, P. J. (1995). Effects of pH on responses to adenosine, CGS 21680, carbachol and nitroprusside in the isolated perfused superior mesenteric arterial bed of the rat. *Br J Pharmacol* **116**, 2641-2646.

Hill, P. A., Buttle, D. J., Jones, S. J., Boyde, A., Murata, M., Reynolds, J. J., and Meikle, M. C. (1994). Inhibition of bone resorption by selective inactivators of cysteine proteinases. *J Cell Biochem* **56**, 118-130.

Hill, P. A., Docherty, A. J. P., Bottomley, K. M. K., O'Connell, J. P., Morphy, J. R., Reynolds, J. J., and Meikle, M. C. (1995). Inhibition of bone resorption *in vitro* by selective inhibitors of gelatinase and collagenase. *Biochem J* **308**, 167-175.

Hogevold, H. E., Grogaard, B., and Reikeras, O. (1992). Effects of short-term treatment with corticosteroids and indomethacin on bone healing. A mechanical study of osteotomies in rats. *Acta Orthop Scand* **63**, 607-611.

Holt, I., and Marshall, M. J. (1998). Integrin subunit beta 3 plays a crucial role in the movement of osteoclasts from the periosteum to the bone surface. *J Cell Physiol* **175**, 1-9.

Holton, P. (1959). The liberation of adenosine triphosphate on antidromic stimulation of sensory nerves. *J Physiol* **145,** 494-504.

Horton, M. A., Rimmer, E. F., Lewis, D., Pringle, J. A., Fuller, K., and Chambers, T. J. (1984). Cell surface characterisation of the human osteoclast: phenotypic relationship to other bone marrow-derived cell types. *J Pathol* **144,** 281-294.

Horton, M. A., Lewis, D., McNulty, K., Pringle, J. A., and Chambers, T. J. (1985). Monoclonal antibodies to osteoclastomas (giant cell bone tumours): definition of osteoclast-specific cellular antigens. *Cancer Res* **45,** 5663-5669.

Horton, M. A., Taylor, M. L., Arnett, T. R., and Helfrich, M. H. (1991). Arg-Gly-Asp (RGD) peptides and the anti-vitronectin receptor antibody 23C6 inhibit dentine resorption and cell spreading by osteoclasts. *Exp Cell Res* **195**, 368-375.

Horton, M. A. (1997). The αvβ3 Integrin "Vitronectin Receptor". *Int J Biochem Cell Biol* **29**, 721-725.

Hughes, D. E., Dai, A., Tiffee, J. C., Li, H. H., Mundy, G. R., and Boyce, B. F. (1996). Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-beta. *Nature Medicine* **2**, 1132-1136.

Ibbotson, K. L., Boyce, R. W., Orcutt, C. M., Franks, A. F., and D'Souza, S. M. (1987). Formation of multinucleate osteoclast-like cells in culture of dog and rat bone marrow. *J Bone Miner Res* **2**, 270A (abstract).

Ikegame, M., Rakopoulos, M., Martin, T. J., Moseley, J. M., and Findlay, D. M. (1996). Effects of continuous calcitonin treatment on osteoclast-like cell development and calcitonin receptor expression in mouse bone marrow cultures. *J Bone Miner Res* **11**, 456-465.

Iotsova, V., Caamano, J., Loy, J., Yang, Y., Lewin, A., and Bravo, R. (1997). Osteopetrosis in mice lacking NF-*k*B1 and NF-*k*B2. *Nature Medicine* **3**, 1285-1289.

Jaffe, H. L., Bodansky, A., and Chandler, J. P. (1932). Ammonium chloride decalcification as modified by calcium intake: the relation between generalised osteoporosis and ostitis fibrosa. *J Exp Med* **56**, 823-834.

Jee, W. S. S., Ueno, K., Deng, Y. P., and Woddbury, D. M. (1985). The effects of prostaglandin E_2 in growing rats: increased metaphyseal hard tissue and cortico-endosteal bone formation. *Calcif Tissue Int* **37**, 148-157.

Jee, W. S. S., and Ma, Y. F. (1997). The *in vivo* anabolic actions of prostaglandins in bone. *Bone* **21**, 297-304.

Johnston, R. B. (1988). Monocytes and macrophages. *New Eng J Med* **318**, 747-752.

Jones, S. J., and Boyde, A. (1976). Experimental study of changes in osteoblastic shape induced by calcitonin and parathyroid extract in an organ culture system. *Cell Tissue Res* **169,** 449-465.

Jones, S. J., Boyde, A., and Ali, N. N. (1984). The resorption of biological and non-biological substrates by cultured avian and mammalian osteoclasts. *Anat Embryol Berl* **170**, 247-256.

Jones, S. J., Gray, C., Boyde, A., and Burnstock, G. (1997). Purinergic transmitters inhibit bone formation by cultured osteoblasts. *Bone* **21**, 393-399.

Jorgensen, H. R., Svanholm, H., and Host, A. (1988). Bone formation induced in an infant by systemic prostaglandin-E_2 administration. *Acta Orthop Scand* **59**, 464-466.

Kaji, H., Sugimoto, T., Kanatani, M., Nishiyama, K., and Chihara, K. (1997). Dexamethasone stimulates osteoclast-like cell formation by directly acting on hemopoietic blast cells and enhances osteoclast-like cell formation stimulated by parathyroid hormone and prostaglandin E_2. *J Bone Miner Res* **12**, 734-741.

Kanai, N., Lu, R., Satriano, J. A., Bao, Y., Wolkoff, A. W., and Schuster, V. L. (1995). Identification and characterisation of a prostaglandin transporter. *Science* **268**, 866-869.

Kaplan, A. D., and Dixon, S. J. (1996). Extracellular nucleotides act through P2 purinoceptors to modulate acid production by osteoblastic cells. *J Bone Miner Res* **11**, T344 (abstract).

Kaufman, J. M. (1995). Role of calcium and vitamin D in the prevention and the treatment of postmenopausal osteoporosis: an overview. *Clin Rheumatol* **14** Suppl 3**,** 9-13.

Kawaguchi, H., Raisz, L. G., Voznesensky, O. S., Alander, C. B., Hakeda, Y., and Pilbeam, C. C. (1994). Regulations of the two prostaglandin G/H synthases by parathyroid hormone, interleukin-1, cortisol and prostaglandin E_2 in cultured neonatal mouse calvariae. *Endocrinology* **135**, 1157-1164.

Kawaguchi, H., Pilbeam, C., Harrison, J. R., and Raisz, L. G. (1995). The role of prostaglandins in the regulation of bone metabolism. *Clin Ortho Rel Res* **313**, 36-46.

Kaysinger, K. K., and Ramp, W. K. (1998). Extracellular pH modulates the activity of cultured human osteoblasts. *J Cell Biochem* **68**, 83-89.

Kenessey, A., Nacharaju, P., Ko, L. W., and Yen, S. H. (1997). Degradation of tau by lysosomal enzyme cathepsin D: implication for Alzheimer neurofibrillary degeneration. *J Neurochem* **69**, 2026-2038.

Kenny, A. D. (1985). Role of carbonic anhydrase in bone: plasma acetazolamide concentrations associated with inhibition of bone loss. *Pharmacology* **31**, 97-107.

King, B. F., Ziganshina, L. E., Pintor, J., and Burnstock, G. (1996). Full sensitivity of $P2X_2$ purinoceptor to ATP revealed by changing extracellular pH. *Br J Pharmacol* **117**, 1371-1373.

Kitajima, S., Sakakibara, R., and Uyeda, K. (1983). Significance of phosphorylation of phosphofructokinase. *J Biol Chem* **258**, 13292-13298.

Klein, D. C., and Raisz, L. G. (1970). Prostaglandins: stimulation of bone resorption in tissue culture. *Endocrinology* **86**, 1436-1440.

Kolliker, A. (1873). "Die normale resorption des Knochengewebes und ihre bedeutung fur die entstehung der typischen knochenformen." F. C. W. Vogel, Leipzig.

Kraut, J. A., Mishler, D. R., and Kurokawa, K. (1984). Effect of colchicine and calcitonin on calcemic response to metabolic acidosis. *Kidney Int* **25**, 608-612.

Krieger, N. S., Sessler, N. E., and Bushinsky, D. A. (1992). Acidosis inhibits osteoblastic and stimulates osteoclastic activity *in vitro*. *Am J Physiol* **262**, F442-448.

Krieger, N. S., Riordon, D., and Bushinsky, D. A. (1997). Both protein kinase C and protein kinase A signal transduction pathways mediate acid-induced bone resorption. *J Bone Miner Res* **12**, S289 (abstract).

Kumagai, H., Sakamoto, H., Guggino, S., Filburn, C. R., and Sacktor, B. (1989). Neurotransmitter regulation of cytosolic calcium in osteoblast-like bone cells. *Calcif Tissue Int* **45**, 251-254.

Kumagai, H., Sacktor, B., and Filburn, C. R. (1991). Purinergic regulation of cytosolic calcium and phosphoinositide metabolism in rat osteoblast-like osteosarcoma cells. *J Bone Miner Res* **6**, 697-708.

Kurumbail, R. G., Stevens, A. M., Gierse, J. K., McDonald, J. J., Stegeman, R. A., Pak, J. Y., Gildehaus, D., Miyashiro, J. M., Penning, T. D., Seibert, K., Isakson, P. C., and C., S. W. (1996). Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. *Nature* **384**, 644-648.

Lacey, D. L., Timms, E., Tan, H. L., Kelley, M., Dunstan, C. R., Burgess, T., Elliott, R., Colombero, A., Elliott, G., Scully, S., Chang, M. S., Hsu, H., Sullivan, J., Hawkins, N., Davy, E., Capparelli, C., Eli, A., Qian, Y. X., Kaufman, S., Sarosi, I., Shalhoub, V., Senaldi, G., Guo, J., Delaney, J., and Boyle, W. J. (1998). Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* **93**, 165-176.

Lader, C. S., and Flanagan, A. M. (1998). Prostaglandin E_2, interleukin 1 α, and tumour necrosis factor-α increase human osteoclast formation and bone resorption *in vitro*. *Endocrinology* **139**, 3157-3164.

Laitala-Leinonen, T., Howell, M. L., Dean, G. E., and Vaananen, H. K. (1996). Resorption-cycle-dependent polarisation of mRNAs for different subunits of V-ATPase in bone-resorbing osteoclasts. *Mol Biol Cell* **7**, 129-142.

Lakkakorpi, P. T., and Vaananen, H. K. (1990). Calcitonin, prostaglandin E_2, and dibutyryl cyclic adenosine 3',5'-monophosphate disperse the specific microfilament structure in resorbing osteoclasts. *J Histochem Cytochem* **38**, 1487-1493.

Lakkakorpi, P. T., Horton, M. A., Helfrich, M. H., Karhukorpi, E. K., and Vaananen, H. K. (1991). Vitronectin receptor has a role in bone resorption but does not mediate tight sealing zone attachment of osteoclasts to the bone surface. *J Cell Biol* **115**, 1179-1186.

Lakkakorpi, P. T., and Vaananen, H. K. (1996). Cytoskeletal changes in osteoclasts during the resorption cycle. *Microsc Res Tech* **33**, 171-181.

Lanyon, L. E. (1993). Osteocytes, strain detection, bone modeling and remodelling. *Calcif Tissue Int* **53** Suppl 1, S102-106.

Lee, S. K., Goldring, S. R., and Lorenzo, J. A. (1994). Calcitonin receptor mRNA and protein are markers of the differentiated osteoclast. *J Bone Miner Res* **9**, S243 (abstract).

Lehenkari, P. P., Laitala-Leinonen, T., Linna, T. J., and Vaananen, H. K. (1997). The regulation of pHi in osteoclasts is dependent on the culture substrate and on the stage of the resorption cycle. *Biochem Biophys Res Commun* **235**, 838-844.

Lehenkari, P., Hentunen, T. A., Laitala-Leinonen, T., Tuukkanen, J., and Vaananen, H. K. (1998). Carbonic anhydrase II plays a major role in osteoclast differentiation and bone resorption by effecting the steady state intracellular pH and Ca^{2+}. *Exp Cell Res* **242,** 128-137.

Lennox, B. (1975). Observations on the accuracy of point counting including a description of a new graticule. *J Clin Pathol* **28**, 99-103.

Leong, W. S., Russell, R. G., and Caswell, A. M. (1994). Stimulation of cartilage resorption by extracellular ATP acting at P2-purinoceptors. *Biochim Biophys Acta* **1201**, 298-304.

Lerner, U. (1980). Indomethacin inhibits bone resorption and lysosomal enzyme release from bone in organ culture. *Scand J Rheumatol* **9**, 149-156.

Lerner, U. H., Ransjo, M., and Ljunggren, O. (1987). Prostaglandin E_2 causes a transient inhibition of mineral mobilisation, matrix degradation, and lysosomal enzyme release from mouse calvaria bones *in vitro*. *Calcif Tissue Int* **40**, 323-331.

Liebermann, D. A., Gregory, B., and Hoffman, B. (1998). AP-1 (Fos/Jun) transcription factors in hematopoietic differentiation and apoptosis. *Int J Oncology* **12**, 685-700.

Luo, L. C., Yu, H., and Ferrier, J. (1997). Differential purinergic receptor signalling in osteoclasts and osteoblastic cells. *Cell Signal* **9**, 603-607.

Maciel, F. M., Sarrazin, P., Morisset, S., Lora, M., Patry, C., Dumais, R., and de Brum Fernandes, A. J. (1997). Induction of cyclooxygenase-2 by parathyroid hormone in human osteoblasts in culture. *J Rheumatol* **24**, 2429-2435.

Manolagas, S. C. (1995). Role of cytokines in bone resorption. *Bone* **17**, 63S-67S.

Marshall, M. J., Holt, I., and Davie, W. J. (1995). The number of tartrate-resistant acid phosphatase-positive osteoclasts on neonatal mouse parietal bones is decreased when prostaglandins synthesis is inhibited and increased in response to prostaglandin E_2, parathyroid hormone, and 1,25-Dihydroxyvitamin D_3. *Calcif Tissue Int* **56**, 240-245.

Marshall, M. J., Holt, I., and Davie, M. W. J. (1996). Inhibition of prostaglandin synthesis leads to a change in adherence of mouse osteoclasts from bone to periosteum. *Calcif Tissue Int* **59**, 207-213.

Martin, T. J., Findlay, D. M., Heath, J. K., and Ng, K. W. (1993). Osteoblasts: differentiation and function. *In* "Physiology and Pharmacology of Bone" (G. R. Mundy & T. J. Martin, Eds.), pp.149-173. Springer Verlag, Berlin

Martinson, J., and Muren, A. (1963). Excitatory and inhibitory effects of vagus stimulation on gastric motility in the cat. *Acta Physiol Scand* **57**, 309-316.

Massey, H. M., Rae, A., and Flanagan, A. M. (1998). A minor population of cells with a macrophage phenotype gives rise to osteoclasts. *Bone* **22**, 30S (abstract).

Matayoshi, A., Brown, C., DiPersio, J. F., Haug, J., Abu-Amer, Y., Liapis, H., Kuestner, R., and Pacific, R. (1996). Human blood-mobilised hematopoietic precursors differentiate into osteoclasts in the absence of stromal cells. *Proc Natl Acad Sci USA* **93**, 10785-10790.

Matsukura, M., Shinozuka, K., Zon, G., Mitsuya, H., Reitz, M., Cohen, J. S., and Broder, S. (1987). Phosphorothioate analogs of oligodeoxynucleotides: inhibitors of replication and cytopathic effects of human immunodeficiency virus. *Proc Natl Acad Sci USA* **84,** 7706-7710.

Matsuzaki, K., Udagawa, N., Takahashi, N., Yamaguchi, K., Yasuda, H., Shima, N., Morinaga, T., Toyama, Y., Yabe, Y., Higashio, K., and Suda, T. (1998). Osteoclast differentiation factor (ODF) induces osteoclast-like cell formation in human peripheral blood mononuclear cell cultures. *Biochem Biophys Res Commun* **246,** 199-204.

McQueney, M. S., Amegadzie, B. Y., D'Alessio, K., Hanning, C. R., McLaughlin, M. M., McNulty, D., Carr, S. A., Ijames, C., Kurdyla, J., and Jones, C. S. (1997). Autocatalytic activation of human cathepsin K. *J Biol Chem* **272**, 13955-13960.

McSheehy, P. M., and Chambers, T. J. (1986a). Osteoblastic cells mediate osteoclastic responsiveness to parathyroid hormone. *Endocrinology* **118**, 824-828.

McSheehy, P. M., and Chambers, T. J. (1986b). Osteoblast-like cells in the presence of parathyroid hormone release a soluble factor that stimulates osteoclastic bone resorption. *Endocrinology* **119**, 1654-1659.

Meghji, S., Sandy, J. R., Scutt, A. M., Harvey, W., and Harris, M. (1988). Stimulation of bone resorption by lipoxygenase metabolites of arachidonic acid. *Prostaglandins* **36**, 139-149.

Meghji, S., Henderson, B., Morrison, M. S. and Arnett, T. R. (1996). Ca^{2+} release from cultured mouse calvaria is very sensitive to ambient pH. *J Bone Miner Res* **11**, 1824 (abstract).

Meghji, S., Morrison, M. S., Henderson, B. and Arnett, T. R. (1997). H^{+} stimulates osteoclastic bone resorption in tissue culture. *J Dental Res.* **76**, 1025 (abstract).

Meghji, S., Morrison, M. S., Heron, K., Henderson, B., and Arnett, T. R. (1998). Extracellular ATP stimulates calvarial bone resorption. *J Bone Miner Res, in press* (abstract).

Mencken, H. L. (1919). Exeunt Omnes. *The Smart Set* **60**, 138-145.

Mercer, B., Markland, F., and Minkin, C. (1998). Contortrostatin, a homodimeric snake venom disintegrin, is a potent inhibitor of osteoclast attachment. *J Bone Miner Res* **13**, 409-414.

Minkin, C., and Jennings, J. M. (1972). Carbonic anhydrase and bone remodelling: sulfonamide inhibition of bone resorption in organ culture. *Science* **176**, 1031-1033.

Minkin, C. (1982). Bone acid phosphatase: tartrate-resistant acid phosphatase as a marker of osteoclast function. *Calcif Tissue Int* **34**, 285-290.

Mitchell, J. A., Akarasereenont, P., Thiemermann, C., Flower, R. J., and Vane, J. R. (1994). Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. *Proc Natl Acad Sci* USA **90**, 11693-11697.

Miyamoto, A., Kunisada, T., Hemmi, H., Yamane, T., Yasuda, H., Miyake, K., Yamazaki, H., and Hayashi, S. (1998). Establishment and characterisation of an immortal macrophage-like cell line inducible to differentiate to osteoclasts. *Biochem Biophys Res Commun* **242**, 703-709.

Modderman, W. E., Tuinenburg Bol Raap, A. C., and Nijweide, P. J. (1991). Tartrate-resistant acid phosphatase is not an exclusive marker for mouse osteoclasts in cell culture. *Bone* **12**, 81-87.

Morrison, M. S. and Arnett, T. R. (1996a). Indomethacin and aspirin stimulate pit formation by rat osteoclasts. *J Bone Miner Res* **11**, S186 (abstract).

Morrison, M. S. and Arnett, T. R. (1996b). Indomethacin and ibuprofen stimulate pit formation by rat osteoclasts. *J Bone Miner Res* **11**, 1825 (abstract).

Morrison, M. S. and Arnett, T. R. (1997a). Effects of extracellular pH on resorption pit formation by chick osteoclasts. *J Bone Miner Res* **12**, F258 (abstract).

Morrison, M. S. and Arnett, T. R. (1997b). ATP stimulates resorption pit formation by rat osteoclasts. *J Bone Miner Res* **12**, O8 (abstract).

Morrison, M. S. and Arnett. T. R. (1997c). The novel cyclooxygenase II inhibitor NS-398 stimulates pit formation by rat osteoclasts. *Bone* **20**,111S (abstract).

Morrison, M. S. and Arnett, T. R. (1998). pH effects on osteoclast formation and activation. *Bone* **22**, 30S (abstract).

Morrison, M. S., Turin, L., King, B. F., Burnstock, G., and Arnett, T. R. (1998). ATP is a potent stimulator of the activation and formation of rodent osteoclasts. *J Physiol* **511**, 495-500.

Mullender, M. G., and Huiskes, R. (1997). Osteocytes and bone lining cells: which are the best candidates for mechano-sensors in cancellous bone? *Bone* **20**, 527-532.

Murrills, R. J., Stein, L. S., and Dempster, D. W. (1993). Stimulation of bone resorption and osteoclast clear zone formation by low pH: a time-course study. *J Cell Physiol* **154**, 511-518.

Murrills, R. J., Dempster, D. W., and Arnett, T. R. (1998). Isolation and culture of osteoclasts and osteoclast resorption assays. *In* "Methods in Bone Biology" (T. R. Arnett and B. Henderson, Eds.), pp. 64-105. Chapman and Hall, London.

Nakamura, I., Takahashi, N., Sasaki, T., Jimi, E., Kurokawa, T., and Suda, T. (1996a). Chemical and physical properties of the extracellular matrix are required for the actin ring formation in osteoclasts. *J Bone Miner Res* **11**, 1873-1879.

Nakamura, I., Gailit, J., and Sasaki, T. (1996b). Osteoclast integrin alpha v beta 3 is present in the clear zone and contributes to cellular polarisation. *Cell Tissue Res* **286**, 507-515.

Nakamura, I., Sasaki, T., Tanaka, S., Takahashi, N., Jimi, E., Kurokawa, T., Kita, Y., Ihara, S., Suda, T., and Fukui, Y. (1997a). Phosphatidylinositol-3 kinase is involved in ruffled border formation in osteoclasts. *J Cell Physiol* **172**, 230-239.

Nakamura, I., Takahashi, N., Udagawa, N., Moriyama, Y., Kurokawa, T., Jimi, E., Sasaki, T., and Suda, T. (1997b). Lack of vacuolar proton ATPase association with the cytoskeleton in osteoclasts of osteosclerotic (oc/oc) mice. *FEBS Lett* **401**, 207-212.

Narbaitz, R., Stumpf, W., and Sar, M. (1983). Autoradiographic demonstration of target cells for 1,25-dihydroxycholecalciferol in bones from fetal rats. *Calcif Tissue Int* **35**, 177-182.

Neary, J. T., and Burnstock, G. (1996). Purinoceptors in the regulation of cell growth and differentiation. *Drug Dev Res* **39**, 407-412.

Nesbitt, S. A., and Horton, M. A. (1997). Trafficking of matrix collagens through bone-resorbing osteoclasts. *Science* **276**, 266-269.

Nicholson, G. C., Moseley, J. M., Sexton, P. M., Mendelsohn, F. A., and Martin, T. J. (1986). Abundant calcitonin receptors in isolated rat osteoclasts. Biochemical and autoradiographic characterisation. *J Clin Invest* **78**, 355-360.

Nicholson, G. C., Moseley, J. M., Sexton, P. M., and Martin, T. J. (1987). Chicken osteoclasts do not possess calcitonin receptors. *J Bone Miner Res* **2**, 53-59.

Nijweide, P. J., Modderman, W. E., and Hagenaars, C. E. (1995). Extracellular adenosine triphosphate. A shock to hemopoietic cells. *Clin Orthop Rel Res* **313**, 92-102.

Noble, B. S., Stevens, H., Loveridge, N., and Reeve, J. (1997). Identification of apoptotic changes in osteocytes in normal and pathological human bone. *Bone* **20**, 273-282.

Nordstrom, T., Rotstein, O. D., Romanek, R., Asotra, S., Heersche, J. N., Manolson, M. F., Brisseau, G. F., and Grinstein, S. (1995). Regulation of cytoplasmic pH in osteoclasts. Contribution of proton pumps and a proton-selective conductance. *J Biol Chem* **270**, 2203-2212.

Nordstrom, T., Shrode, L. D., Rotstein, O. D., Romanek, R., Goto, T., Heersche, J. N., Manolson, M. F., Brisseau, G. F., and Grinstein, S. (1997). Chronic extracellular acidosis induces plasmalemmal vacuolar type H^+ ATPase activity in osteoclasts. *J Biol Chem* **272**, 6354-6360.

Norrdin, R. W., Jee, W. S., and High, W. B. (1990). The role of prostaglandins in bone *in vivo*. *Prostaglandins Leukot Essent Fatty Acids* **41**, 139-149.

Ohlsson, C., Bengtsson, B. A., Isaksson, O. G., Andreassen, T. T., and Slootweg, M. C. (1998). Growth hormone and bone. *Endocrine Reviews* **19,** 55-79.

Okahashi, N., Nakamura, I., Jimi, E., Koide, M., Suda, T., and Nishihara, T. (1997). Specific inhibitors of vacuolar H^+-ATPase trigger apoptotic cell death of osteoclasts. *J Bone Miner Res* **12**, 1116-1123.

Onoe, Y., Miyaura, C., Kaminakayashiki, T., Nagai, Y., Noguchi, K., Chen, Q. R., Seo, H., Ohta, H., Nozawa, S., Kudo, I., and Suda, T. (1996). IL-13 and IL-4 inhibit bone resorption by suppressing cyclooxygenase-2-dependent prostaglandin synthesis in osteoblasts. *J Immunol* **156**, 758-764.

Ousler, M. J., Pederson, L., Fitzpatrick, L., Riggs, B. L., and Spelsberg, T. (1994). Human giant cell tumours of bone (osteoclastomas) are estrogen target cells. *Proc Natl Acad Sci USA* **91,** 5227-5231.

Owens, J. M., Gallagher, A. C., and Chambers, T. J. (1996). Bone cells required for osteoclastic resorption but not for osteoclastic differentiation. *Biochem Biophys Res Commun* **222**, 225-229.

Pacifici, R. (1995). Cytokines and osteoclast activity. *Calcif Tissue Int* **56,** S27-S28.

Pacifici, R. (1998). Editorial: Cytokines, estrogen and postmenopausal osteoporosis - the second decade. *Endocrinology* **139**, 2659-2661.

Pead, M. J., and Lanyon, L. E. (1989). Indomethacin modulation of load-related stimulation of new bone formation *in vivo*. *Calcif Tissue Int* **45**, 34-40.

Peck, W. A. (1984). The effects of glucocorticoids on bone cell metabolism and function. *Adv Exp Med Biol* **171,** 111-119.

Pilbeam, C. C., Kawaguchi, H., Hakeda, Y., Voznesensky, O., Alander, C. B., and Raisz, L. G. (1993). Differential regulation of inducible and constitutive prostaglandin endoperoxide synthase in osteoblastic MC3T3-E1 cells. *J Biol Chem* **268**, 25643-25649.

Pilbeam, C. C., Raisz, L. G., Voznesensky, O., Alander, C. B., Delman, B. N., and Kawaguchi, H. (1995). Autoregulation of inducible prostaglandin g/h synthase in osteoblastic cells by prostaglandins. *J Bone Miner Res* **10**, 406-414.

Pilbeam, C. C., Rao, Y., Voznesensky, O., Kawaguchi, H., Alander, C. B., Raisz, L. G., and Herschman, H. (1997). Transforming growth factor-beta1 regulation of prostaglandin G/H synthase-2 expression in osteoblastic MC3T3-E1 cells. *Endocrinology* **138**, 4672-4682.

Piper, K., Boyde, A., and Jones, S. J. (1992). The relationship between the number of nuclei of an osteoclast and its resorptive capability in vitro. *Anat Embyol Berl* **186**, 291-299.

Piper, K., Boyde, A., and Jones, S. J. (1995). Volumes of chick and rat osteoclasts cultured on glass. *Calcif Tissue Int* **56**, 382-389.

Puscas, I., and Coltau, M. (1995). Prostaglandins with vasodilating effects inhibit carbonic anhydrase while vasoconstrictive prostaglandins and leukotrienes B4 and C4 increase CA activity. *Int J Clin Pharmacol Ther* **33**, 176-181.

Puscas, I., Coltau, M., and Pasca, R. (1996). Nonsteroidal antiinflammatory drugs activate carbonic-anhydrase by a direct mechanism of action. *J Pharm Exp Thera* **277**, 1464-1466.

Quinn, J. M. W., McGee, J. O., and Athanasou, N. A. (1994). Cellular and hormonal factors influencing monocyte differentiation to osteoclastic bone-resorbing cells. *Endocrinology* **134,** 2416-2423.

Rabadjija, L., Brown, E. M., Swartz, S. L., Chen, C. J., and Goldhaber, P. (1990). H(+)-stimulated release of prostaglandin E_2 and cyclic adenosine 3',5'-monophosphoric acid and their relationship to bone resorption in neonatal mouse calvaria cultures. *Bone Miner* **11**, 295-304.

Raisz, L. G., and Koolemans-Beyen, A. R. (1974). Inhibition of bone collagen synthesis by prostaglandin E_2 in organ culture.

Raisz, L. G., and Kream, B. E. (1983). Regulation of bone formation. *N Engl J Med* **309**, 29-35 & 83-89.

Raisz, L. G., Simmons, H. A., Thompson, W. J., Shepard, K. L., Anderson, P. S., and Rodan, G. A. (1988). Effects of a potent carbonic anhydrase inhibitor on bone resorption in organ culture. *Endocrinology* **122**, 1083-1086.

Raisz, L. G., Simmons, H. A., and Fall, P. M. (1989). Biphasic effects of nonsteroidal anti-inflammatory drugs on prostaglandin production by cultured rat calvariae. *Prostaglandins* **37**, 559-565.

Raisz, L. G., and Fall, P. M. (1990). Biphasic effects of prostaglandin E_2 on bone formation in cultured fetal rat calvariae: interaction with cortisol. *Endocrinology* **126**, 1654-1659.

Raisz, L. G. (1995). Physiologic and pathologic roles of prostaglandins and other eicosanoids in bone metabolism. *J Nutr* **125**, 2024S-2027S.

Ralston, S. H. (1997). The Michael Mason Prize Essay 1997. Nitric oxide and bone: what a gas! *Br J Rheumatol* **36,** 831-838.

Ramp, W. K., Lenz, L. G., and Kaysinger, K. K. (1994). Medium pH modulates matrix, mineral, and energy metabolism in cultured chick bones and osteoblast-like cells. *Bone Miner* **24**, 59-73.

Reddy, S. V., Takahashi, S., Dallas, M., Williams, R. E., Neckers, L., and Roodman, G. D. (1994). Interleukin-6 antisense deoxyoligonucleotides inhibit bone resorption by giant cells from human giant cell tumours of bone. *J Bone Miner Res* **9**, 753-757.

Rice, D. P. C., Kim, H.-J., and Thesleff, I. (1997). Detection of gelatinase B expression reveals osteoclastic bone resorption as a feature of early calvarial bone development. *Bone* **21**, 479-486.

Robinson, D. R., Tashjian Jr, A. H., and Levine, L. (1975). Prostaglandin-stimulated bone resorption by rheumatoid synovia - possible mechanism for bone destruction in rheumatoid arthritis. *J Clin Invest* **56,** 1181-1187.

Roodman, G. D., Ibbotson, K. J., MacDonald, B. R., Kuehl, T. J., and Mundy, G. R. (1985). 1,25-Dihydroxyvitamin D_3 causes formation of multinucleated cells with several osteoclast characteristics in cultures of primate marrow. *Proc Natl Acad Sci USA* **82,** 8213-8217.

Roodman, G. D. (1995). Application of bone marrow cultures to the study of osteoclast formation and osteoclast precursors in man. *Calcif Tissue Int* **56**, Suppl 1, S22-23.

Roodman, G. D. (1996). Advances in bone biology: the osteoclast. *Endocrine Reviews* **17**, 308-332.

Rosner, M. J., and Becker, D. P. (1984). Experimental brain injury: successful therapy with the weak base, tromethamine. With an overview of CNS acidosis. *J Neurosurg* **60**, 961-971.

Roussel, M. F., Downing, J. R., Rettenmier, C. W., and Sherr, C. J. (1988). A point mutation in the extracellular domain of the human CSF-1 receptor (c-fms proto-oncogene product) activates its transforming potential. *Cell* **55,** 979-88.

Roux, S., Pichaud, F., Quinn, J., Lalande, A., Morieux, C., Jullienne, A., and de Vernejoul, M. C. (1997). Effects of prostaglandins on human hematopoietic osteoclast precursors. *Endocrinology* **138**, 1476-1482.

Rubin, J., Biskobing, D., Fan, X., Rubin, C., McLeod, K., and Taylor, W. R. (1997). Pressure regulates osteoclast formation and M-CSF expression in marrow culture. *J Cell Physiol* **170**, 81-87.

Saadoun, S., Lluch, M., Rodriguez-Alvarez, J., Blanco, I., and Rodriguez, R. (1998). Extracellular acidification modifies Ca^{2+} fluxes in rat brain synaptosomes. *Biochem Biophys Res Commun* **242**, 123-128.

Saino, H., Matsuyama, T., Takada, J., Kaku, T., and Ishii, S. (1997). Long-term treatment of indomethacin reduces vertebral bone mass and strength in ovariectomised rats. *J Bone Miner Res* **12**, 1844-1850.

Sakai, D., Tong, H. S., and Minkin, C. (1995). Osteoclast molecular phenotyping by random cDNA sequencing. *Bone* **17**, 111-119.

Salo, J., Metsikko, K., Palokangas, H., Lehenkari, P., and Vaananen, H. K. (1996). Bone-resorbing osteoclasts reveal a dynamic division of basal plasma membrane into two different domains. *J Cell Sci* **109**, 301-307.

Salo, J., Lehenkari, P., Mulari, M., Metsikko, K., and Vaananen, H. K. (1997). Removal of osteoclast bone resorption products by transcytosis. *Science* **276**, 270-273.

Sarma, U., and Flanagan, A. M. (1996). Macrophage colony-stimulating factor induces substantial osteoclast generation and bone resorption in human bone marrow cultures. *Blood* **88**, 2531-2540.

Sarma, U., Edwards, M., Motoyoshi, K., and Flanagan, A. M. (1998). Inhibition of bone resorption by 17β-Estradiol in human bone marrow cultures. *J Cell Physiol* **175**, 99-108.

Sato, M., Sardana, M. K., Grasser, W. A., Garsky, V. M., Murray, J. M., and Gould, R. J. (1990). Echistatin is a potent inhibitor of bone resorption in culture. *J Cell Biol* **111**, 1713-1723.

Schlesinger, P. H., Mattsson, J. P., and Blair, H. C. (1994). Osteoclastic acid transport: mechanism and implications for physiological and pharmacological regulation. *Miner Electrolyte Metab* **20**, 31-39.

Schofl, C., Cuthbertson, K. S. R., Walsh, C. A., Mayne, C., Cobbold, P., Von Zur Muhlen, A., Hesch, R., and Gallagher, J. A. (1992). Evidence for P_2-Purinoceptors on human osteoblast-like cells. *J Bone Miner Res* **7**, 485-490.

Sebastian, A., Harris, S. T., Ottaway, J. H., Todd, K. M., and Morris, R. C., Jr. (1994). Improved mineral balance and skeletal metabolism in postmenopausal women treated with potassium bicarbonate. *N Engl J Med* **330**, 1776-1781.

Sebastian, A., and Morris, R. C., Jr. (1994). Improved mineral balance and skeletal metabolism in postmenopausal women treated with potassium bicarbonate [letter; comment]. *N Engl J Med* **331**, 279.

Segre, G. V., Perkins, A. S., Witters, L. A., Potts, J. T., Jr. (1981). Metabolism of parathyroid hormone by isolated rat Kupffer cells and hepatocytes. *J Clin Invest* **67**, 449-457.

Selander, K. S., Harkonen, P. L., Valve, E., Monkkonen, J., Hannuniemi, R., and Vaananen, H. K. (1996). Calcitonin promotes osteoclast survival *in vitro*. *Molecular Cellular Endocrin.* **122**, 119-129.

Shibutani, T., and Heersche, J. N. (1993). Effect of medium pH on osteoclast activity and osteoclast formation in cultures of dispersed rabbit osteoclasts. *J Bone Miner Res* **8**, 331-336.

Shimegi, S. (1996). ATP and adenosine act as a mitogen for osteoblast-like cells (MC3T3-E1). *Calcif Tissue Int* **58**, 109-113.

Shuto, T., Kukita, T., Hirata, M., Jimi, E., and Koga, T. (1994). Dexamethasone stimulates osteoclast-like cell formation by inhibiting granulocyte-macrophage colony-stimulating factor production in mouse bone marrow cultures. *Endocrinology* **134**, 1121-1126.

Silver, I. A., Murrills, R. J., and Etherington, D. J. (1988). Microelectrode studies on the acid microenvironment beneath adherent macrophages and osteoclasts. *Exp Cell Res* **175**, 266-276.

Simonet, W. S., Lacey, D. L., Dunstan, C. R., Kelley, M., Chang, M. S., Luthy, R., Nguyen, H. Q., Wooden, S., Bennett, L., Boone, T., Shimamoto, G., DeRose, M., Elliott, R., Colombero, A., Tan, H. L., Trail, G., Sullivan, J., Davy, E., Bucay, N., Renshaw Gegg, L., Hughes, T. M., Hill, D., Pattison, W., Campbell, P., Sander, S., Van, G., Tarpley, J., Derby, P., Lee, R., Program, A. E., and Boyle, W. J. (1997). Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* **89**, 309-319.

Sims, S. M., and Dixon, S. J. (1989). Inwardly rectifying K+ current in osteoclasts. *Am J Physiol* **256**, C1277-1282.

Skerry, T. M., Bitensky, L., Chayen, J., and Lanyon, L. E. (1989). Early strain-related changes in enzyme activity in osteocytes following bone loading *in vivo*. *J Bone Miner Res* **4**, 783-788.

Sly, W. S., Whyte, M. P., Sundaram, V., Tashian, R. E., Hewett Emmett, D., Guibaud, P., Vainsel, M., Baluarte, H. J., Gruskin, A., Al Mosawi, M., Sakati, N., and Ohlsson, A. (1985). Carbonic anhydrase II deficiency in 12 families with the autosomal recessive syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification. *N Engl J Med* **313**, 139-145.

Smith, W. L. (1992). Prostanoid biosynthesis and mechanisms of action. *Am J Physiol* **263**, F181-F191.

Soekanto, A., Ohya, K., and Ogura, H. (1994). The effect of sodium salicylate on the osteoclast-like cell formation and bone resorption in a mouse bone marrow culture. *Calcif Tissue Int* **54**, 290-295.

Spowage, M., and Arnett, T. R. (1995). Time dependence of proton-activated osteoclastic resorption. *Calcif Tissue Int* **56**, 445 (abstract).

Sprague, S. M., Krieger, N. S., and Bushinsky, D. A. (1994). Greater inhibition of *in vitro* bone mineralisation with metabolic than respiratory acidosis. *Kidney Int* **46**, 1199-1206.

Steen, K. H., Reeh, P. W., Anton, F., and Handwerker, H. O. (1992). Protons selectively induce lasting excitation and sensitisation to mechanical stimulation of nociceptors in rat skin, *in vitro*. *J Neurosci* **12**, 86-95.

Steen, K. H., Steen, A. E., and Reeh, P. W. (1995). A dominant role of acid pH in inflammatory excitation and sensitisation of nociceptors in rat skin, *in vitro*. *J Neurosci* **15**, 3982-3989.

Stein, C. A., Tonkinson, J., and Yakubov, L. (1991). Phosphorothioate oligonucleotides - antisense inhibitors of gene expression? *Pharmacol Therapeut* **52**, 365-384.

Stein, C. A., and Krieg, A. M. (1994). Problems in interpretation of data derived from *in vitro* and *in vivo* use of antisense oligodeoxynucleotides [editorial]. *Antisense Res Dev* **4**, 67-69.

Suda, K., Takahashi, N., and Martin, T. J. (1992). Modulation of osteoclast differentiation. *Endocrine Reviews* **13**, 251-267.

Suda, T., Udagawa, N., Nakamura, I., Miyaura, C., and Takahashi, N. (1995a). Modulation of osteoclast differentiation by local factors. *Bone* **17**, 87S-91S.

Suda, K., Takahashi, N., and Martin, T. J. (1995b). Modulation of osteoclast differentiation: Update 1995. *Endocrine Reviews* **4**, 266-270.

Sundquist, K., Lakkakorpi, P., Wallmark, B., and Vaananen, K. (1990). Inhibition of osteoclast proton transport by bafilomycin A1 abolishes bone resorption. *Biochem Biophys Res Commun* **168**, 309-313.

Surprenant, A., Rassendren, F., Kawashima, E., North, R. A., and Buell, G. (1996). The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7). *Science* **272**, 735-738.

Suzuki, A., Kotoyori, J., Oiso, Y., and Kozawa, O. (1993). Prostaglandin E_2 is a potential mediator of extracellular ATP action in osteoblast-like cells. *Cell Adhes Commun* **1**, 113-118.

Suzuki, A., Shinoda, J., Oiso, Y., and Kozawa, O. (1995). Mechanism of phospholipase D activation induced by extracellular ATP in osteoblast-like cells. *J Endocrinol.* **145**, 81-86.

Tai, H., Miyaura, C., Pilbeam, C. C., Tamura, T., Ohsugi, Y., Koishihara, Y., Kubodera, N., Kawaguchi, H., Raisz, L. G., and Suda, T. (1997). Transcriptional induction of cyclooxygenase-2 in osteoblasts is involved in interleukin-6-induced osteoclast formation. *Endocrinology* **138**, 2372-2379.

Takahashi, N., Yamana, H., Yoshiki, S., Roodman, G. D., Mundy, G. R., Jones, S. J., Boyde, A., and Suda, T. (1988a). Osteoclast-like cell formation and its regulation by osteotropic hormones in mouse bone marrow cultures. *Endocrinology* **122**, 1373-1382.

Takahashi, N., Akatsu, T., Udagawa, N., Sasaki, T., Yamaguchi, A., Moseley, J. M., Martin, T. J., and Suda, T. (1988b). Osteoblastic cells are involved in osteoclast formation. *Endocrinology* **123**, 2600-2602.

Takahashi, N., Kukita, T., MacDonald, B. R., Bird, A., Mundy, G. R., McManus, L. M., Miller, M., Boyde, A., Jones, S., and Roodman, G. D. (1989). Osteoclast-like cells form in long-term human bone marrow but not in peripheral blood. *J Clin Invest* **84**, 543-550.

Tamura, T., Takahashi, N., Akatsu, T., Sasaki, T., Udagawa, N., Tanaka, S., and Suda, T. (1993). New resorption assay with mouse osteoclast-like multinucleated cells formed *in vitro*. *J Bone Miner Res* **8**, 953-960.

Tanaka, S., Takahashi, N., Udagawa, N., Tamura, T., Akatsu, T., Stanley, E. R., Kurokawa, T., and Suda, T. (1993). Macrophage colony-stimulating factor is indispensable for both proliferation and differentiation of osteoclast progenitors. *J Clin Invest* **91**, 257-263.

Tanaka, S., Amling, M., Neff, L., Peyman, A., Uhlmann, E., Levy, J. B., and Baron, R. (1996). c-Cbl is downstream of c-Src in a signalling pathway necessary for bone resorption. *Nature* **383**, 528-531.

Testa, N. G., Allen, T. D., Lajtha, L. G., Onions, D., and Jarret, O. (1981). Generation of osteoclasts *in vitro*. *J Cell Sci* **47**, 127-137.

Teti, A., Blair, H. C., Teitelbaum, S. L., Kahn, A. J., Koziol, C., Konsek, J., Zambonin Zallone, A., and Schlesinger, P. H. (1989a). Cytoplasmic pH regulation and chloride/bicarbonate exchange in avian osteoclasts. *J Clin Invest* **83**, 227-233.

Teti, A., Blair, H. C., Schlesinger, P., Grano, M., Zambonin Zallone, A., Kahn, A. J., Teitelbaum, S. L., and Hruska, K. A. (1989b). Extracellular protons acidify osteoclasts, reduce cytosolic calcium, and promote expression of cell-matrix attachment structures. *J Clin Invest* **84**, 773-780.

Tondravi, M. M., McKercher, S. R., Anderson, K., Erdmann, J. M., Quiroz, M., Maki, R., and Teitelbaum, S. L. (1997). Osteopetrosis in mice lacking haematopoietic transcription factor PU.1. *Nature* **386**, 81-84.

Traianedes, K., Dallas, M. R., Garrett, I. R., Mundy, G. R., and Bonewald, L. F. (1998). 5-Lipoxygenase metabolites inhibit bone formation *in vitro*. *Endocrinology* **139**, 3178-3184.

Turner, R. T., Riggs, B. L., and Spelsberg, T. C. (1994). Skeletal effects of estrogen. *Endocrine Reviews* **15**, 129-154.

Turner, R. T., Riggs, B. L., and Spelsberg, T. C. (1995). Skeletal effects of estrogen: Update 1995. *Endocrine Reviews* **4**, 155-158.

Uy, H. L., Guise, T. A., De La Mata, J., Taylor, S. D., Story, B. M., Dallas, M. R., Boyce, B. F., Mundy, G. R., and Roodman, G. D. (1995). Effects of parathyroid hormone (PTH)-related protein and PTH on osteoclasts and osteoclast precursors *in vivo*. *Endocrinology* **136**, 3207-3212.

Vaananen, H. K., and Parvinen, E. K. (1983). High active isoenzyme of carbonic anhydrase in rat calvaria osteoclasts. Immunohistochemical study. *Histochemistry* **78**, 481-485.

Vaananen, H. K., Karhukorpi, E. K., Sundquist, K., Wallmark, B., Roininen, I., Hentunen, T., Tuukkanen, J., and Lakkakorpi, P. (1990). Evidence for the presence of a proton pump of the vacuolar H(+)-ATPase type in the ruffled borders of osteoclasts. *J Cell Biol* **111**, 1305-1311.

Vaananen, H. K., and Horton, M. (1995). The osteoclast clear zone is a specialised cell-extracellular matrix adhesion structure. *J Cell Sci* **108**, 2729-2732.

Vaananen, H. K. (1996). Osteoclast function: Biology and mechanisms. *In* "Principles of Bone Biology" (J. P. Bilezikia, L. G. Raisz, and G. A. Rodan, Eds.), Vol. 1, pp. 103-113. Academic Press Inc., San Diego.

Vaes, G. (1968). On the mechanisms of bone resorption: The action of parathyroid hormone on the excretion and synthesis of lysosomal enzymes and on the extracellular release of acid by bone cells. *J Cell Biol* **39,** 676-697.

Vaes, G. (1972). The release of collagenase as an inactive proenzyme by bone explants in culture. *Biochem J* **126,** 275-89.

Vanderschueren, D., and Bouillon, R. (1995). Androgens and bone. *Calcif Tissue Int* **56,** 341-346.

Vane, J. R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature* **231**, 232-235.

Vane, J., and Botting, R. (1987). Inflammation and the mechanism of action of anti-inflammatory drugs. *FASEB-Journal* **1**, 89-96.

Virgino, C., Church, D., North, R. A., and Surprenant, A. (1997). Effects of divalent cations, protons and calmidazolium at the rat P2X7 receptor. *Neuropharm* **36**, 1285-1294.

Wachman, A., and Bernstein, D. S. (1968). Diet and osteoporosis. *Lancet* **1**, 958-959.

Wagner, R. W. (1994). Gene inhibition using antisense oligodeoxynucleotides. *Nature* **372,** 333-5.

Wagstaff, S. C., Birch, M. A., Bowler, W. B., Wlodarski, B. W., Dillon, J. P., Roberts, E. R., Carron, J. A., Gallagher, J. A., and Bilbe, G. (1998). Osteoclasts express a novel member of the ATP binding cassette superfamily. *Bone* **22**, 35S (abstract).

Walker, D. G. (1973). Osteopetrosis in mice cured by temporary parabiosis. *Science* **180**, 875-880.

Walsh, C. A., Dawson, W. E., Birch, M. A., and Gallagher, J. A. (1990). The effects of extracellular pH on bone resorption by avian osteoclasts *in vitro*. *J Bone Miner Res* **5,** 1243-1247.

Walsh, C. A., Beresford, J. N., Birch, M. A., Boothroyd, B., and Gallagher, J. A. (1991). Application of reflected light microscopy to identify and quantitate resorption by isolated osteoclasts. *J Bone Miner Res* **6**, 661-671.

Wang, H. J., Tong, H., and Minkin, C. (1997). The single osteoclast pit assay: individual micro-isolated osteoclasts form pits on dentine slices. *J Bone Miner Res* **12**, F257 (abstract).

Weidema, A. F., Barbera, J., Dixon, S. J., and Sims, S. M. (1997). Extracellular nucleotides activate non-selective cation and Ca^{2+}-dependent K^+ channels in rat osteoclasts. *J Physiol* **503.2**, 303-315.

Weidema, A. F., Dixon, S. J., and Sims, S. M. (1998). Extracellular nucleotides activate P2X and P2Y purinoceptors on mammalian osteoclasts. *J Dent Res* **77**, 904 (abstract).

Weinstein, A. J., Fan, D., and Biskobing, D. (1998). Calcitonin receptor expression in osteoclasts is modulated by changes in culture medium pH. *J Invest Med* **46**, 33 (abstract).

Wildman, S. S., King, B. F., and Burnstock, G. (1997). Potentiation of ATP-responses at a recombinant P2X2 receptor by neurotransmitters and related substances. *Br J Pharmacol* **120**, 221-224.

Yamate, T., Mocharla, H., Taguchi, Y., Igietseme, J. U., Manolagas, S. C., and Abe, E. (1997). Osteopontin expression by osteoclast and osteoblast progenitors in the murine bone marrow: demonstration of its requirement for osteoclastogenesis and its increase after ovariectomy. *Endocrinology* **138**, 3047-3055.

Yasuda, H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, M., Mochizuki, S., Tomoyasu, A., Yano, K., Goto, M., Murakami, A., Tsuda, E., Morinaga, T., Higashio, K., Udagawa, N., Takahashi, N., and Suda, T. (1998). Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci USA* **95,** 3597-3602.

Yoshida, H., Hayashi, S., Kunisada, T., Ogawa, M., Nishikawa, S., Okamura, H., Sudo, T., Shultz, L. D., and Nishikawa, S. (1990). The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature* **345,** 442-444.

Yu, H. H., and Ferrier, J. (1993a). Osteoblast-like cells have a variable mixed population of purino / nucleotide receptors. *FEBS Lett* **328**, 209-214.

Yu, H., and Ferrier, J. (1993b). ATP induces an intracellular calcium pulse in osteoclasts. *Biochem Biophys Res Commun* **191**, 357-363.

Yu, H., and Ferrier, J. (1994). Mechanisms of ATP-induced Ca^{2+} signalling in osteoclasts. *Cell Signal* **6**, 905-914.

Yu, H., and Ferrier, J. (1995). Osteoclast ATP receptor activation leads to a transient decrease in intracellular pH. *J Cell Sci* **108**, 3051-3058.

Zaidi, M., Moonga, B., Moss, D. W., and MacIntyre, I. (1989). Inhibition of osteoclastic acid phosphatase abolishes bone resorption. *Biochem Biophys Res Commun* **159**, 68-71.

Zaman, G., Suswillo, R. F., Cheng, M. Z., Tavares, I. A., and Lanyon, L. E. (1997). Early responses to dynamic strain change and prostaglandins in bone-derived cells in culture. *J Bone Miner Res* **12**, 769-777.

Zanovello, P., Bronte, V., Rosato, A., Pizzo, P., and Di Virgilio, F. (1990). Response of mouse lymphocytes to extracellular ATP. II. Extracellular ATP causes cell type-dependent lysis and DNA fragmentation. *J Immunol* **145**, 1545-1550.

Zheng, L. M., Zychlinsky, A., Liu, C., Ojcius, D., and Young, J. D. (1991). Extracellular ATP as a trigger for apoptosis or programmed cell death. *J Cell Biol* **112**, 279-288.

APPENDIX 1

Abbreviations and Common names

Abbreviation / common name	Full / systematic name
1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)	1,25-dihydroxycholecalciferol
Adenosine	9-β-D-ribofuranosyladenine
Adenosine deaminase	Adenosine aminohydrolase; EC 3.5.4.4
Antibiotic / antimycotic	Penicillin G sodium (100u/ml) Streptomycin sulphate (100μg/ml) Amphotericin B (0.25μg/ml)
Apyrase	Adenosine 5′-triphosphatase / adenosine 5′-diphosphatase; EC 3.6.1.5
Aspirin	Acetylsalicylic acid
ATP	Adenosine 5′-triphosphate (disodium salt)
BGJ	Biggers, Gwatkin and Heyner medium
Dexamethasone	9α-fluoro-16α-methyl-prednisolone
FCS	Foetal calf serum,
D-(+) glucose	Dextrose
L-glutamine	L-2-aminoglutaramic acid
HBSS	Hanks' balanced salt solution
Ibuprofen	α-methyl-4-[2-methylpropyl]-benzeneacetic acid
Indomethacin	1-[p-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid
MEM	Minimum essential medium with

	Earle's salts, without L-glutamine
NS-398	N-[2-cyclohexyloxy-4-nitrophenyl] methanesulfonamide)
PBS	Phosphate buffered saline (Dulbecco's), without sodium bicarbonate
Prostaglandin E_2 (PGE_2)	[5Z,11α,13E,15S]-11,15-dihydroxy-9-oxoprosta-5,13-dienoic acid
PTH	Parathyroid hormone
Suramin	Germanin; 8,8′-[carbonyl-bis[imino-3,1-phenylcarbonyl-imino(4-methyl-3,1-phenylene)-carbonylimino]]bis-1,3,5-naphthalene trisulfonic acid hexasodium salt
TRAP	Tartrate-resistant acid phosphatase

APPENDIX 2

Materials and Suppliers

1,25-dihydroxyvitamin D3	Dr K.W. Colston St. George's Hospital Medical School, London, U.K.
Adenosine	Sigma, Poole, Dorset, U.K.
Adenosine deaminase	Sigma, Poole, Dorset, U.K.
Antibiotic / Antimycotic	GIBCO, Paisley, U.K.
Apyrase	Sigma, Poole, Dorset, U.K.
Aspirin	Sigma, Poole, Dorset, U.K.
ATP	Sigma, Poole, Dorset, U.K.
Bacharach Fyrite kit	Jencons (Scientific) Ltd Leighton Buzzard, U.K.
BGJ	Flow Laboratories, Irvine, U.K.
Dexamethasone	Sigma, Poole, Dorset, U.K.
FCS	GIBCO, Paisley, U.K.
D-(+) glucose	Sigma, Poole, Dorset, U.K.
L-glutamine	GIBCO, Paisley, U.K.
Glycerol jelly	BDH, Poole, Dorset, U. K.
Ibuprofen	Sigma, Poole, Dorset, U.K.
Indomethacin	Sigma, Poole, Dorset, U.K.
MEM	GIBCO, Paisley, U.K.
MK886	Dr S. Meghji Eastman Dental Institute London, U.K.
Minitab	Minitab Inc., State College Pennsylvania, USA.
NS-398	ICN Biomedicals Ltd Thame, U.K.
Oligonucleotides	Genosys Biotechnologies Ltd Pampisford, Cambridgeshire, U.K.

Parathyroid hormone (bovine fragment 1-34)	Sigma, Poole, Dorset, U.K.
PBS	GIBCO, Paisley, U.K.
Prostaglandin E_2	Sigma, Poole, Dorset, U.K.
Stainless steel grids	Expanded Metal Co., West Hartlepool, U.K.
Suramin	ICN Biomedicals Ltd Thame, U.K.
TRAP stain	Sigma, Poole, Dorset, U.K.
Untreated elephant ivory	HM Customs, London Heathrow Airport.

APPENDIX 3

Published Papers

Rapid Report

ATP is a potent stimulator of the activation and formation of rodent osteoclasts

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1. There is increasing evidence that extracellular ATP acts directly on bone cells via P2 receptors. In normal rat osteoclasts, ATP activates both non-selective cation channels and Ca^{2+}-dependent K^+ channels. In this study we investigated the action of ATP on the formation of osteoclasts and on the ultimate function of these cells, namely resorption pit formation.

2. We found that ATP stimulated resorption pit formation up to 5·6-fold when osteoclast-containing bone cell populations from neonatal rats were cultured for 26 h on ivory discs, with a maximum effect occurring at relatively low concentrations (0·2–2 μM). The stimulatory effect of ATP was amplified greatly when osteoclasts were activated by culture in acidified media (pH 6·9–7·0). Pit formation by acid-activated osteoclasts in the absence of ATP was inhibited by apyrase, an ecto-ATPase and by suramin, an antagonist of P2 receptors.

3. Over the same concentration range at which rat osteoclast activation occurred (0·2–2 μM), ATP also enhanced osteoclast formation in 10 day mouse marrow cultures, by up to 3·3-fold, with corresponding increases in resorption pit formation. Higher concentrations of ATP (20–200 μM) reduced or blocked osteoclast formation. Adenosine, a P1 receptor agonist, was without effect on either osteoclast activation or formation.

4. These results suggest that low levels of extracellular ATP may play a fundamental role in modulating both the resorptive function and formation of mammalian osteoclasts.

ATP and other extracellular nucleotides are now recognized as important messenger molecules for cell–cell communication. Cell membrane receptors for ATP are classified into two main groups: the P2Y receptor family couples to G-proteins to stimulate phospholipases, activating a series of intracellular signalling pathways, including IP_3-dependent mobilization of intracellular Ca^{2+}; the P2X receptor family gates cation channels permeable to calcium, sodium, potassium and, most probably, hydrogen ions. Seven main subtypes of each family have been identified to date (Burnstock & King, 1996). ATP is released into the extracellular space via synaptic vesicles from nerve cells, as the result of cell damage and also by active secretion via 'ATP binding cassette' transport proteins such as P-glycoproteins and sulphonylurea receptors (reviewed by Burnstock, 1997).

There is increasing evidence that ATP may play an important role in bone as a signalling agent. In osteoclasts, the polarized multinucleate cells responsible for the resorption of bone and other mineralized tissues, exogenous ATP induces an intracellular Ca^{2+} pulse (Yu & Ferrier, 1993, 1994) and causes a transient intracellular pH decrease that is Ca^{2+} independent (Yu & Ferrier, 1995). Recent electrophysiological experiments have provided indirect evidence for the co-expression on osteoclasts of both P2Y and P2X receptors for ATP (Weidema *et al.* 1997). Histochemical evidence exists for the expression of the $P2Y_2$ (P2U) receptor on osteoclasts derived from human giant cell tumours (Bowler *et al.* 1995, 1998*a*). Bowler and co-workers have also shown that ATP exerts a small stimulatory effect on resorption pit formation by giant cell tumour osteoclasts but that this effect is not mediated by $P2Y_2$ receptors (Bowler *et al.* 1998*a*). Following our initial reports in abstract form (Morrison & Arnett, 1997, 1998*a*), the present study provides the first description of the potent stimulatory effects of ATP on the formation and resorptive function of normal mammalian osteoclasts.

METHODS

Materials

1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) was provided by Dr K. W. Colston (St George's Hospital Medical School, London). Culture media were purchased from Gibco. All other reagents were from Sigma. Fresh stock solutions of ATP, adenosine, apyrase and suramin were prepared in phosphate-buffered saline (PBS) for each experiment; stock solutions of dexamethasone and $1,25(OH)_2D_3$ were prepared in ethanol and stored for short periods at −20 °C. ATP and adenosine solutions, both of which are acidic, were titrated to pH 7·0 with NaOH immediately before use to avoid unwanted pH effects on osteoclast function (Arnett & Spowage, 1996). Untreated elephant ivory was kindly provided by HM Customs and Excise (London Heathrow Airport).

Resorption pit formation assay

The effects of extracellular ATP and adenosine on resorption pit formation by mature rat osteoclasts were studied using modifications of an assay described previously (Arnett & Spowage, 1996). All experiments were performed using standard minimum essential medium supplemented with Earle's salts, 10% fetal calf serum, 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin and 0·25 $\mu g\ ml^{-1}$ amphotericin B (complete mixture abbreviated to 'MEM'). In some experiments, MEM was acidified by the direct addition of small amounts of concentrated hydrochloric acid (10 mequiv l^{-1} H^+, equivalent to 85 μl of 11·5 M HCl per 100 ml medium). This has the effect of reducing HCO_3^- concentration and producing an operating pH close to 6·95 in a 5% CO_2 environment, which is optimal for resorption pit formation (Murrills *et al.* 1998). Elephant ivory was prepared by cutting 250 μm thick transverse wafers using a low speed diamond saw (Buehler, Coventry, UK); 5 mm diameter discs were cut from wet ivory wafers using a standard paper punch, washed extensively by sonication in distilled water and stored dry at room temperature. Before use, ivory discs were sterilized by brief immersion in ethanol, allowed to dry and then rinsed in sterile PBS.

Mixed cell populations containing osteoclasts were obtained by mincing rapidly the pooled long bones of 2-day-old rat pups, killed by cervical dislocation ($n = 5$), in 5 ml MEM, followed by vortexing for 20 s. The resulting cell suspension was allowed to sediment for 45 min onto 5 mm ivory discs, pre-wetted with 50 μl MEM, in 96-well plates (100 μl cell suspension per disc). Discs were rinsed twice in PBS before transfer to the pre-equilibrated test culture media in a 6-well plate. Each test or control well contained 4 ml of acidified MEM and five replicate ivory discs; cultures were incubated for 26 h in a humidified atmosphere of 5% CO_2–95% air. At the end of the experiment, medium pH and P_{CO_2} were measured using a blood gas analyser (Radiometer, Copenhagen, Denmark), taking careful precautions to prevent CO_2 loss. Ivory discs were removed and fixed in 2% glutaraldehyde, then stained for tartrate-resistant acid phosphatase (TRAP) using Sigma kit 387-A. The numbers of TRAP-positive multinucleated osteoclasts (two or more nuclei), and the number of stromal cells were assessed 'blind' using transmitted light microscopy. Discrete resorption pits were counted 'blind' by scanning the entire surface of each disc using reflected light microscopy after restaining in 1% Toluidine Blue in 1% sodium borate for 2 min.

Osteoclast formation assay

Long bones of 8-week-old mice ($n = 2$), killed by cervical dislocation, were fragmented in 5 ml unmodified MEM, followed by vortexing for 1 min. The resulting cell suspension was allowed to sediment for 24 h onto sterile 5 mm diameter ivory discs, pre-wetted with 50 μl MEM, in 96-well plates (100 μl cell suspension per disc). Ivory discs were then removed and placed in test or control medium in a 6-well plate. Each test or control well contained 4 ml of non-acidified MEM with 10 nM $1,25(OH)_2D_3$ and 10 nM dexamethasone, and six replicate ivory discs; cultures were incubated in a humidified atmosphere of 5% CO_2–95% air, with medium changes every 2 days. MEM was not acidified for these experiments because low pH conditions are inhibitory for osteoclast formation (Morrison & Arnett, 1998*b*). Medium pH and P_{CO_2} were monitored during and at the end of experiments using a blood gas analyser, as above. After 10 days incubation, the discs were fixed in 2% glutaraldehyde, and stained for tartrate-resistant acid phosphatase (TRAP) using Sigma kit 387-A. A control group of ivory discs was also removed, fixed and stained after 3 days incubation to check for the presence of any mature osteoclasts that might have been released during the initial cell preparation. The total area occupied by TRAP-positive multinucleated osteoclasts and resorption pits was assessed 'blind' by transmitted and reflected light microscopy, via a colour video image output, using standard 'dot count' morphometry. Area measurements, rather than discrete cell and pit counts, were necessary because this assay system requires high cell densities to function, resulting in large, semi-contiguous groups of TRAP-positive osteoclasts associated with extensive, often conjoined areas of resorption.

Statistics

Statistical comparisons were made by one-way analysis of variance of log-transformed data, using Bonferroni's correction for multiple comparisons; representative data are presented as means ± S.E.M. for five or six replicates. Significance was assumed at $P < 0·05$. Each experiment was repeated 3 or 4 times.

RESULTS

Effect of ATP on mature rat osteoclasts

Extracellular ATP exerted a reproducible, biphasic effect on resorption pit formation by rat osteoclasts in low density, acid-activated 26 h cultures (pH 6·94 ± 0·016). At low concentrations (0·2 and 2 μM ATP) striking stimulations were observed, with up to a 3·5-fold increase in pit number (Fig. 1).

At higher concentrations, resorption was progressively reduced (Fig. 1); this was largely due to a selective cytotoxic effect of ATP (associated with cell vacuolation) on osteoclasts. However, numbers of mononuclear cells (i.e. cells of osteoblastic/fibroblastic morphology) were unaltered by ATP: treatment with 0, 0·2, 2, 20, 200 and 2000 μM ATP resulted in 1230 ± 95, 1323 ± 213, 1200 ± 114, 1228 ± 167, 1390 ± 151 and 1001 ± 143 mononuclear cells per ivory disc. Time lapse video microscopy, using methods similar to those described by Arnett *et al.* (1996), revealed that 60–90 min following addition of 2 mM ATP to freshly isolated mature rat osteoclasts, the cell abruptly retracts and dies in a manner reminiscent of apoptosis (data not shown). Adenosine, over a similar concentration range, was without significant effect on either resorption pit formation or osteoclast numbers except at the highest dose (2 mM), where some cytotoxicity was observed (data not shown).

The interaction between the effects of pH and extracellular ATP was investigated using rat osteoclast cultures incubated for 26 h with 0 or 2 μM ATP in either control (non-acidified)

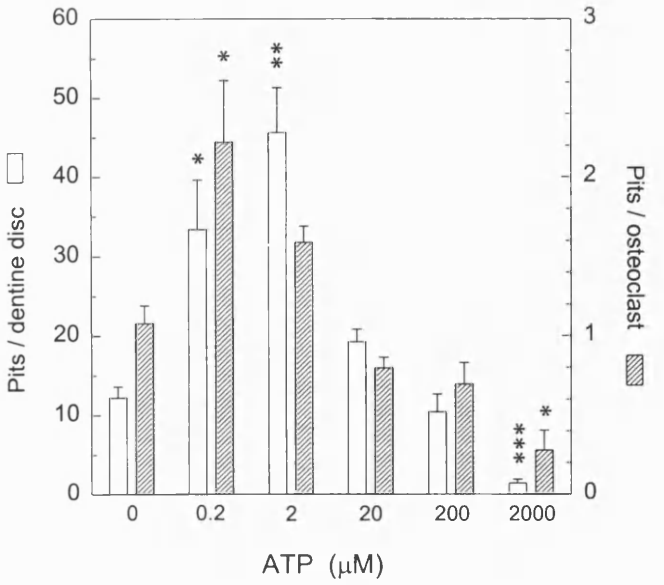

Figure 1. Biphasic effect of ATP on resorption pit formation by rat osteoclasts

Osteoclasts were cultured on 5 mm dentine discs in acidified medium (pH 6·937 ± 0·016) for 26 h. The stimulatory effect of ATP was evident at lower concentrations and a selective cytotoxic effect at the highest concentration. Values are means ± S.E.M. ($n = 5$); * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$ with respect to control.

or acidified MEM (Fig. 2). In the absence of ATP, acidification (pH reduction from 7·166 to 6·945) elicited a modest, 3-fold stimulation of resorption pit formation; and in non-acidified medium ATP caused a 2·5-fold stimulation of pit formation. However, when osteoclasts were cultured with 2 μM ATP in acidified MEM, resorption was stimulated 17-fold compared with control. Thus, the stimulatory effect of ATP was enhanced greatly at low pH and vice versa.

To study further the possible dependence of the acid activation of resorption on low levels of extracellular ATP, osteoclasts were cultured for 26 h with apyrase, which hydrolyses extracellular ATP to AMP. In this experiment, acidification resulted in a 3·5-fold stimulation of pit formation; apyrase inhibited acid-activated resorption, with a near-maximal effect at 0·1 units ml^{-1} (Fig. 3). There was no evidence that apyrase was cytotoxic at any dose. A similar, non-toxic, inhibitory effect was also observed reproducibly when acid-activated osteoclasts were cultured with suramin, an antagonist of P2 receptors (resorption levels at pH 7·216, pH 7·013 and pH 7·009 + 10 μM suramin were 4·40 ± 0·67, 15·40 ± 3·39 and 6·20 ± 1·39* pits per dentine disc, respectively; * $P < 0{\cdot}05$ *vs.* pH 7·013 group; osteoclast and mononuclear cell numbers were unaltered by any treatment).

Effect of ATP on osteoclast formation in mouse marrow cultures

In 10 day mouse marrow cultures, extracellular ATP at low concentrations stimulated the formation of TRAP-positive osteoclasts and resorption pits reproducibly. As was the case in the mature rat osteoclast assay, peak effects were observed in the range 0·2–2 μM ATP. In the presence of

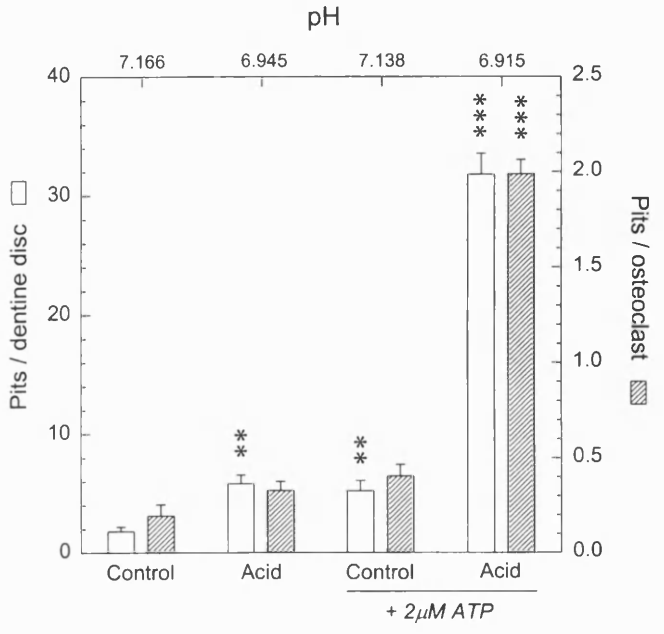

Figure 2. Comparison of the effects of ATP on resorption pit formation by rat osteoclasts cultured in unmodified medium (Control) or in acidified medium (Acid) for 26 h

The figure shows potentiation of ATP-stimulated resorption at low pH. Values are means ± S.E.M. ($n = 5$); ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$ with respect to control.

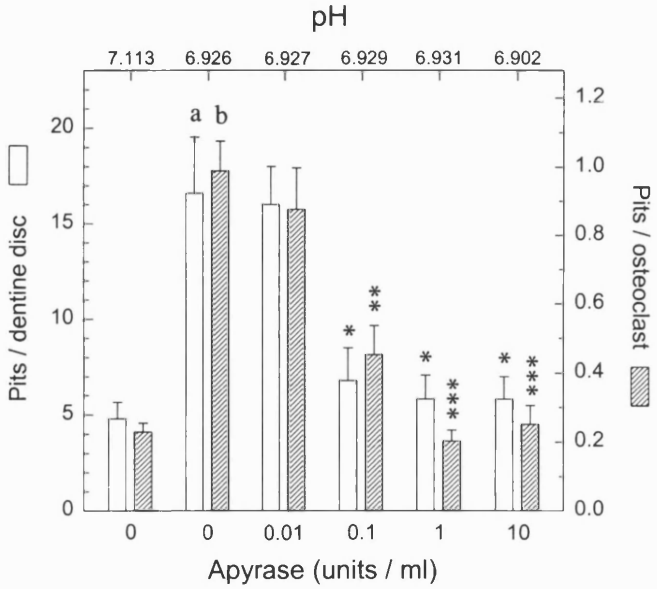

Figure 3. Stimulation of resorption pit formation by rat osteoclasts cultured for 26 h in acidified medium and inhibition of acid-stimulated pit formation by apyrase

Values are means ± S.E.M. ($n = 5$). [a] $P < 0{\cdot}01$; [b] $P < 0{\cdot}001$ with respect to pH 7·113 control value. * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$ with respect to pH 6·926 control value.

2 μM ATP, osteoclast formation was stimulated 3·3-fold and resorption increased 4-fold compared with control. Higher concentrations either inhibited (20 μM) or completely blocked (200 μM) osteoclast formation and resorption (Fig. 4). Adenosine over the same concentration range was without significant effect.

In the control groups that were fixed and stained for TRAP after 3 days incubation, osteoclasts and resorption pits were never observed, indicating that the osteoclasts and resorption pits observed after 10 days in culture resulted entirely from formation of new osteoclasts.

DISCUSSION

The modulation of bone resorption by local factors is a complex process that is still not well understood (Roodman, 1996). Our results indicate that extracellular ATP, which causes striking stimulation of both resorption pit formation and osteoclast recruitment at submicromolar concentrations, must be considered as a potentially important paracrine or autocrine agent for bone.

It is unclear from the present data whether exogenous ATP stimulates resorption pit formation via direct effects on mature osteoclasts, indirect effects on cells such as osteoblasts, or both. Electrophysiological evidence (Yu & Ferrier, 1993, 1994, 1995; Weidema *et al.* 1997) and histochemical evidence (Bowler *et al.* 1995, 1998*a*) exists for the expression of P2X and P2Y receptors on normal or tumour-derived osteoclasts. P2Y receptors are also present on osteoblast-like cells (Bowler *et al.* 1995, 1998*b*). Pure populations of normal mammalian osteoclasts are not routinely available for study and multinucleate osteoclasts constitute only about 1–2% of the cells in the low density rat bone cell cultures used in our experiments. Additionally, our results do not distinguish the mode of action of ATP in increasing osteoclast formation in 10 day marrow cultures. ATP could be acting directly on osteoclast precursor cells, as well as indirectly on the stromal component of marrow

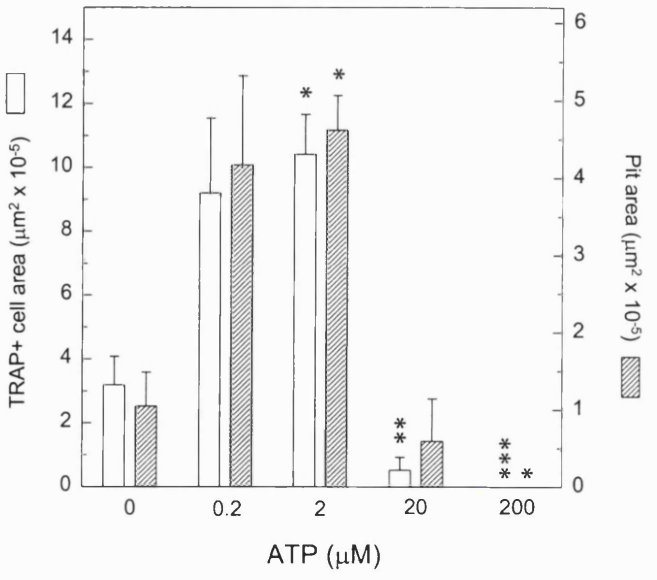

Figure 4. Biphasic effect of ATP on osteoclast formation and excavation of resorption pits in mouse marrow cultures maintained for 10 days on 5 mm dentine discs in unmodified medium (pH 7·212 ± 0·010)

Values are means ± S.E.M. ($n = 6$); * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$ with respect to control.

cultures; it is possible also that the stimulatory effects of ATP in these long term cultures were due, in some degree, to enhanced osteoclast survival.

The rapid, selective cytotoxic action of ATP at millimolar concentrations on osteoclasts is in agreement with the findings of Nijweide *et al.* (1995); this effect may be mediated by the P2Z ($P2X_7$) receptor for ATP^{4-}, activation of which results in the formation of cytolytic pores (reviewed by Burnstock, 1997). Interestingly, we found that resorption pit formation by osteoclasts derived from late chick embryos was not enhanced by ATP, although the high dose inhibitory effect was still evident (M. S. Morrison & T. R. Arnett, unpublished observations). Chick osteoclasts also differ from mammalian osteoclasts in that they resorb bone more avidly and fail to show an inhibitory response to calcitonin (Arnett & Dempster, 1987). The lack of effect of adenosine suggests that P1 receptors are not involved in the modulation of osteoclastic function, in line with the earlier findings of Yu & Ferrier (1993), who demonstrated that adenosine exerted no significant effect on intracellular Ca^{2+} in osteoclasts.

Previous work has demonstrated the remarkable sensitivity of rat osteoclasts to stimulation by extracellular protons (Arnett & Dempster, 1986; Arnett & Spowage, 1996). The pH response occurs within a relatively limited range, such that shifts in the range pH 7·25–7·00 act as a reversible 'off–on switch' for resorptive activity, with the major change associated with a pH difference of as little as 0·1 unit (Arnett & Spowage, 1996). Embryonic chick osteoclasts exhibit similar responses (Arnett & Dempster, 1987), as do mouse calvarial cultures (Meghji *et al.* 1996) and osteoclasts formed from mouse marrow cultures (Morrison & Arnett, 1998*b*). An important feature of H^+-stimulated resorption is that it does not exhibit desensitization: indeed, the magnitude of the response of mature rat osteoclasts appears to increase with time in culture, such that after 7 days, pH drops of ~0·1 unit are associated with 15- to 20-fold enhancements of pit formation (M. S. Morrison & T. R. Arnett, in preparation). The present work demonstrates that there is a powerful synergy between the stimulatory effects of low-dose ATP and protons on the resorptive activity of mature rat osteoclasts, i.e. the full sensitivity of osteoclasts to activation by ATP is only evident at low pH (~6·9–7·0), and vice versa. In the case of the osteoclast formation experiments, ATP appeared to enhance resorptive efficiency only slightly; this was probably a consequence of the non-acidified medium (pH ~7·2) used to maximize osteoclast recruitment in this assay (Morrison & Arnett, 1998*b*).

The effect of apyrase (which hydrolyses ATP to AMP) on acid-stimulated pit formation was to inhibit resorption without affecting cell viability or morphology, suggesting that the 'pH effect' may be dependent on trace levels of either free or bound extracellular ATP. The concentrations of ATP in question may be quite low, given the powerful stimulatory effect seen in the presence of 200 nM ATP. There are two main possibilities for the origin of extracellular ATP in osteoclast-containing bone cell cultures. First, ATP (intracellular concentration ~2 mM) could be released from cells that were damaged during the relatively vigorous procedures used to isolate osteoclasts from bones. Second, ATP may be exported from intact cells via transport proteins; for example, osteoclasts have been reported to express a novel member of the ATP binding cassette superfamily (Wagstaff *et al.* 1998). There is also recent evidence for constitutive release of ATP by osteoblast-like cells, a process that appears to be stimulated by fluid shear forces (Bowler *et al.* 1998*c*). Interestingly, McSheehy & Chambers (1986) reported that osteoblasts released an unknown soluble factor of relatively low molecular weight that stimulated osteoclasts.

Striking recent experiments have shown that extracellular acidification is required for the $P2X_2$ receptor subtype to show its full sensitivity to extracellular ATP (King *et al.* 1996; Wildman *et al.* 1997). Indeed, the pH-activation profile of the recombinant $P2X_2$ receptor expressed in *Xenopus* is remarkably similar to that for resorption pit formation by rat osteoclasts (Arnett & Spowage, 1996). No other ATP receptors of the P2X or P2Y families are known to exhibit such pH sensitivity. Although our data do not distinguish conclusively between P2 receptor type(s) which may be mediating the stimulatory effects of low concentrations of ATP on rodent osteoclasts, the apparent lack of desensitization to ATP, suramin antagonism and acid activation are consistent with the possible involvement of the $P2X_2$ receptor subtype. When the recombinant $P2X_2$ receptor is activated by ATP and extracellular H^+, it opens a non-selective cation/proton channel. Opening such a channel in the osteoclast (dorsal/basolateral) cell membrane could increase critically the intracellular availability of H^+ (which is then actively pumped out across the ruffled border), so as to facilitate resorption pit formation (Arnett & King, 1997). Such a mechanism would augment the intracellular proton supply thought to be derived via osteoclastic carbonic anhydrase. Alternatively, the putative 'pH receptor' on osteoclasts may be distinct from ATP receptors.

In addition to increasing resorption, ATP may additionally exert negative effects on bone formation. Recent experiments have shown that ATP, albeit at concentrations somewhat higher than those optimal for stimulation of resorption, inhibits appositional bone formation by cultured primary osteoblasts (Jones *et al.* 1997). ATP also induces cartilage resorption *in vitro* (Leong *et al.* 1994). In inflamed tissue, ATP may be released from damaged cells, mast cells or platelets (King *et al.* 1996); inflammation is also associated with local acidification (Burnstock, 1997), as is the case at sites of fracture and surgery (Brueton *et al.*, 1993). It is conceivable that ATP could also play in role in tumour osteolysis, given the propensity of transformed cells to release ATP (Burnstock, 1997) and cause local acidification. The present findings suggest a new mechanism by which localized bone destruction could occur when ATP is released in acidified tissues.

ARNETT, T. R. & DEMPSTER, D. W. (1986). Effect of pH on bone resorption by rat osteoclasts *in vitro*. *Endocrinology* **119**, 119–124.

ARNETT, T. R. & DEMPSTER, D. W. (1987). A comparative study of disaggregated chick and rat osteoclasts *in vitro*: effects of calcitonin and prostaglandins. *Endocrinology* **120**, 602–608.

ARNETT, T. R. & KING, B. F. (1997). ATP as an osteoclast regulator? (Perspectives in Physiology). *Journal of Physiology* **503**, 236.

ARNETT, T. R., LINDSAY, R., KILB, J. M., MOONGA, B. S., SPOWAGE, M. & DEMPSTER, D. W. (1996). Selective toxic effects of tamoxifen on osteoclasts: comparison with the effects of oestrogen. *Journal of Endocrinology* **149**, 503–508.

ARNETT, T. R. & SPOWAGE, M. (1996). Modulation of the resorptive activity of rat osteoclasts by small changes in extracellular pH near the physiological range. *Bone* **18**, 277–279.

BOWLER, W. B., BIRCH, M. A., GALLAGHER, J. A. & BILBE, G. (1995). Identification and cloning of human P2U purinoceptor present in osteoclastoma, bone and osteoblasts. *Journal of Bone and Mineral Research* **10**, 1137–1145.

BOWLER, W. B., LITTLEWOOD-EVANS, A., BILBE, G., GALLAGHER, J. A. & DIXON, C. J. (1998*a*). $P2Y_2$ receptors are expressed by human osteoclasts of giant cell tumor but do not mediate ATP-induced bone resorption. *Bone* **22**, 195–200.

BOWLER, W. B., MAIER, R., BILBE, G. & GALLAGHER, J. A. (1998*b*). Osteoblasts express multiple G-protein coupled P2Y receptor subtypes. *Bone* **22**, 2S (abstract).

BOWLER, W. B., TATTERSALL, J. A., HUSSEIN, R., DIXON, C. J., COBBOLD, P. H. & GALLAGHER, J. A. (1998*c*). Release of ATP by osteoblasts: modulation by fluid shear forces. *Bone* **22**, 3S (abstract).

BRUETON, R. N., REVELL, W. J. & BROOKES, M. (1993). Haemodynamic changes during bone healing in a stable fracture. In *Bone and Bone Marrow Circulation in Normal and Pathological Conditions*, ed. SCHOUTENS, A., ARLET, J., GARDINIERS, J. W. M. & HUGHES, S. P. F., pp. 121–128. Plenum, New York.

BURNSTOCK, G. (1997). The past, present and future of purine nucleotides as signalling molecules. *Neuropharmacology* **36**, 1127–1139.

BURNSTOCK, G. & KING, B. F. (1996). Numbering of cloned P2 purinoceptors. *Drug Development Research* **38**, 67–71.

JONES, S. J., GRAY, C., BOYDE, A. & BURNSTOCK, G. (1997). Purinergic transmitters inhibit bone formation by cultured osteoblasts. *Bone* **21**, 393–399.

KING, B. F., ZIGASHINA, L. E., PINTOR, J. & BURNSTOCK, G. (1996). Full sensitivity of P2X2 purinoceptor to ATP revealed by changing extracellular pH. *British Journal of Pharmacology* **117**, 1371–1373.

LEONG, W. S., RUSSELL, R. G. G. & CASWELL, A. M. (1994). Stimulation of cartilage resorption by extracellular ATP acting at P2-purinoceptors. *Biochimica et Biophysica Acta* **1201**, 298–304.

MCSHEEHY, P. M. J. & CHAMBERS, T. J. (1986). Osteoblast-like cells in the presence of parathyroid hormone release a soluble factor that stimulates osteoclastic bone resorption. *Endocrinology* **119**, 1654–1659.

MEGHJI, S., HENDERSON, B., MORRISON, M. S. & ARNETT, T. R. (1996). Ca^{2+} release from cultured mouse calvaria is very sensitive to ambient pH. *Journal of Bone and Mineral Research* **11**, 1824 (abstract).

MORRISON, M. S. & ARNETT, T. R. (1997). ATP stimulates resorption pit formation by rat osteoclasts. *Journal of Bone and Mineral Research* **12**, 1520 (abstract).

MORRISON, M. S. & ARNETT, T. R. (1998*a*). ATP stimulates osteoclast formation. *Journal of Bone and Mineral Research* **13**, 525 (abstract).

MORRISON, M. S. & ARNETT, T. R. (1998*b*). pH effects on osteoclast formation and activation. *Bone* **22**, 30S (abstract).

MURRILLS, R. M., DEMPSTER, D. W. & ARNETT, T. R. (1998). Isolation and culture of osteoclasts and osteoclast resorption assays. In *Methods in Bone Biology*, ed. ARNETT, T. R. & HENDERSON, B., pp. 64–105. Chapman and Hall, London.

NIJWEIDE, P. J., MODDERMAN, W. E. & HAGENAARS, C. E. (1995). Extracellular adenosine triphosphate: a shock to hemopoietic cells. *Clinical Orthopaedics and Related Research* **313**, 92–102.

ROODMAN, G. D. (1996). Advances in bone biology: the osteoclast. *Endocrine Reviews* **17**, 308–332.

WAGSTAFF, S. C., BIRCH, M. A., BOWLER, W. B., WLODARSKI, B. W., DILLON, J. P., ROBERTS, E. R., CARRON, J. A., GALLAGHER, J. A. & BILBE, G. (1998). Osteoclasts express a novel member of the ATP binding cassette superfamily. *Bone* **22**, 35S (abstract).

WEIDEMA, A. F., BARBERA, J., DIXON, S. J. & SIMS, S. M. (1997). Extracellular nucleotides activate non-selective cation and Ca^{2+} dependent K^+ channels in rat osteoclasts. *Journal of Physiology* **503**, 303–315.

WILDMAN, S. S., KING, B. F. & BURNSTOCK, G. (1997). Potentiation of ATP-responses at a recombinant $P2X_2$ receptor by neurotransmitters and related substances. *British Journal of Pharmacology* **120**, 221–224.

YU, H. & FERRIER, J. (1993). ATP induces an intracellular calcium pulse in osteoclasts. *Biochemical and Biophysical Research Communications* **191**, 357–363.

YU, H. & FERRIER, J. (1994). Mechanisms of ATP-induced Ca^{2+} signaling in osteoclasts. *Cellular Signaling* **6**, 905–914.

YU, H. & FERRIER, J. (1995). Osteoclast ATP receptor activation leads to a transient decrease in extracellular pH. *Journal of Cell Science* **108**, 3051–3058.

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