Purinergic signalling in bone cells

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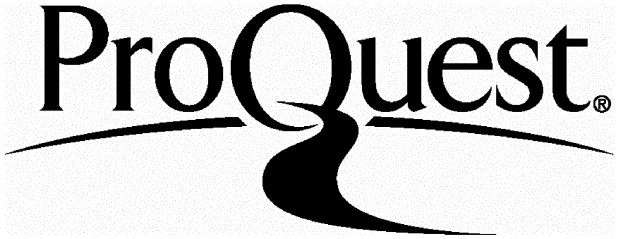

ABSTRACT

There is increasing evidence that ATP and other extracellular nucleotides, signalling through P2 receptors, play an important role in bone remodelling. I used immunohistochemistry and *in situ* hybridisation to study the expression of P2 receptors in rat bone sections and on cultured bone cells: osteoclasts, the multinucleated cells responsible for bone resorption, osteoblasts, the bone-forming cells, and chondrocytes were all shown to express a number of P2 receptors, both of the ionotropic P2X family and the metabotropic P2Y family.

ATP has previously been shown to be a potent stimulator of bone resorption, however, it was not known which receptor subtypes mediate this stimulatory effect. Three different models were used to study the effects of a wider range of P2 receptor agonists on osteoclast recruitment and resorption: the 26 hour disaggregated rat osteoclast assay, 72 hour whole organ mouse calvaria cultures, and 10 day mouse marrow cultures. My main discovery is that extracellular ADP, the first degradation product of ATP, is a powerful stimulator, at nanomolar concentrations, of osteoclast activation and recruitment. Furthermore, evidence is provided, using subtype-selective agonists and antagonists, that these ADP effects are probably mediated via the $P2Y_1$ receptor. It is suggested that *in vivo*, ADP could be involved in the mediation of inflammatory bone loss.

The effects of nucleotides on osteoblast function were studied using the bone nodule assay by culturing primary rat calvarial osteoblasts for 3 weeks and by analysing the formation of mineralised bone nodules. Both ATP and UTP, a $P2Y_2$ and $P2Y_4$ receptor agonist, significantly inhibited bone nodule number at concentrations $\geq$ 1-10 μM, whereas ADP showed no effect. Taken together, these results suggest subtype-specific roles for P2 receptors present in bone cells: ADP is a powerful stimulator of bone resorption signalling via the $P2Y_1$ receptor, whereas UTP, signalling via the $P2Y_2$ receptor, could play a role as an inhibitor of bone formation.

In parallel, further functional experiments were carried out to assess the interactions of low pH and of osteolytic stimuli, and to investigate whether human osteoclasts are as acid-sensitive as rodent and avian osteoclasts. Interestingly, both nucleotides (ADP and ATP) and the cytokine RANKL require low pH to show their full stimulatory effect on bone resorption, suggesting that low pH is a key requirement for resorption to occur. In conclusion, this is one of the first studies linking specific P2 receptor subtypes to key functional actions of extracellular nucleotides on bone.

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PREFACE

In this preface, I would like to give a short outline of the different chapters in this thesis. The work presented here has focused mainly on purinergic signalling in osteoblasts and osteoclasts, in addition to investigating some new aspects of the effects of acidosis on bone structure and function.

Extracellular nucleotides are now recognised as important signalling molecules mediating a wide range of functions. They act via two types of receptors: P2X receptors, which are ligand-gated ion channels, and P2Y receptors, which are G protein-coupled receptors raising cytosolic free calcium ($[Ca^{2+}]_i$).

My project was based on a large number of pharmacological and electrophysiological studies suggesting the presence of P2 receptors on bone cells, and on relatively few functional studies reporting that adenosine 5'-triphosphate (ATP) had an effect on both formation and activation of osteoclasts, the multinucleated cells responsible for bone resorption, and also on bone formation by osteoblasts. However, ATP is a potent agonist at most P2 receptor subtypes, and it was neither known which P2 receptor subtypes were present on bone cells, nor which of these receptors might be mediating effects on osteoclast and osteoblast function.

Chapter 1 provides a general introduction: (1) to the bone field, introducing the composition of bone, the three major types of bone cells (osteoblasts, osteocytes, osteoclasts), the composition and cells of cartilage, which factors control bone remodelling, and a brief overview on bone diseases; (2) to the rapidly expanding field of purinergic signalling, introducing the history, classification and characterisation of receptor subtypes, and the source, breakdown and physiological roles of extracellular nucleotides; (3) to the current knowledge about P2 receptors in bone, although more detailed descriptions of P2 receptors on bone cells are given in the introduction to each experimental chapter.

In the first experimental chapter (Chapter 2), the expression of P2 receptors was studied on frozen sections of rat bone, and on cultured rat osteoclasts and osteoblasts, using immunohistochemistry and *in situ* hybridisation. Evidence is presented that osteoblasts, osteoclasts and chondrocytes express a wide range of P2 receptors, both of the P2X and P2Y families.

The second experimental chapter (Chapter 3) explored which receptor subtype(s) might be involved in mediating the osteolytic effects of ATP on bone, using P2 receptor subtype- selective agonists and antagonists in three different assays: disaggregated rat osteoclast resorption assays, whole organ cultures of mouse calvariae, and mouse marrow cultures.

Chapter 4, the third experimental chapter, explored which receptors might play a role in bone formation, again using a range of P2 receptor agonists, and an *in vitro* model of bone formation (bone nodule assay), in addition to two different assays to assess changes in proliferation of osteoblastic cells after nucleotide application.

Chapter 5, the fourth experimental chapter, investigated a few aspects of acidosis and bone, since extracellular acidification has long been known to have a powerful stimulatory effects on osteoclasts. However, it is still unclear by which mechanism these deleterious effects are mediated. First, it was investigated whether human osteoclasts are as acid-sensitive as rat, rabbit and chick osteoclasts, then the interaction between RANKL and low pH was studied, and I looked for the expression of acid-sensing ion channels on osteoclasts.

A detailed discussion of results is provided at the end of each experimental chapters. In the General Discussion (Chapter 6), the major findings of this thesis will be summarised, the results of each chapter discussed in a broader context, and I speculate on the potential physiological /pathophysiological relevance of my findings and propose future directions of research.

During my first year, I performed collaborative work with another PhD student at the Autonomic Neuroscience Institute, Mina Ryten, on the sequential expression of three P2X receptor subtypes ($P2X_2$, $P2X_5$, $P2X_6$) in developing rat skeletal muscle. This work is now published (see Appendix II), but has not been included as an experimental chapter, since it does not relate to the bone field and the other chapters in this thesis.

CHAPTER 1

GENERAL INTRODUCTION

BONE

Bone composition

Bone is a highly specialised form of connective tissue that, together with cartilage, makes up the skeletal system. It is composed of: (1) inorganic mineral salts deposited within an organic collagen matrix; (2) thin layers of non-mineralised collagen matrix (called osteoid); and (3) three major cell types: osteoclasts, osteoblasts and osteocytes.

The skeleton's major functions are to provide structural support for the body, to protect vital internal organs and house the bone marrow, and to act as the main reservoir of mineral salts for the body, thus playing a crucial role in calcium (Ca^{2+}) and phosphate homeostasis. 99% of the body's calcium and 85-90% of the body's phosphorus are stored in the skeleton.

The organic matrix makes up about 30% of the total skeletal mass, and consists mainly of type I collagen embedded in a glycosaminglycan gel containing non-collagenous proteins such as osteocalcin, osteonectin, osteopontin and bone sialoprotein. The organic component also contains traces of growth factors and cytokines that may have an important local regulatory role in bone remodelling. Deposition of mineral salts into the organic matrix gives bone its characteristic rigidity and functional strength. The major inorganic components are hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) crystals. Sodium (Na^+) and small amounts of magnesium and carbonate are also present in bone.

Most bones have a basic architecture of an outer layer of compact (or cortical) bone surrounding an inner trabecular (or spongy) zone, with the spaces in between the

trabeculae often occupied by bone marrow. Compact bone, composing about 80% of the skeleton and mainly found in the shafts of long bones and surfaces of flat bones, is much denser and less metabolically active than spongy bone; nutrients are provided via Haversian canals containing blood vessels, lymphatic tissue and nerves. Mineralised matrix is arranged in concentric layers around each canal and forms cylinders called osteons or Haversian systems. In contrast, trabecular bone is found mainly at the ends of long bones and in the inner parts of flat bones. The potential for metabolic activity is much higher, since more bone surface is freely exposed; nutrients can diffuse from the extracellular fluid into the trabeculae.

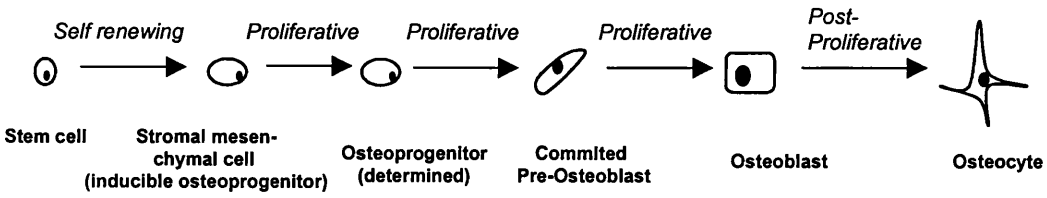
There are three major cell types that maintain the bone structure: bone-forming osteoblasts, bone-resorbing osteoclasts and osteocytes.

Osteoblasts

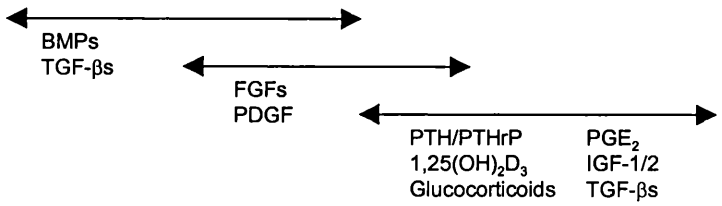
Origin and differentiation

Osteoblasts are mononuclear cells of mesenchymal origin responsible for bone formation. They resemble fibroblasts in their morphological appearance, but are unique in that they are able to form a mineralised collagenous extracellular matrix. The periosteum and bone marrow are important sources of mesenchymal osteoprogenitor cells; the stromal bedding of bone marrow gives additionally rise to other mesenchymal cells such as fibroblasts, chondrocytes, myoblasts and adipocytes. A summary of the developmental stages of the osteoblastic phenotype and characteristic features of each stage is shown in Figure 1.1 (**Fig. 1.1**). The progression of osteoblast maturation requires the sequential activation and suppression of genes encoding phenotype-specific and regulatory proteins, as indicated in Figure 1.1 C.

A: Developmental progression



B: Factors contributing to phenotype



C: Temporal expression levels of phenotype-related genes

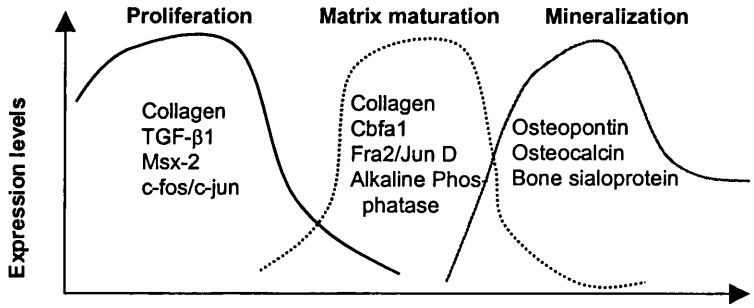


Figure 1.1

Regulation of osteoblast growth and differentiation.

(**A**) illustrates the morphological features of differentiating osteoblasts at each stage from stem cell to osteocyte. (**B**) lists the factors that promote differentiation of the precursor cell populations. (**C**) shows temporal expression of cell growth and osteoblast phenotype-related genes during an *in vi*tro bone formation culture. Adapted from Lian *et al.* (1999).

Interestingly, only very few osteoblast-specific genes, that do not exist in other cell types, have been identified to date: one example is osteocalcin, a vitamin K-dependent protein secreted exclusively by osteoblasts. The heterodimeric transcription factor 'core binding factor a1' (Cbfa1), also called Runx2, was first thought to be specific for osteoblasts, but it has now also been identified as a hypertrophic chondrocyte differentiation factor (Takeda *et al.*, 2001). However, this role is independent from its role as osteoblast an differentiation factor (Ducy *et al.*, 1997; Otto *et al.*, 1997). Cbfa1 is expressed in all mesenchymal condensations before osteoblast differentiation has been initiated. Later in development, Cbfa1 expression increases in cells of the osteoblast lineage. Study of Cbfa1-deficient mice revealed its crucial role in skeletogenesis: these mice develop a normally patterned skeleton that is made exclusively of cartilage because osteoblast differentiation never occurs (Otto *et al.*, 1997).

Additionally, Cbfa1-deficient mice lack osteoclasts since, as discussed later, osteoclastogenesis requires the presence of osteoblasts. Overexpression of Cbfa1 leads to ectopic endochondral bone formation. Cbfa1 is involved in the regulation of several osteoblastic genes such as osteocalcin, osteopontin and type I collagen, and many growth factors controlling the osteoblast differentiation pathway seem to interact with Cbfa1 (Ducy *et al.*, 1997). A recent study reported that Cbfa1 can also regulate the expression, and thus secretion, of the osteoclast-inhibiting factor osteoprotegerin (OPG) in osteoblasts, favouring a role for Cbfa1 in inhibiting bone resorption, and providing a molecular link between bone formation and bone resorption (Thirunavukkarasu *et al.*, 2000). The transcription factors acting upstream of Cbfa1 to control its expression remain to be identified; likely candidates include the homeobox genes *Msx2* and *Bapx1* (Satokata *et al.*, 2000). As shown in Fig. 1.1 C, other key regulatory factors necessary for expression of osteoblast phenotypic genes are transcription factors of the activator protein-1 (AP-1) complex, including c-fos and c-jun, proteins encoded by the immediate early genes *c-fos* and *c-jun*. *C-fos* is expressed in osteoprogenitor cells and periosteal tissues, but not in the mature osteoblast. Overexpression results in osteosarcoma development, whereas deletion of *c-fos* leads to osteopetrosis (see osteoclast chapter).

Additionally, mice deficient in the non-receptor tyrosine kinase c-Abl are osteoporotic due to a defect in early osteoblast differentiation, adding *c-Abl* to the genes known to be important in osteoblast maturation (Li *et al.*, 2000a). Cadherin-mediated cell-cell adhesion has also been shown to be essential for the commitment of cells to the osteoblast lineage, as well as for subsequent matrix mineralisation and for osteoblast survival (Hunter *et al.*, 2001).

Committed pre-osteoblasts are recognisable in bone by their close proximity to surface osteoblasts, and by histochemically detectable levels of alkaline phosphatase, one of the earliest markers of the osteoblast phenotype. In their final differentiation stage, osteoblasts are defined by their biosynthesis, secretion and organisation of bone extracellular matrix. Active, osteoid-secreting osteoblasts are large cells of cuboidal shape with a prominent protein synthesising apparatus, whereas quiescent osteoblasts (bone lining cells) have a flat morphology.

Bone deposition

The active osteoblast secretes type I collagen and non-collagenous proteins as osteoid towards the mineralising front of the tissue, with an osteoid width normally in the range of 10 μm. To support mineral deposition, collagen fibres must mature by forming intra- and intermolecular, bone-tissue specific, covalent cross-links. Urine-measurements of collagen cross-links are used clinically to assess the degree of bone resorption (Apone *et al.*, 1997). Osteoblasts synthesise three classes of non-collagenous proteins: proteoglycans, glycoproteins, some of them containing an RGD (Arg-Gly-Asn)-sequence, and γ-carboxylated glutamate (gla) proteins. The major bone glycoprotein, alkaline phosphatase, remains bound to the osteoblast, but can be cleaved off and found within the mineralised matrix. Alkaline phosphatase probably plays a role in the mineralisation process by hydrolysing phosphate esters, and thus providing a source of phosphate, or in the removal of phosphate-containing inhibitors of apatite growth. Osteonectin, a second glycoprotein, can also be induced in non-skeletal tissues, both transiently in tissues undergoing rapid proliferation or remodelling, and constitutively in epithelial cells and

platelets. The exact function of osteonectin in bone remodelling remains to be determined, but osteonectin-deficient mice develop osteopenia with decreased bone formation (Delany *et al.*, 2000).

RGD-glycoproteins contain the cell-attachment consensus sequence RGD that binds to the integrin class of cell-surface molecules. Besides collagen I, several RGD-containing cell-attachment proteins are synthesised in bone, amongst them thrombospondin, fibronectin, vitronectin, fibrillin, osteopontin and bone sialoprotein. However, only bone sialoprotein is specific to bone and appears to be tightly correlated with the mineralisation process. Osteopontin mediates both cell-cell interactions and cell-matrix interactions. It binds strongly to hydroxyapatite, possibly explaining its abundance in bone matrix and suggesting a role in matrix mineralisation. Not only is it produced by osteoblasts, but also expressed at high levels in osteoclasts. *In vitro* experiments have shown that osteopontin can promote attachment of cells and stimulate signalling events in osteoclasts. Osteopontin-deficient mice revealed no major bone phenotype, but recent studies suggest that it might be required under circumstances of accelerated, pathological bone loss, induced by conditions such as ovariectomy and mechanical stress (Yoshitake *et al.*, 1999; Asou *et al.*, 2001; Ishijima *et al.*, 2001).

The gla-containing proteins matrix-gla-protein and osteocalcin, also called bone-gla-protein, are posttranslationally carboxylated in a vitamin-K dependent manner, changing glutamine to glutamate residues, which have been implied in improved binding to matrix Ca^{2+}. Matrix-gla-protein-deficient mice develop calcification in extraskeletal sites such as the aorta, and osteocalcin-deficient mice have increased bone mineral density compared to wild-types, but the proteins' exact functions in the mineralisation process remain unclear (Ducy *et al.*, 1996; Luo *et al.*, 1997a). Serum osteocalcin measurements are commonly used as markers for bone formation/turnover and high levels of serum undercarboxylated osteocalcin have been correlated with increased risk of hip fracture (Szulc *et al.*, 1993).

The mechanism by which mineral deposition into the collagenous matrix is initialised is still debated. Bone mineral is initially deposited at discrete sites and as bone

matures, the initially laid down small hydroxyapatite crystals become larger and contain fewer impurities such as carbonate and magnesium. After the formation of the first stable crystal ('critical nucleus'), growth is due to both addition of ions to crystals and crystal aggregation, but the final size of bone crystals is still very small (maximally up to ~200Å in dimension) compared to large hydroxyapatite crystals occurring geologically. Membrane-bound extracellular bodies, known as extracellular matrix vesicles, are released from osteoblasts and chondrocytes and may play a role in accumulating Ca^{2+} and phosphate ions, thus facilitating initial mineral deposition, but their exact role remains unclear. Several promoters (nucleators) of mineralisation have been identified based on solution studies, including bone sialoprotein (Hunter and Goldberg, 1993). Additionally, some enzymes that regulate phosphoprotein phosphorylation and dephosphorylation have also been associated with the mineralisation process. Alkaline phosphatase increases local phosphate concentrations by hydrolysing phosphate esters, and cells that lack alkaline phosphatase do not mineralise in culture systems (Lian *et al.*, 1999).

Some osteoblasts remain behind the advancing mineralising bone surface and become trapped in lacunae, where they are called osteocytes (see below).

Control of osteoblastic function

Osteoblasts and osteocytes have receptors for some key regulators of bone remodelling, including cytokines, parathyroid hormone (PTH), 1,25-dihydroxyvitaminD_3 (1,25-$(OH)_2D_3$), estrogens and a number of growth factors, as listed in Figure 1.1 B. For a detailed review of individual growth factor effects, see Lian *et al.* (1999), and the chapter on bone remodelling. A surprising recent finding was that leptin is an important regulator of differentiated osteoblasts. Leptin, generally known as a starvation and adiposity signal, is synthesised by adipocytes and binds to receptors primarily located in the hypothalamus. Mice deficient in leptin, or its receptor, are not only obese, but have significantly higher bone mass than wild-type mice. This was unexpected, because the absence of leptin causes sterility and thus hypogonadism which, through estrogen deficiency, should normally accelerate bone loss. Interestingly, leptin does not directly

target osteoblasts, but studies rather suggest that inhibition of bone formation through leptin involves a central, probably hypothalamic component, and provides an explanation for the protection that obesity gives against osteoporosis (Ducy *et al.*, 2000).

Another group of newly identified bone formation-stimulators are statins, commonly prescribed drugs for lowering serum cholesterol levels. Their anabolic effect on bone was associated with increased expression of bone morphogenetic protein-2 (BMP-2) gene in bone cells and might have therapeutic applications for osteoporosis treatment (Mundy *et al.*, 1999).

Members of the transforming growth factor-β (TGF-β) superfamily, including TGF-β itself and BMPs, play important roles in osteoblast differentiation.

Osteocytes

Osteocytes are the most numerous cells in bone, yet their exact function is still debated. They are able to communicate with each other and with cells on the bone surface via gap junction-coupled cytoplasmic processes extending through canaliculi in the bone matrix. Osteocyte function has been difficult to study because they are embedded in mineralised tissue and difficult to obtain in reasonable numbers and purity. However, recent studies suggested that osteocytes function as mechanosensors in the early stage of bone remodelling. Strain-derived flow of interstitial fluid through canaliculi seems to mechanically activate osteocytes, as well as ensuring transport of cell signalling molecules, nutrients and waste products. Osteocytes appear to use signalling pathways such as the generation of nitric oxide and prostaglandins in addition to cell-cell communication via gap junctions. In ageing bone, empty lacunae are observed suggesting that osteocytes may undergo apoptosis. It has been proposed that osteocyte apoptosis may direct the removal of damaged or redundant bone by releasing signals to attract osteoclasts (Noble and Reeve, 2000).

Osteoclasts

Origin and differentiation

Osteoclasts are multinucleated cells formed by the fusion of haematopoietic, mononuclear progenitors of the monocyte/macrophage lineage, and are responsible for mineralised substrates, such as bone and dentine. When migrating to sites of bone resorption from bone marrow or peripheral blood, osteoclast precursors encounter vascular endothelium. Osteoclast precursors have recently been shown to selectively adhere to microvascular endothelium pre-activated by exposure to the pro-resorptive cytokines interleukin-1β (IL-1β) and tumour necrosis factor (TNF)-α, probably through up-regulation of receptors and adhesion molecules (McGowan *et al.*, 2001).

The osteoclast differentiation pathway is summarised in Figure 1.2 (**Fig. 1.2**). The transcription factor PU-1 has been found to regulate the initial stages of myeloid differentiation into the macrophage/osteoclast lineage, and its deletion leads to the earliest developmental form of osteopetrosis yet described due to a lack of osteoclastogenesis (Tondravi *et al.*, 1997). The theory of a common osteoclast/ macrophage precursor is supported by experimental data showing that the absence of *c-fos* results in altered haematopoiesis with arrested osteoclastogenesis, and a lineage shift from osteoclasts to macrophages, thus leading to increased numbers of bone marrow macrophages and osteopetrosis (Wang *et al.*, 1992; Grigoriadis *et al.*, 1994). In addition, human osteoclasts derive largely from CD14-positive monocytes; CD14 is expressed by human macrophages, but not mature osteoclasts, demonstrating again that osteoclast and macrophages share a common monocyte precursor (Massey and Flanagan, 1999).

As first noted in 1990, osteoclasts can not only derive from immature monocyte/macrophage progenitors, but also from mature tissue macrophages when a suitable microenvironment prepared by bone marrow-derived stromal cells or osteoblasts is provided (Udagawa *et al.*, 1990). Two molecules, produced by stromal cells, have now been identified and shown to be both essential and sufficient for osteoclastogenesis, thus greatly improving our understanding of the origin, differentiation and activation of

osteoclasts: macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B (NF-κB) ligand (= RANKL, also called osteoprotegerin ligand (OPGL), tumour necrosis factor-related activation-induced cytokine (TRANCE) or osteoclast differentiation factor (ODF)). M-CSF, secreted by stromal cells, osteoblasts and T-cells, binds to its receptor, c-Fms, on osteoclast precursors to induce signals required for both proliferation and differentiation into mature osteoclasts (Tanaka *et al.*, 1993). This was highlighted by the finding that osteoclast deficiency in the osteopetrotic *(op/op)* mouse is due to the failure of haematopoietic stromal cells to release functionally active M-CSF because of a single base-pair insertion in the coding region of the M-CSF gene (Yoshida *et al.*, 1990; Hattersley *et al.*, 1991; Takahashi *et al.*, 1991). Contrary to the PU-1 deficiency-caused osteopetrosis, this defect lies in the local stromal microenvironment, and not in osteoclast precursors, and can thus not be cured by bone marrow transplantation, but only by *in vivo* administration of M-CSF.

In contrast to M-CSF, RANKL is not a secreted, but a surface-residing molecule expressed on osteoblasts, T-cells and bone marrow stromal cells, that binds to its receptor, RANK, on osteoclast precursors. This explains why osteoclast progenitors require close contact with stromal cells to differentiate into mature osteoclasts. RANKL has been shown to activate both NF-κB and AP-1 in target cells (Wei *et al.*, 2001) through receptor interaction with TNF receptor-associated factors (TRAFs), especially TRAF6. Both NF-κB- and TRAF6-deficient mice develop osteopetrosis due to defects in osteoclast differentiation and function, respectively (Franzoso *et al.*, 1997; Iotsova *et al.*, 1997; Lomaga *et al.*, 1999; Kobayashi *et al.*, 2001). Mature osteoclasts also express the receptor RANK, and bone resorption is induced by RANKL expressed on osteoblasts/stromal cells, explaining why osteoclastic resorptive activity involves cell-to-cell contact with stromal cells (Jimi *et al.*, 1996). The osteolytic actions of RANKL will be explored in more detail in Chapter 5. Several cells and tissues secrete a soluble decoy-receptor for RANKL, called osteoprotegerin (OPG). OPG strongly inhibits osteoclast formation *in vivo* and *in vitro* by preventing the binding of RANKL to its receptor, RANK, on osteoclast precursors (Yasuda *et al.*, 1998; Hsu *et al.*, 1999). Both RANKL deficiency and OPG overexpression lead to osteopetrosis, whereas loss of OPG renders

mice osteoporotic due to enhanced osteoclastogenesis (Bucay *et al.*, 1998; Kong *et al.*, 1999), suggesting that OPG is a physiologically important inhibitor of osteoclast formation, and that a critical ratio of RANKL/OPG expression levels is required for physiological bone resorption.

TNF-α is among the most potent osteoclastogenic cytokines produced in inflammation and can induce RANKL expression in stromal cells. Although earlier studies suggested that TNF-α, acting directly on precursors, could act independently of RANKL to induce osteoclast formation (Azuma *et al.*, 2000; Kobayashi *et al.*, 2000), recent efforts reveal that TNF-α alone fails to induce complete osteoclastogenesis, but rather suggest a synergism between these two cytokines (Komine *et al.*, 2001). Pure osteoclast precursors need to be primed by pre-exposure to permissive levels of RANKL (and these are levels insufficient to induce osteoclast formation), before TNF-α can be osteoclastogenic (Lam *et al.*, 2000; Zhang *et al.*, 2001). Thus, TNF-α targets both marrow stromal cells and osteoclast precursors. The synergism and coupling between TNF-α and RANKL to enhance osteoclastogenesis explains part of the severe osteolysis occurring at sites of inflammation and infection, in addition to other characteristics of inflammation that might contribute to bone loss, such as acidic pH, prostaglandins, hypoxia and increased release of nucleotides. Conversely, T-cells are also able to counterbalance RANKL action by production of interferon-γ (IFN-γ), which induces rapid degradation of TRAF6 and consequently inhibits osteoclastogenesis (Takayanagi *et al.*, 2000).

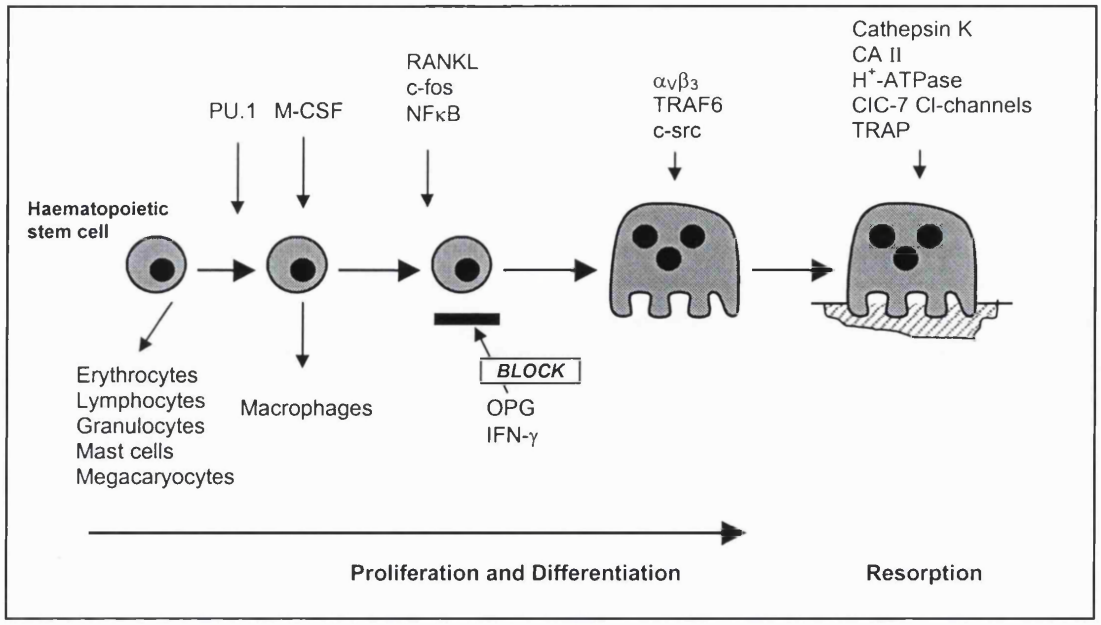


Figure 1.2

Osteoclast differentiation pathway

The discovery of the essential roles of M-CSF and RANKL greatly facilitated *in vitro* osteoclastogenesis. Purified cells of the monocyte/macrophage lineage, obtained from bone marrow or peripheral blood, can now be differentiated *in vitro* into mature osteoclasts by adding pure M-CSF and RANKL to the culture medium, replacing the requirement for stromal cells. Many standard osteoclastogenic and osteolytic agents such as PTH, 1,25-$(OH)_2D_3$ and prostaglandin E_2 (PGE_2) are now known to exert their effects indirectly through up-regulation of RANKL expression on stromal cells (Tsukii *et al.*, 1998; Lee and Lorenzo, 1999), whereas others stimulate M-CSF secretion by stromal cells. However, a recent study on integrins demonstrates that osteoclasts generated in stromal cell-free cultures retained some macrophage-associated integrins that were absent in osteoclast generated in stromal cell (but not RANKL expressing)-rich cultures. Both cultures were dependent on the addition of soluble RANKL, but the study demonstrates that osteoclasts generated in the absence of stromal cells may not completely reflect the *in vivo* situation, and may potentially produce misleading data because the osteoclast

phenotype is normally associated with a loss of macrophage-associated integrins (Lader *et al.*, 2001).

In osteoclast cultures, tartrate resistant acid phosphatase (TRAP) is widely used as a histological marker for the osteoclastic phenotype, in addition to multinuclearity and expression of calcitonin and vitronectin receptors. TRAP and multinuclearity are reliable markers for osteoclasts in bone. However, TRAP is not an exclusive marker for osteoclasts in cultures, since, in bone marrow cultures, macrophages can become TRAP-positive (Hattersley and Chambers, 1989; Modderman *et al.*, 1991). Reports of large numbers of 'osteoclast-like' cells in cultures in which there was virtually no bone resorption suggest that the presence of stained multinucleated cells is not a reliable marker for osteoclasts generated *in vitro*. Therefore, care should be taken when assessing TRAP-stained cultures, and only bone resorption, associated with TRAP-positive multinuclear cells, provides unequivocal evidence that osteoclasts are present *in vitro*.

How do osteoclasts resorb bone?

Attachment

After proliferation of immature osteoclast precursors and commitment to the osteoclast phenotype, the mature osteoclast is unique in being able to resorb mineralised substrates. The sequence of events required for bone resorption is known as the 'resorption cycle'. It involves migration of the osteoclast to the site of resorption, attachment to the bone matrix, polarisation and formation of four membrane domains, the dissolution of bone mineral and subsequent degradation of the organic matrix, removal of degraded products from the resorption lacunae, and finally the return to a quiescent state, or osteoclast apoptosis.

When activated upon attachment to bone surfaces, osteoclasts polarise, driven by profound re-organisation of the cytoskeleton. The primary attachment sites of the osteoclast are represented by podosomes, several attachment structures containing a filamentous (F)-actin core surrounded by vinculin and talin. Accumulation of podosomes

precedes bone resorption. In actively resorbing osteoclasts, microfilaments are reorganised in a specific ring structure, where vinculin and talin form a 'double circle' with F-actin sandwiched in between (Lakkakorpi *et al.*, 1989). This 'actin ring' is often used as a marker for actively resorbing osteoclasts in culture. It represents an organelle-free area of tight cell membrane-bone surface attachment, and is thus called 'sealing' or 'clear zone'. The sealing zone encloses the resorption lacunae and prevents leakage of bone degradation products. The part of the plasma membrane enclosed by the sealing zone enlarges into a highly convoluted 'ruffled border', from which proteolytic enzymes and acid are secreted to dissolve the bone. Recent data propose a new, more dynamic model for the sealing zone, where this barrier is not impermeable as suggested, but open to limited diffusion of negatively charged molecules with molecular weight up to 10000 (Stenbeck and Horton, 2000).

The recognition of bone matrix appears to be mainly controlled by integrins. Human osteoclasts express $\alpha_v\beta_3$, $\alpha_v\beta_1$ and $\alpha_2\beta_1$ integrins (Nesbitt *et al.*, 1993). The 'vitronectin receptor' $\alpha_v\beta_3$ has been suggested to be the major osteoclast attachment molecule, possibly through recognising RGD-containing osteopontin in the bone matrix, but has recently also been implicated in other intracellular signalling events derived from receptors at the basolateral membrane (Nakamura *et al.*, 2001; Sanjay *et al.*, 2001). Interference with integrin function by RGD-containing peptides, or the snake venom-derived disintegrin echistatin, or anti-receptor antibodies, inhibits osteoclast attachment and spreading and interrupts resorption *in vitro* and *in vivo* (Sato *et al.*, 1990; Horton *et al.*, 1991; Lakkakorpi *et al.*, 1991; Nakamura *et al.*, 1999). Osteoclasts deficient in the β_3 integrin subunit differentiate normally, but are dysfunctional (McHugh *et al.*, 2000). However, localisation studies have shown that $\alpha_v\beta_3$ is distributed throughout the osteoclast membrane except in the sealing zone (Lakkakorpi *et al.*, 1991; Lakkakorpi *et al.*, 1993; Helfrich *et al.*, 1996), suggesting that other integrins, *e.g.* $\alpha_2\beta_1$, or mechanisms mediate the tight attachment at the sealing zone. In this context, cadherin-mediated cell-matrix adhesion has recently been implicated in the formation of the sealing zone (Ilvesaro *et al.*, 1998).

The ruffled border represents a specific acid- and protease-secreting and phagocytosing membrane domain, the osteoclast's 'resorptive organelle'. It is formed by fusion of intracellular acidic vesicles with the region of plasma membrane facing the bone, and has been proposed to resemble lysosomes due to the targeted, mannose 6-phosphate-regulated transport of lysosomal enzymes to the ruffled border, followed by release into the acidic environment (Baron, 1989). Inhibition of bone resorption by calcitonin is associated with disruption of this targeted transport (Baron *et al.*, 1990). However, although facing the extracellular matrix, there is evidence for the presence of characteristic late endosomal membrane markers at the ruffled border (Palokangas *et al.*, 1997).

Over the last ten years there has been considerable progress in identifying the mechanism responsible for the ruffled border formation, probably a process similar to exocytosis since it may represent the transport of acidifying vesicles along microtubules and their polarised insertion into the membrane (Abu-Amer *et al.*, 1997). The tyrosine kinase $pp60^{c\text{-}src}$, gene product of the protooncogene *c-src*, has been shown to be essential for ruffled border formation (Boyce *et al.*, 1992). *C-src*-deficient mice develop osteopetrosis (Soriano *et al.*, 1991). Downstream substrates of $pp60^{c\text{-}src}$ include c-Cbl, $p130^{Cas}$, paxillin, the tyrosine kinase Pyk2 as well as the phosphatidylinositol-3 kinase (PI-3 kinase) (Tanaka *et al.*, 1996; Lakkakorpi *et al.*, 1997; Nakamura *et al.*, 1998; Lakkakorpi *et al.*, 1999). Wortmannin, a potent inhibitor of PI-3 kinase, blocks osteoclastic bone resorption by preventing fusion of precursor vacuoles with the ruffled border membrane, and by inhibiting actin rearrangement (Nakamura *et al.*, 1995; Nakamura *et al.*, 1997). Additionally, kinase-independent signalling pathways of $pp60^{c\text{-}src}$ such as its translocation to the cytoskeleton upon attachment, and its involvement in integrin signalling, might be responsible for the osteoclast defect observed in *c-src*-deficient mice (Schwartzberg *et al.*, 1997; Sanjay *et al.*, 2001).

Degradation of bone matrix

Osteoclasts resorb both organic and inorganic bone material. Dissolution of the inorganic mineral phase precedes enzymatic degradation of the organic phase. The demineralisation process involves acidification of the isolated compartment to a pH of 4.5, which is sufficient to dissolve the strongly basic hydroxyapatite:

$$[Ca_{10}(PO_4)_6(OH)_2] + 8\ H^+ \rightarrow 10\ Ca^{2+} + 6\ HPO_4^{2-} + 2\ H_2O$$

This is accomplished by very high expression of the vacuolar (V-type) electrogenic H^+-ATPase (Väänänen *et al.*, 1990), both at the ruffled border and in intracellular vesicles, which are transported to the ruffled border prior and during resorption. Activity of this H^+-pump is an energy-intensive process and the energy source for acid secretion appears to be almost exclusively glucose (Williams *et al.*, 1997). H^+-ATPase is sensitive to bafilomycin A1, which effectively inhibits bone resorption *in vitro* and *in vivo* (Sundquist and Marks, 1994). To maintain electroneutrality, H^+-transport is charge-balanced by a chloride (Cl^-) channel expressed at the ruffled border. This Cl^- channel has only recently been identified at the molecular level to be the ClC-7 Cl^- channel (Kornak *et al.*, 2001).

The source of protons for H^+-ATPase is carbonic acid (H_2CO_3), generated intracellularly from carbon dioxide (CO_2) and water (H_2O), a process facilitated by high expression of carbonic anhydrase type II (CA II). To maintain intracellular pH within physiological limits, hydrochloric acid (HCl) secretion at the ruffled border is counterbalanced by passive Cl^-/bicarbonate (HCO_3^-) exchange in the basolateral membrane, which at the same time provides Cl^- required for the Cl^- channel (Teti *et al.*, 1989b).

Several proteolytic enzymes secreted into the resorption lacunae then degrade the exposed organic components. The roles of these enzymes are controversial and not fully understood. Two major classes of proteolytic enzymes have been mostly studied: lysosomal cystein proteinases and matrix metalloproteinases (MMPs: collagenases and gelatinases). The thiol-proteinase cathepsin K and MMP-9 (gelatinase B) are the

predominant proteinases in human osteoclasts (Drake *et al.*, 1996), but other thiol-proteinases (cathepsin B and L), acid proteinases (cathepsin D) and metalloproteinases might also play an important role. MMP-9 cleaves denatured type I collagen amongst other collagen types. It can be released from osteoblasts, and prior to osteoclastic resorption, it may act with other collagenases to remove the non-mineralised osteoid layer covering the bone surface (Chambers *et al.*, 1985). MMPs may also have a distinct role in migration of pre-osteoclasts from the periosteum to the developing bone marrow cavity (Blavier and Delaissé, 1995). However, MMP-9 knockout mice have only transient disturbances of bone resorption (Vu *et al.*, 1998).

The crucial role of these different processes involved in the osteoclast's ability to degrade bone is highlighted by a number of osteopetrotic cases in mice and humans: mutations and/or deletions of specific H^+-ATPase subunits, of ClC-7 Cl^- channels, of CA II and of cathepsin K all result in osteopetrosis (Sly *et al.*, 1983; Gowen *et al.*, 1999; Frattini *et al.*, 2000; Kornak *et al.*, 2001) (see also **Fig. 1.2**).

The basolateral membrane in polarised osteoclasts was always thought to be homogenous, but recent studies, using viral glycoproteins as a tool to investigate protein targeting, suggest a division into two functionally distinct domains (Salo *et al.*, 1996). The centrally located novel basolateral domain has now been suggested to be the site of exocytosis of transcytosed degradation products, thus also being called 'functional secretory domain'. The fate and trafficking of matrix degradation products has long been debated, but it is now evident that products are endocytosed by the osteoclast, transcytosed to the functional secretory domain and released into the extracellular fluid (Nesbitt and Horton, 1997; Salo *et al.*, 1997). Quantitative data are still missing, but clearly large amounts of degraded matrix material have to be transported through the resorbing osteoclast because the volume of resorption pits can easily exceed the volume of the entire cell. In addition, it is still unknown to which extent matrix degradation occurs extracellularly in the resorption lacunae and/or intracellularly in the transcytotic compartments. In this context, a new function for TRAP has been suggested: it is localised in transcytotic vesicles and has been implicated in the generation of highly

destructive oxygen species, which are able to destroy collagen. Thus, TRAP may facilitate fragmentation and further destruction of matrix degradation products in transcytotic vesicles (Halleen *et al.*, 1999).

Figure 1.3 summarises the processes involved in bone degradation **(Fig. 1.3)**.

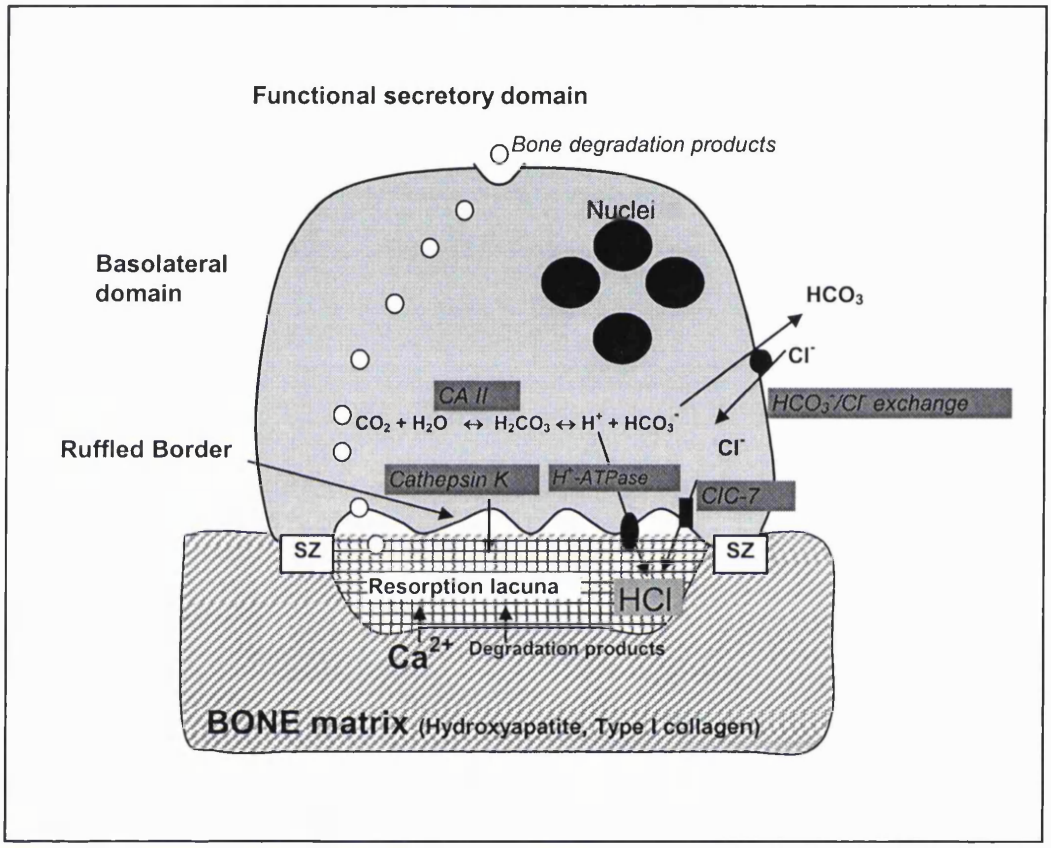


Figure 1.3

How does the osteoclast resorb bone?

SZ = sealing zone; CA II = carbonic anhydrase II.

Maintenance of 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* maintain intracellular pH via a Na^+/H^+ exchanger, which has been shown to be essential for the induction, but not for the maintenance of resorption and cytoplasmic spreading (Hall *et al.*, 1992). The presence of a pH- and membrane potential-sensitive H^+ conductance in osteoclast membranes has also been reported (Nordström *et al.*, 1995). Additionally, a Na^+/Ca^{2+} exchanger, NCX-1, has recently been identified in osteoclasts and was suggested to play a role in bone resorption by coupling H^+ extrusion with Ca^{2+} fluxes during bone resorption (Moonga *et al.*, 2001).

Osteoclasts appear to 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 mainly regulated by the Na^+/H^+ exchanger. Osteoclast pH_i also varies according to the substrate, with a more alkaline pH_i when cultured on bone compared to glass (Lehenkari *et al.*, 1997). Similar substrate dependency has been reported when studying potassium (K^+) conductance in osteoclasts (Arkett *et al.*, 1994). The significant differences in osteoclast pH_i regulation depending on the substrate raise 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 pH_i 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.

Regulation of resorption

Osteoclast differentiation and activation are regulated by several systemic (PTH, $1,25(OH)_2D_3$, calcitonin and estrogens) and local factors, most of which are discussed in the 'Bone Remodelling' section. Because bone remodelling occurs in discrete packets throughout the skeleton, it seems probably that cellular events are mostly controlled by

local factors generated in the bone microenvironment, either by cells or released from bone matrix during resorption (*e.g.* TGF-β). Numerous cytokines, growth factors and other small mediators such as protons, nitric oxide and nucleotides have been shown to locally regulate osteoclasts. Some factors differ in their effects depending on the culture system, *e.g.* PGE_2 stimulates resorption in organ cultures, but inhibits the activity of isolated osteoclasts.

Nitric oxide (NO) has been identified as a potent multifunctional signalling molecule with widespread, and partly controversial actions, in bone. It is a short-lived gas generated from L-arginine by the action of nitric oxide synthase (NOS) isoenzymes, and produced by bone cells in response to diverse stimuli such as pro-inflammatory cytokines, mechanical strain and sex hormones. Endothelial NOS (eNOS) is the isoform most widely expressed in bone; eNOS-deficient mice show impaired osteoblast function and a defective anabolic response to exogenous estrogen (Aguirre *et al.*, 2001; Armour *et al.*, 2001). Additionally, NO has bi-directional effects on osteoclast function, depending on the underlying stimulus and concentration: NOS inhibitors protect against inflammatory bone loss, but accelerate bone loss in normal and ovariectomized animals, and cytokine-induced bone resorption is potentiated by low NO concentrations, whereas high NO concentrations inhibit osteoclast formation and function. NO may be involved in the pathogenesis of bone disease and tissue damage associated with inflammatory conditions such as rheumatoid arthritis (Ralston, 1997).

Bisphosphonates, chemical analogues of inorganic pyrophosphate that are characterised by a P-C-P structure with varying side chains linked to the central carbon atom, are very important inhibitors of osteoclastic bone resorption *in vivo* and widely used as anti-resorptive drugs in the treatment of metabolic bone diseases associated with increased bone resorption. They have high affinity for Ca^{2+} and target to bone mineral at active sites of remodelling, where they appear to be internalised by osteoclasts and to inhibit their function. According to their molecular mechanism of action, bisphosphonates can be divided into two pharmacological groups. Nitrogen-containing bisphosphonates (*e.g.* alendronate, pamidronate) act by inhibiting the mevalonate

pathway in osteoclasts, thereby preventing prenylation of small guanosine 5'-triphosphate (GTP)ase signalling proteins required for normal osteoclast function. In contrast, nitrogen-lacking bisphosphonates (*e.g.* clodronate, etidronate) have a different mode of action that may involve the intracellular incorporation into non-hydrolysable, cytotoxic ATP analogues in osteoclasts, or inhibition of protein tyrosine phosphorylation. Ultimately, bisphosphonates therefore result in loss of osteoclast function and osteoclast apoptosis (for a recent review, see Rogers *et al.*, 2000).

Mature osteoclast isolation

In vitro short-term bone resorption models, using isolated primary, mature osteoclasts and mineralised bone or dentine matrix as a substrate, were developed almost twenty years ago (Boyde *et al.*, 1984; Chambers *et al.*, 1984). Beforehand, progress in the understanding of osteoclast biology had been hampered for several reasons: osteoclasts are usually few in number relative to other cell types in bone; they are contained in a hard tissue; additionally, they are at the end of their proliferation and differentiation cycle, presenting major difficulties for the creation of osteoclast cell lines. Data from such short-term cultures complements that obtained from bone organ culture resorption models and long-term cultures of osteoclast-forming haematopoietic stem cells derived from marrow or peripheral blood.

Boyde, Jones, Chambers and colleagues developed the disaggregated osteoclast resorption assay in 1984. Variants of these assays were then widely adopted to study osteoclasts isolated from neonatal rat, rabbit or chick long bones. The method used in each case is simple: osteoclasts are relatively abundant in the bones of neonatal animals (reflecting the requirement for rapid growth modelling) and can be released mechanically by fragmenting the bones, which are not yet fully mineralised, in a suitable medium. The resulting cell suspension is settled onto bone or dentine discs and, after rinsing in saline to remove non-adherent cells, cultured for about 24 hours. Under suitable conditions, osteoclasts can then excavate resorption lacunae. Although this model has several limitations in attempts to study the whole physiological cascade of bone resorption, it

provides an excellent tool for detailed studies of the cellular mechanisms involved in the destruction of mineralised bone matrix, especially since the application of confocal microscopy to study osteoclasts cultured on bone or dentine slices. Because osteoclasts sediment and adhere more rapidly than other cell types present in the mixed cell population released from fragmented bones, "functionally purified" osteoclast populations may be generated by careful adjustment of settling times and washing methods. One of the most important factors in this assay system is to obtain adequate basal levels of resorption. This can be accomplished by the use of slightly acidified culture medium, as first described by Arnett & Dempster (1986).

Assessment of resorption is typically achieved by counting the number of multinuclear (> 3 nuclei) osteoclasts, stained histochemically for TRAP, and the number and/or area of resorption pits, using the technique of reflected light microscopy, after staining the discs with toluidine blue to visualise pits (Walsh *et al.*, 1991). This replaced the more complicated use of scanning electronic microscopy to study resorption. Other groups have suggested that measuring the volume of each individual pit rather than discrete number or resorbed area is a more accurate method of assessing resorption (Boyde and Jones, 1991).

Bone growth and remodelling

Bone is a dynamic, living tissue, with continuous modelling and remodelling by bone cells, allowing the skeleton to grow and adapt.

Bone modelling involves both the growth and the shaping of bones. During growth, two types of ossification can be distinguished. Intramembranous ossification occurs in the developing flat bones of the skull: mesenchymal cells of the embryonic connective tissue differentiate into osteoblasts, which lay down an amorphous ground substance that becomes subsequently mineralised. In contrast, endochondral bone formation, occurring in long bones, involves a slow degradation of hyaline cartilage structures that become transformed into bone by ossification. Endochondral ossification permits elongation and

thickening of the bone during foetal development and throughout childhood. Bone modelling also occurs as part of the fracture healing process.

Throughout life, bone continues to be remodelled to maintain mechanical integrity of the adult human skeleton. This occurs mainly as local processes carried out in small areas by population of cells called bone-remodelling units (BMUs). A cycle of bone resorption by osteoclasts and subsequent bone formation by osteoblasts in a unit takes about 100 days. The remodelling, where about 5-20% of the human adult skeleton is remodelled by 2 million BMUs at any time, is strongly related to mechanical stresses and strains imposed on the skeleton and occurs mainly in areas of trabecular bone due to its larger surface area rather than in compact bone. The signals which initiate bone remodelling at a specific site have not yet been fully elucidated.

Regulation of bone remodelling

Bone remodelling is regulated by a complex interplay between systemic hormones, mechanical stimuli and locally produced cytokines, growth factors and other mediators.

Systemic hormones

Four hormones are primarily concerned with the regulation of bone metabolism: 1,25-$(OH)_2D_3$, PTH, calcitonin and estrogen, plus a number of other hormones, including androgens, growth hormone, thyroid hormones, glucocorticoids and PTH-related protein (PTHrP). Many of these hormones affect bone remodelling via the production of local factors.

Vitamin D_3

Vitamin D_3 is a secosteroid produced in the skin by the action of ultraviolet sunlight on precursors [provitamin D_3 (= 7-dehydrocholesterol) => previtamin D_3 => vitamin D_3 (cholecalciferol)]. Vitamin D_3 is biologically inert and must undergo two successive hydroxylations in the liver and kidney to become the biologically active 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$, or calcitriol). Despite two hydroxylation steps, 1,25-$(OH)_2D_3$ is still very lipid soluble and acts like a steroid hormone: it binds to a nuclear vitamin D receptor (VDR) which exposes a DNA-binding region, resulting in increased and/or inhibited transcription of certain mRNAs. VDRs are found in mainly in the intestine, kidney and bone, but also in a number of other tissues. The main biological effect of 1,25-$(OH)_2D_3$ is to maintain the serum Ca^{2+} levels within the normal range of 2.5 mmol/L. This is accomplished by increasing dietary uptake of Ca^{2+} in the intestine, by facilitating Ca^{2+} reabsorption in the kidneys and by enhancing the mobilisation of Ca^{2+} stores from bone. 1,25-$(OH)_2D_3$ increases the number of mature osteoclasts by inducing stem cells to differentiate into osteoclasts. However, this appears to be regulated indirectly through its action on osteoblasts, since mature osteoclasts do not express VDRs (Suda *et al.*, 1992).

Parathryoid hormone (PTH)

PTH is synthesised and secreted by the chief cells of parathyroid glands. After synthesis as preproPTH, a leader sequence is removed upon entry into the endoplasmic reticulum to form proPTH and an additional six amino acid (AA) residues are cleaved off in the Golgi apparatus before the active 84 AA polypeptide PTH is secreted. The 1-34 amino terminal fragment is the biologically active fragment.

PTH acts via at least three different receptors. PTH receptors couple to both G_q and G_S, thereby initiating both Ca^{2+}- and cAMP-driven responses. PTH can stimulate both osteoclasts and osteoblasts, but the effect on osteoclasts appears to be predominating resulting in a net Ca^{2+} efflux from bone. Interestingly, prolonged intermittent

administration of PTH has been shown to increase bone formation, a property for which it is being explored clinically as an anabolic agent. However, as is the case for 1,25-$(OH)_2D_3$, the stimulatory action on osteoclasts seems to be mediated via receptors on osteoblasts. PTH also increases formation of 1,25-$(OH)_2D_3$, thus indirectly enhancing Ca^{2+} absorption from the intestine (for a review on PTH: Jüppner *et al.*, 1999).

Calcitonin

Calcitonin is a 32 AA polypeptide synthesised by the parafollicular cells in the thyroid glands. Receptors for calcitonin are found in kidney and bone, and its main action is to lower circulating Ca^{2+} and phosphate levels by inhibiting bone resorption and increasing Ca^{2+} excretion in the urine. In contrast to PTH and 1,25-$(OH)_2D_3$, calcitonin acts directly on osteoclasts and the expression of calcitonin receptors is often used as a marker for osteoclasts. It can affect multiple stages in the mammalian osteoclast lineage, including inhibition of osteoclast formation and of resorption, and is thus used clinically in the treatment of osteoporosis. Interestingly, osteoclasts 'escape' from calcitonin-induced inhibition during continuous exposure to the hormone, probably due to down-regulation of calcitonin receptor mRNA expression (Takahashi *et al.*, 1995).

Sex steroids

Estrogens, members of the gonadal steroid family, have been shown to play an important role in preventing osteoporosis and maintaining bone mass. Naturally occurring estrogens are 17β-estradiol, estrone and estriol, secreted primarily by ovarian follicles, corpus luteum and placenta. 17β-estradiol is the most potent of the three. Two intracellular estrogen receptor (ER) subtypes exist, ERα and ERβ; both have been found in osteoblasts, osteoclasts and osteocytes. Many of the effects are thought to be mediated by a genomic pathway involving ligand/receptor interaction, like for other members of the steroid hormone family. However, a potent anti-apoptotic effect of estrogens on osteoblasts and osteocytes has recently been reported that is due to a distinct, rapid activation of Src/Shc/ERK signalling pathways via a non-genotropic, sex-nonspecific action of the classical receptors (Kousteni et al., 2001), providing a whole new concept.

The bone-preserving action of estrogens is probably mediated predominantly through effects on osteoclastogenesis (Sarma *et al.*, 1998), and probably osteoclast apoptosis (Compston, 2001). These effects may be largely mediated by cells in the bone microenvironment through cytokine production, rather than by direct effects on osteoclasts, *e.g.* increased levels of pro-osteoclastogenic cytokines including IL-1, IL-6, TNF-α, M-CSF and PGE_2, have been reported in estrogen deficiency (Riggs, 2000; Compston, 2001). It has recently been shown that ovariectomy failed to induce bone loss in T-cell-deficient mice. This establishes T-cells as essential mediators of increased osteoclastic bone resorption in estrogen deficiency, and TNF-α, produced by T-cells, as a key mediator of ovariectomy-induced bone loss (Cenci *et al.*, 2000). Additionally, estrogens can down-regulate osteoclastogenesis by decreasing the responsiveness of osteoclast precursors to RANKL (Srivastava *et al.*, 2001).

There is increasing evidence that *androgens*, mainly testosterone, play an important role in bone development and homeostasis, both in males and females. The main effects appear to be stimulation of bone formation. Some of the effects may also be mediated by metabolites produced by enzymes present in bone, *e.g.* conversion of testosterone to estradiol through aromatases (Compston, 2001).

In contrast to the effects of estrogens and androgens, there is little and controversial evidence of direct effects of *progesterone*, the third member of gonadal steroids, on bone, although progesterone receptors have been identified on osteoblasts (Compston, 2001).

Other systemic hormones

Several other systemic factors are important in regulating skeletal growth and bone remodelling.

Glucocorticoids are steroids secreted by the adrenal cortex. Their effect on bone remodelling remains controversial and appears to depend on exposure time and concentrations. Although they can inhibit bone resorption in organ cultures *in vitro*, they have now been recognised as the major cause for accelerated bone loss accompanying

patients who take glucocorticoids as a rheumatoid arthritis treatment. This is due to a number of systemic and local effects on bone metabolism, including an indirect increase in PTH secretion through impaired intestinal Ca^{2+} absorption (secondary hyperparathyroidism), a decrease in the recruitment of osteoblasts from their progenitors, and accelerated osteoblast apoptosis. Interestingly, physiological concentrations of glucocorticoids are required for the late stages of osteoclast differentiation and function during development, but their greatest effect postnatally is inhibition of bone formation (for a review on glucocorticoids and bone, see Lukert, 1999).

The *thyroid hormones*, triiodothyronine and thyroxine, can also stimulate bone resorption and formation, and are critical for maintenance of normal bone remodelling (Mundy, 1999).

Growth hormone, a peptide hormone secreted by the pituitary gland, can stimulate bone formation and resorption, mostly mediated indirectly by both systemic and local production of insulin-like growth factors (IGFs) (Ohlsson *et al.*, 1998).

Parathyroid hormone-related protein/peptide (PTHrP) is a protein with PTH activity, but compared to PTH it represents a longer polypeptide (140 AA compared to 84 AA), it is encoded by a gene on a different chromosome, and it is produced by many different tissues in the body. However, both PTH and PTHrP are able to bind to a common receptor, and consequently they have similar biological activities. PTHrP is involved in cartilage development, but additionally, it can be secreted by specific tumours and is therefore regarded as a major hypercalcaemia-causing factor in malignancy (Guise *et al.*, 1996). Thus, it can have both hormone-like and local effects.

Vasoactive intestinal peptide (VIP), a member of the secretin family of gastrointestinal hormones, has also been shown to inhibit osteoclast formation (Lerner, 2000).

Local regulators

A large number of locally produced cytokines, growth factors and other small mediators affecting bone cells have now been identified. Since bone remodelling occurs in discrete units throughout the skeleton, it seems likely that the cellular events are mainly controlled by factors generated in the microenvironment of bone. Stimulators and inhibitors can influence bone cells either directly, or indirectly by inducing production of local factors by surrounding cells, including osteoblasts and osteoclasts themselves, or by T-cells, monocytes, macrophages, endothelial cells and nerve cells, demonstrating the high degree of interaction and coupling between cells in the bone microenvironment. Interestingly, most inhibitors of osteoclast activity act directly on osteoclasts, whereas most of the stimulators act indirectly via receptors on osteoblasts.

Table 1 summarises the cytokines, growth factors and other mediators known to be implicated in bone metabolism (adapted from Greenfield *et al.*, 1999; Mundy, 1999; Compston, 2001). Some of these factors will be discussed in more detail later, but for the sake of brevity I will not give an extensive review of all factors.

Table 1.1

Local factors affecting bone remodelling

Cytokine / Growth factor / Other	Abbreviation
Stimulators of bone resorption (osteoclast formation, activity ↑ and/or apoptosis ↓)	
Interleukins-1 (α,β), -6, -8, -11	IL-1(α,β), -6, -8, -11
Tumour necrosis factors and Lymphotoxin	TNFs and LT
Epidermal growth factor	EGF
Platelet-derived growth factor	PDGF
Fibroblast growth factors	FGFs
Leukaemia inhibitory factor	LIF
Vascular endothelial growth factor	VEGF
Macrophage-colony stimulating factor	M-CSF
Granulocyte/macrophage-colony stimulating factor	GM-CSF
Transforming growth factor-β (*can also inhibit*)	TGF-β
Insulin-like growth factors	IGFs
Prostaglandin E_2 *(but inhibits isolated osteoclasts)*	PGE_2
Receptor activator of NF-κB ligand	RANKL
PTH-related protein	PTHrP
Leukotrienes	LT
Annexin II	/
Adenosine 5'-triphosphate + Adenosine 5'-diphosphate	ATP + ADP
Hydrogen Ions / Extracellular pH ≤ 7.0	H^+
Hypoxia/ Low oxygen tension (≤ 5% O_2)	Po_2
Inhibitors of bone resorption	
Interferon-γ	IFN-γ
Interleukin-4, -13, -18	IL-4, -13, -18
Osteoprotegerin	OPG
Transforming growth factor-β (*can also stimulate*)	TGF-β
Hepatocyte growth factor	HGF
High extracellular Phosphate and Calcium	Ca^{2+} and PO_4^-
Endothelins	/
Stimulators of bone formation (osteoblast proliferation and/or differentiation ↑)	
Insulin-like growth factors	IGFs
Transforming growth factor-β	TGF-β
Fibroblast growth factors	FGFs
Platelet-derived growth factor	PDGF
Bone morphogenetic proteins	BMPs
Nitric oxide	NO

Pathophysiology of bone remodelling / Bone diseases

Abnormalitities of bone remodelling can produce a variety of skeletal disorders. *Primary osteoporosis* is the most common metabolic disorder of the skeleton. It is defined as a condition characterised by low bone mass and disruption of bone architecture due to a net excess of bone resorption over bone formation, resulting in reduced bone strength and increased risk of fragility fracture. It remains asymptomatic unless structural collapse or fracture of bone occurs. Damage is particularly likely to occur in the dorsal vertebrae, femoral neck/hip and distal radius since all these areas have a high content of trabecular bone, which is more metabolically active and therefore lost more rapidly. The pathogenesis of osteoporosis is multifactorial and determined by both genetic and environmental factors affecting the peak bone mass, but the most common form is associated with advancing age and menopause. Bone loss after menopause is primarily caused by estrogen deficiency. In addition to these primary causes of osteoporosis, there are a number of disorders leading to *secondary osteoporosis*, *e.g.* hyperparathyroidism or glucocorticoid therapy amongst many others (for a review, see Compston, 2000).

In contrast, *osteopetrosis* is characterised by a steady increase in bone density due to impaired formation of osteoclasts or loss of osteoclast function. This can result in haematological abnormalities, due to crowding out of the bone marrow cavities, and also in neurological defects, due to narrowing of spaces in the bone through which nerves could normally pass. As discussed in the osteoclast section, several phenotypes and causes of osteopetrosis have been identified to date (osteoclast differentiation defects: *c-fos*, PU-1, NF-κB, M-CSF, RANKL; osteoclast dysfunction: TRAF6, *c-src*, H^+-ATPase subunits, ClC-7 Cl^--channels, CA II and cathepsin K).

Paget's disease is marked by an irregular increase in osteoclast numbers and activity, often affecting multiple sites throughout the skeleton. This increased bone resorption is met by a compensatory increase in bone formation and local bone turnover, leading to unorganised and reparative woven bone. Thus, in Paget's disease, there may be an increased bone density, but because of the irregular architecture, bone strength is

decreased and bones are more prone to fracture. The cause of this disease is still largely unknown. For many years, a slow viral infection had been suggested as the main cause, in addition to genetic abnormalities. However, a recent study on a large group of patients does not support the hypothesis that a viral infection is a necessary step in the pathogenesis (Helfrich *et al.*, 2000).

Osteomalacia describes a disease characterised by impaired osteoid mineralisation resulting in soft bones with an increased tendency to fractures. In children this condition is known as *rickets*, often caused by nutritional deficiency in vitamin D, Ca^{2+} or phosphate.

Inflammatory bone loss is a symptom of rheumatoid arthritis and periodontal disease. In the inflammatory state, production of inflammatory cytokines can lead to increased osteoclast activation and formation from precursors and macrophages present in the rheumatoid synovial fluid, and therefore to excessive osteoclastic resorption.

Similarly, several *cancers* have profound effects upon the skeleton, causing an increase in osteoclast formation and activity either systemically, as in humoral hypercalcaemia of malignancy, or locally, as in bone metastases, *e.g.* breast cancer derived. One major product of tumour cells is PTHrP, which promotes osteoclast formation, additionally to other pro-resorptive cytokines (Guise, 2000).

Cartilage

Cartilage is a special support tissue, devoid of blood vessels, and composed mainly of proteoglycan aggregates (mainly sulphated glycosaminglycans) associated with collagen (mainly type II), and elastic fibres, accounting for the solid, but flexible consistency. Matrix is deposited by chondrocytes, which are derived from mesenchymal stem cells. Of the three types of cartilage – hyaline, elastic and fibrocartilage – only hyaline cartilage plays an important role in bone biology. It forms the temporary skeleton scaffold during endochondral ossification, and also the articular surfaces in

joints. During growth of long bones, specialised areas at the end of each bone, the epiphyses, are separated from the shaft of the bone, the diaphysis, by a growth plate of actively proliferating cartilage. There, chondrocytes can be divided, according to their differentiation state, into resting, proliferative, or hypertrophic chondrocytes. Epiphyseal growth plate chondrocytes proliferate in columns towards the diaphysis, becoming hypertrophied as they deposit cartilage matrix. This uni-directional proliferation is largely responsible for the longitudinal growth of bones. As chondrocyte hypertrophy occurs, perichondrial cells differentiate into osteoblasts to form a bone collar around the cartilaginous core. The matrix around the most advanced hypertrophic chondrocytes becomes mineralised, before these cells undergo apoptosis.

In recent years, significant progress has been made into the understanding of the molecular control and the mechanisms of endochondral ossification. Briefly, it is now known that Cbfa1 and other transcription factors promote the differentiation of type II collagen-producing cells, present in the skeletal mesenchymal condensations, into hypertrophic chondrocytes that secrete the growth factor Indian hedgehog (Ihh). Ihh acts on cells of the perichondrium, stimulating PTHrP production, which then inhibits hypertrophy, and also on cells in the bone collar, where Ihh induces Cbfa1 expression. Cbfa1 can then fulfill its crucial role as an osteoblast differentiation factor. Additionally, hypertrophic chondrocytes secrete the angiogenic factor vascular endothelial growth factor (VEGF), promoting vascular invasion of the skeletal structures, enabling osteoclasts to form and to resorb the ossified cartilaginous matrix. Osteoblasts derived from the bone collar replace this matrix with a bone matrix rich in type I collagen (de Crombrugghe *et al.*, 2000; Karsenty, 2001; Takeda *et al.*, 2001).

RECEPTORS FOR EXTRACELLULAR NUCLEOTIDES

History

The concept of purines as extracellular signalling molecules was first proposed by Drury and Szent-Györgyi more than 80 years ago, who showed that adenosine and adenosine 5'-monophosphate (AMP) have biological effects on the mammalian heart (Drury and Szent-Györgyi, 1929). A large number of studies followed, confirming the role of nucleotides in the cardiovascular system. Five years later, another report illustrated different actions of adenosine and ATP on vasodilatation, hypotension and ileum contraction, indicating for the first time the existence of multiple purine receptors (Gillespie, 1934). However, ATP had long been established to be the main intracellular energy source and to be involved in various metabolic cycles; thus, its additional role as an extracellular signalling molecule took a long time to be widely accepted. In 1972, Burnstock proposed for the first time the concept of purinergic neurotransmission (Burnstock, 1972), suggesting that ATP was a transmitter involved in non-adrenergic, non-cholinergic nerve-mediated responses of smooth muscle in the gastrointestinal tract and in the bladder. Additionally, in 1976, the concept of co-transmission was introduced (Burnstock, 1976; see section on 'Short-term purinergic signalling'). However, both concepts have only over the last ten years been generally accepted, and extracellular purines and pyrimidines have since been implied in a wide range of biological processes, including smooth muscle contraction, exocrine and endocrine secretion, inflammation, platelet aggregation and pain, amongst many others (for a review, see Ralevic and Burnstock, 1998).

The term 'purinergic receptors' was first formally introduced in 1978 (Burnstock, 1978). Receptors were divided into 'P_1-purinoreceptors', with adenosine as main ligand, and 'P_2-purinoreceptors', with adenosine 5'-diphosphate (ADP) and ATP as main ligands. In principal, this classification remains true; however, the terms 'P_1/P_2-purino-receptors' have been replaced by 'P1-' and 'P2-receptors', and receptors for pyrimidines, *e.g.* for uridine 5'-triphosphate (UTP), are now included in the P2-receptor family

(Fredholm *et al.*, 1994). With further discovery and cloning of mammalian P2 receptor subtypes, a new nomenclature was needed: P2 receptors are now divided according to their molecular structure into ionotropic $P2X_n$ and metabotropic $P2Y_n$ receptors, replacing the older nomenclature into P_{2X}, P_{2Y}, P_{2T}, P_{2Z} and P_{2U} subtypes (Abbracchio and Burnstock, 1994; Burnstock and King, 1996).

P1 receptors

Four members of the adenosine/P1 receptor family have now been cloned and characterised from a variety of species: A_1, A_{2A}, A_{2B}, A_3, and, with the exception of the A_{2A} subtype, selective agonists and antagonists have been identified. All P1 receptors couple to G proteins, and modulate adenylate cyclase activity in an inhibitory (A_1, A_3) or stimulatory (A_{2A}, A_{2B}) fashion, resulting in cyclic AMP (cAMP) changes. However, they show distinct tissue distributions and pharmacological profiles. Adenosine plays a major role as a vasodilator in the heart and as a neuromodulator acting as a general central nervous system (CNS) depressant, *e.g.* the stimulatory effects of caffeine and theophylline are produced by inhibition of adenosine actions. Many diseases have been envisioned as candidates for treatment with compounds acting upon the adenosine regulatory system, including asthma, Parkinson's disease and psychiatric disorders.

Since this thesis will focus on the role of members of the P2 receptor family, the reader is referred to recent reviews for a summary of the distribution, pharmacology and physiology of P1 receptors (Ralevic and Burnstock, 1998; Fredholm *et al.*, 2000).

P2X receptors

Seven mammalian members of the family of P2X receptors have been cloned so far ($P2X_1$ – $P2X_7$). The $P2X_{1-7}$ subunits share an overall sequence identity, ranging from 35% to 48%, and their sizes range from 379 to 595 AA. The $p2x_8$ receptor subtype has recently been cloned from embryonic chick skeletal muscle (Bo *et al.*, 2000). P2X receptors define a novel class of ligand-gated cation channels (**Fig. 1.4 A**), consisting of

two transmembrane domains, separated by an extensive *N*-glycosylated extracellular loop, always containing 10 cysteine residues, and short intracellular amino (N)-and carboxy (C)-termini, with the exception of $P2X_2$ and $P2X_7$, which have a more extended C-terminal tail. ATP represents the major ligand and induces rapid and selective permeability to cations (Na^+, K^+, Ca^{2+}), explaining their abundant distribution on excitable cells such as neurons, smooth muscle and glial cells, and their role as mediators of fast excitatory neurotransmission in both the central and peripheral nervous system. Although all P2X receptors mediate fast signalling, their localisation, function and pharmacological characteristics are different, the latter mainly regarding the kinetics of desensitisation, and the response to the agonists α,β-methyleneATP (α,β-meATP) and the antagonists suramin and PPADS (pyridoxal-5'-phophate-6-azophenol-2',4'-disulphonate). Functional P2X channels are homomultimers or heteromultimers formed by the association of at least three subunits (**Fig. 1.4 B**). For a summary of possible P2X subunit combinations, see Torres *et al.* (1999). It is not yet known how the properties of different P2X subunits influence the phenotype of the heteromultimeric receptors. Alternative splicing and species-differences may increase heterogeneity of P2X receptors. Although P2X receptors play important physiological and pathophysiological roles in a variety of biological processes, subtype-selective agonists and antagonists are still rare and have yet to be discovered.

The following paragraphs will give a brief overview over distribution and most important characteristics of each receptor subtype.

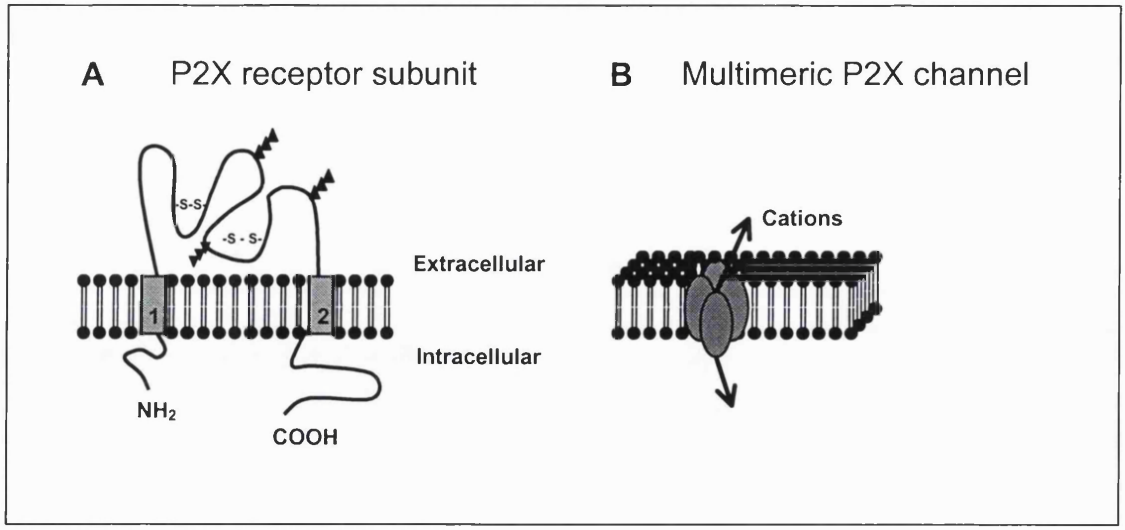


Figure 1.4

Structure of P2X receptors

(**A**) P2X receptor subunits consist of two hydrophobic transmembrane domains, a large *N*-glycosylated (triangles) extracellular loop, and intracellular N- and C-termini. The extracellular loop includes two to six potential *N*-linked glycosylation sites and 10 conserved cysteine residues, which may form intramolecular disulfide bridges (-S-S-). (**B**) At least three (or four) subunits are thought to form a functional P2X channel, allowing passage of cations.

$P2X_1$ receptor

This rapidly desensitising and α,β-meATP-sensitive receptor seems to be the most significantly expressed P2X subtype in smooth muscle, where it is a target of synaptically released ATP. It is mainly expressed in the bladder, in smooth muscle layers of small arteries, and in the vas deferens. Nerves innervating the vas deferens co-release ATP, acting at $P2X_1$ receptors, and noradrenaline, acting at α_1 adrenoreceptors, causing a rapid phasic contraction and a subsequent tonic contractile response, respectively. Interestingly, targeted deletion of $P2X_1$ leads to reduced fertility in male mice due to defective contraction of the vas deferens in response to sympathetic nerve stimulation (Mulryan *et al.*, 2000).

$P2X_2$ receptor

$P2X_2$ receptors show little or no desensitisation and have significant permeability to Ca^{2+}; however, high Ca^{2+} concentrations will also attenuate responses. They are widely expressed in nervous tissues and other tissues, including bladder and intestine. It is the only P2 receptor subtype that is sensitive to extracellular acidification: acid pH < 7.0 causes a significant increase in ATP-evoked currents with possible implications in nociception (King *et al.*, 1996; Wildman *et al.*, 1997). Additionally, zinc (Zn^{2+}) is a potent potentiator of responses (Wildman *et al.*, 1998). The $P2X_2$ receptor will be discussed in more detail in Chapter 5.

$P2X_3$ receptor

Similar to the $P2X_1$ receptor, the $P2X_3$ receptor is α,β-meATP-sensitive and desensitises rapidly in the continued presence of agonists. Until recently it was thought that $P2X_3$ has a very restricted distribution to a subset of nociceptive sensory neurons (trigeminal, nodose and dorsal root ganglia), and that it is virtually absent from sympathetic, enteric and CNS neurons and all other body tissues, strongly suggesting a role in nociception. However, there is recent evidence that the $P2X_3$ receptor is also expressed on endothelial and epithelial cells (Glass *et al.*, 2000) and possibly motor neurons. It is not clear whether $P2X_3$ exists as a homomultimer or as a $P2X_{2/3}$ heteromultimer, or as both, *in vivo*. As mentioned above, $P2X_2$ receptors are acid-sensitive; thus, a $P2X_{2/3}$ heteromultimer seems likely because the sensitivity of nociceptive neurons is probably enhanced in inflammatory conditions with slow acidosis (for a review on ATP and pain, see Burnstock *et al.*, 2000).

Two recent studies of $P2X_3$-deficient mice highlighted the role of $P2X_3$ in peripheral pain responses, but surprisingly also in pathways controlling urinary bladder volume reflexes: $P2X_3$-deficient mice showed a reduced pain-related behaviour in response to ATP and formalin, but also a markedly diminished response to filling and stretching of the urinary bladder. However, there was no major role for $P2X_3$ in noxious

mechanosensation and acute pain responses. Thus, ATP cannot be considered a general mediator of all painful stimuli (Cockayne *et al.*, 2000; Souslova *et al.*, 2000).

$P2X_4$ receptor

Although this slowly desensitising subtype is generally distributed throughout the body and found extensively in the brain, notable tissue functions have not yet been established; however, it is the only subtype expressed by the acinar cells of salivary glands (Ralevic and Burnstock, 1998).

$P2X_5$ receptor

Transcripts for this receptor have been identified in brain, heart, spinal cord, adrenal medulla, thymus and lymphocytes. Protein expression was recently demonstrated in the proliferating and differentiating cell layers in keratinised and non-keratinised epithelia and in growing hair follicles, implying a role in proliferation and/or differentiation (Gröschel-Stewart *et al.*, 1999; Bardini *et al.*, 2000). Additionally, work from our lab has recently reported a time-course dependent expression in developing chick and rat skeletal muscle, with possible roles in myotube formation (Meyer *et al.*, 1999; Ryten *et al.*, 2001).

$P2X_6$ receptor

$P2X_6$ mRNA is heavily expressed in the CNS, but also present in other tissues, *e.g.* on thymic epithelial cells (Glass *et al.*, 2000). Similarly to $P2X_5$, protein expression has been reported in developing chick and rat skeletal muscle (Meyer *et al.*, 1999; Ryten *et al.*, 2001), but an exact functional role has not been identified.

$P2X_7$ receptor

The $P2X_7$ receptor, previously called P2Z receptor and cloned from rat macrophages and brain in 1996 (Surprenant *et al.*, 1996), is a bi-functional molecule that functions not only as a cation channel, but also, upon prolonged agonist stimulation and low levels of divalent cations, as a cytolytic pore permeable to larger hydrophilic molecules up to 900D (Virginio *et al.*, 1997). Since the latter function is associated with cytotoxicity, the receptor has been connected to lytic and apoptotic events, especially in immunomodulation and inflammation. In this context, the $P2X_7$ receptor on macrophages can trigger release of the pro-inflammatory cytokines IL-1β and IL-6 (Ferrari *et al.*, 1997). $P2X_7$ fails to form heteromultimers with other subunits and reacts preferentially with ATP^{4-} and benzoyl ATP. It has been found in haematopoietic (macrophages, monocytes, lymphocytes, granulocytes) and nervous tissues (microglia) (Collo *et al.*, 1997). Receptor expression has also recently been shown in the exfoliating layers of keratinised epithelium, consistent with an involvement in apoptotic events (Gröschel-Stewart *et al.*, 1999).

P2Y receptors

The P2Y family of receptors is a novel subclass of the superfamily of G protein-coupled receptors, each having 7 transmembrane domains (**Fig. 1.5**). The third intracellular loop and the C-terminus are thought to be involved in G protein-coupling, whereas the third, sixth and seventh transmembrane domains have been implicated in nucleotide binding (Jacobson *et al.*, 1999).

P2Y receptors are widely distributed throughout the body and exhibit more sequence diversity than any other known family of G protein-coupled receptors. Most subtypes couple through G proteins to phospholipase C (PLC), mainly to PLC_β, and subsequent formation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), but inhibition of adenylate cyclase (AC) can also occur (North and Barnard, 1997). IP_3 activates IP_3-sensitive channels on the endoplasmatic reticulum, leading to transient elevation of

cytosolic free Ca^{2+} ($[Ca^{2+}]_i$); both DAG and elevated $[Ca^{2+}]_i$ activate certain forms of protein kinase C (PKC), followed by further downstream signalling events. Recombinant P2Y receptors affect a narrow range of signalling pathways (PLC, AC), whereas endogenous receptors affect a much wider range of intracellular signalling cascades, including phospholipase A_2 (PLA_2), phospholipase D (PLD), mitogen activated protein kinase (MAPK), in addition to PLC and AC (King *et al.*, 2000).

Six mammalian P2Y receptors have been cloned to date: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$ and $P2Y_{12}$. The lower case, p2y, is used for mammalian orphan receptors or functional non-mammalian receptor proteins without a mammalian homologue. The chick $p2y_3$ receptor may be the homologue of the human $P2Y_6$ receptor, whereas a mammalian homologue of the *Xenopus* $p2y_8$ has not yet been identified. The putative $p2y_5$, $p2y_7$, $p2y_9$ and $p2y_{10}$ receptors turned out not to be 'true' members of the P2Y receptor family: $p2y_7$ was found to be identical with the leukotriene B_4 receptor and the others, when expressed in appropriate cell lines with no intrinsic purinoreceptors, lost their sensitivity to ATP (Ralevic and Burnstock, 1998; King *et al.*, 2000).

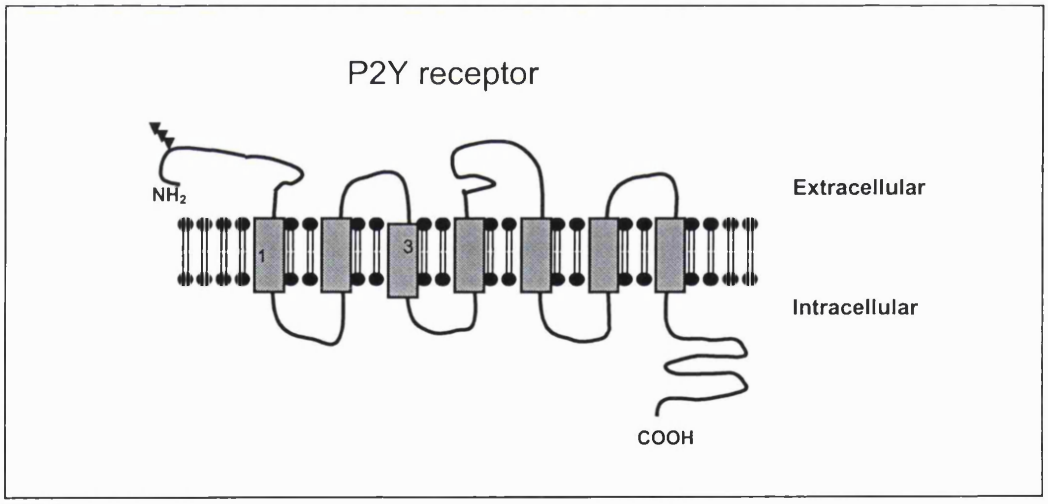


Figure 1.5

Structure of P2Y receptors.

P2Y receptors consist of seven transmembrane domains, with an extracellular N-terminus and an intracellular C-terminus.

$P2Y_1$ receptor

The $P2Y_1$ receptor is a receptor activated by adenine nucleotides (ADP and ATP), but not by uridine nucleotides (uridine 5'-diphosphate (UDP) and UTP). ADP is the most potent naturally occurring agonist, and whether ATP is a full, partial or inactive agonist remains debated and might be species-dependent. It is the only subtype for which significant progress has been made for high-affinity and selective synthetic agonists and antagonists, *e.g.* 2-methylthioADP (2-meSADP) is a highly potent agonist at the $P2Y_1$ receptor. The $P2Y_1$ receptor, which upon agonist binding mainly activates PLC through a $G_{q/11}$-protein, is distributed widely throughout the body. It has been described in vascular, connective, immune and neural tissues. The presence of $P2Y_1$ receptors on vascular endothelium and smooth muscle implies a role in the regulation of vascular tone. A major functional role is the induction of platelet aggregation. Activation of the $P2Y_1$ receptor on platelets leads to platelet shape change, aggregation and $[Ca^{2+}]_i$ rise. ADP, secreted from platelet dense granules, can also potentiate the aggregation response induced by other agents. Targeted disruption of the $P2Y_1$ receptor has been shown to result in impaired platelet aggregation and increased resistance to thrombosis (Fabre *et al.*, 1999; Leon *et al.*, 1999). However, there is also evidence for a second platelet ADP-receptor, coupled to inhibition of AC through G_i proteins, with subsequent alterations in cAMP levels. Selective antagonists of the $P2Y_1$ receptor do not block ADP-induced inhibition of AC in platelets (Ralevic and Burnstock, 1998). The second receptor had tentatively been called $P2Y_{ADP}$ (or P_{2T} or $P2Y_{AC}$), but has recently been cloned and designated $P2Y_{12}$ (see below) (Hollopeter *et al.*, 2001).

$P2Y_2$ receptor

The $P2Y_2$ receptor, formerly called 'P_{2U} receptor', is activated by ATP and UTP with approximately equal potency and is insensitive to nucleoside diphosphates. The receptor is widely distributed in the body. Signal transduction mechanisms are mediated by both $G_{i/o}$ and $G_{q/11}$ proteins. The downstream signalling events seem to depend on the cell type involved, *e.g.* in airway epithelial cells, secondary to PLC activation and $[Ca^{2+}]_i$

mobilisation, Ca^{2+}-sensitive Cl^- channels open up, driving fluid secretion (Cressman *et al.*, 1999). This could be of pathophysiological significance in patients with cystic fibrosis, a condition characterised by a failure to secrete Cl^- ions into the airways. Treatment of cystic fibrosis with the main $P2Y_2$ agonist, UTP, is being pharmacologically explored (Stutts *et al.*, 1992; Cressman *et al.*, 1999).

$P2Y_4$ receptor

Earlier studies on the cloned human $P2Y_4$ receptor suggested that this receptor is highly selective for UTP over ATP, and not activated by nucleoside diphosphates. However, more recent data on the cloned rat $P2Y_4$ have shown that it is equally activated by UTP and ATP, resembling the $P2Y_2$ activation profile. This could imply that some earlier $P2Y_2$-like responses in rat tissues may in fact be partly mediated by the $P2Y_4$ receptor (Bogdanov *et al.*, 1998). $P2Y_4$ seems to have a restricted distribution to the placenta, but mRNA has also been detected in vascular smooth muscle.

$P2Y_6$ receptor

This receptor subtype is activated most potently by UDP, and weakly or not all by nucleoside triphosphates. $P2Y_6$ mRNA is found abundantly in various rat tissues such as thymus, lung, spleen and smooth muscle, but a physiological role has not yet been established.

$P2Y_{11}$ receptor

This receptor seems to be the only cloned receptor selective for ATP, with ADP, UTP and UDP being weakly active or inactive. It couples both to G_q proteins, followed by activation of PLC, and to G_s proteins, followed by stimulation of AC. So far, receptor mRNA has only been detected in spleen and granulocytes.

$P2Y_{12}$ receptor

This receptor was the last mammalian P2Y receptor subtype to be cloned (Hollopeter *et al.*, 2001). It was identified as the second platelet ADP receptor (the first being $P2Y_1$), and couples to inhibition of AC through G_i proteins, a response that can be abolished by pertussis toxin. ADP activation of $P2Y_{12}$ is required for platelet aggregation, and represents the target of efficacious antithrombotic drugs such as clopidrogel. Interestingly, a patient with a bleeding disorder has been shown to have a defect in the gene encoding $P2Y_{12}$. In contrast to $P2Y_1$, the $P2Y_{12}$ receptor has a more selective tissue distribution: it is expressed abundantly in platelets, and to a smaller extent in brain, but absent from all other tissues tested, which makes the receptor an attractive target for the development of new antithrombotic drugs.

Sources and fate of nucleotides

Nucleotides, which are present intracellularly at ~2-5 mM, are released into the extracellular fluid from a variety of sources. Due to its size and high density of charge, ATP cannot permeate membranes, but release of ATP has been observed from a variety of cells. Most of the research into potential mechanisms has focused on release of ATP itself, but ADP- and UTP release have also been reported. A number of cells release ATP upon mechanical stimulation, including tumour cells, platelets, red blood cells, endothelial cells, osteoblasts and chondrocytes (Alkhamis *et al.*, 1990; Bowler *et al.*, 1998b; Lloyd *et al.*, 1999; Pedersen *et al.*, 1999).

There are three general mechanisms by which intracellular ATP can be released: (1) cytolysis; (2) vesicular release; and (3) ATP-binding cassette (ABC) proteins.

ATP release through cytolysis takes place after cell damage or cell death following physical or biological trauma, and can thus contribute to pathophysiological conditions. During trauma, nucleotides have been shown to attain levels as high as 20 μM (Born and Kratzer, 1984), a concentration sufficient to activate all P2 receptor subtypes. In

addition, activated platelets and leukocytes release ADP and ATP at sites of tissue injury and inflammation.

Vesicular release is the way ATP is released from nerve terminals, and possibly from some non-neuronal cells. In nerve terminals, ATP and other transmitters are stored in pre-synaptic vesicles and released through exocytosis during neurotransmission. Recent studies suggest that vascular endothelial cells, when subjected to shear stress, release ATP by vesicular transport (Bodin and Burnstock, 2001a).

ABC proteins have only recently been implicated in the release of ATP. They constitute a highly conserved family of ATP-dependent membrane transporters and consist of two cytoplasmic catalytic ATP-binding domains and two hydrophobic transmembrane domains, which form the translocation pathway for substrates such as amino acids, inorganic ions, sugars and proteins. The most common ABC proteins are the cystic fibrosis conductance regulator (CFTR), the sulfonylurea receptor and P-glycoprotein, product of the multidrug resistance protein gene. Although the concept that ABC proteins can also transport ATP itself across the membrane is still controversial, some studies, using specific blockers of ABC protein channels, suggest their involvement on different types of cells; *e.g.* ATP release by osteoblasts or Ehrlich ascites tumour cells can be inhibited by the addition of glibenclamide, a non-specific CFTR-blocker (Bowler *et al.*, 1998b; Pedersen *et al.*, 1999). For a recent review on ATP release, see Bodin and Burnstock (2001b).

Once released, the action of nucleotides at their receptors is terminated by a cell surface-located enzyme cascade that sequentially degrades nucleoside 5'-triphosphates to their respective nucleoside 5'-di and -monophosphates, nucleosides and free phosphates or pyrophosphate, which can all appear in the extracellular fluid at the same time. In the case of adenine nucleotides, both adenosine and phosphate can be recycled by surrounding cells. If P1 receptors are present on neighbouring cells, adenosine can initiate additional receptor-mediated functions. There is multiplicity of ecto-nucleotidases which belong to at least four different families with various subtypes in each family: ecto-nucleotide diphosphohydrolases (E-NTPDases), ecto-nucleotide pyrophosphatases

(E-NPPs), ecto-alkaline phosphatases and ecto-5'-nucleotidases. To complicate matters, these enzymes have been shown to represent possible targets of P2 receptor antagonists, *e.g.* of suramin; thus, care must be taken in P2 receptor antagonist studies using suramin. For recent reviews on extracellular ATP metabolism, see Zimmermann and Braun (1999) and Zimmermann (2000).

The alternative possibility of ATP synthesis on cell membranes has received little attention to date. However, a new paper reports that combined treatment of endothelial cells with ATP and either AMP or ADP caused unexpected phosphorylation of nucleotides via the backward reactions AMP $\rightarrow$ ADP $\rightarrow$ ATP. This could be mediated by a number of ecto-enzymes, but ecto-nucleoside diphosphate kinase possessed the highest activity and was capable of using both adenine and non-adenine nucleotides as phosphate donors and acceptors. This data demonstrate the co-existence of opposite, ATP-consuming and ATP-generating, pathways on the cell surface, and provide a novel mechanism for regulating the duration and magnitude of purinergic signalling (Yegutkin *et al.*, 2001).

Physiological roles of ATP

Purinergic signalling plays important roles in nervous and non-nervous tissues, including both short-term, fast actions (neurotransmission and secretion), and long-term, slower, trophic actions (development and regeneration) (Burnstock, 1997).

Short-term purinergic signalling

ATP acts as a co-transmitter in many nerves of both peripheral and central nervous system. Sympathetic co-transmission of noradrenaline and ATP has been demonstrated in smooth muscle of the vas deferens and in blood vessels. Similarly, acetylcholine and ATP are co-transmitters in various peripheral and central synaptic terminals, including parasympathetic nerves supplying the urinary bladder and motor nerves of skeletal muscle, especially in developing myotubes. Sensory motor nerves can co-release ATP

and calcitonin gene-related peptide and substance P. Neurones located in the myenteric plexus co-release ATP, NO and VIP, with the proportions varying in different regions of the gut. In the CNS, there is evidence that ATP is co-released with glutamate. For a complete review on purinergic co-transmission, see Burnstock (1999).

Other important short-term signalling mechanisms of ATP include the regulation of ion-transport in epithelial cells, the regulation of vascular tone by ATP acting on endothelial cells through P2Y receptors, the stimulation of insulin secretion by pancreatic β-cells, and also the regulation of immune cell function, *e.g.* modulation of cytokine release (Abbracchio and Burnstock, 1998).

Long-term purinergic signalling

There is increasing evidence that ATP, signalling through P1 and P2 receptors, plays a major role in embryonic development and cell growth, proliferation and differentiation, acting both as a positive and negative regulator.

ATP and adenosine can play important roles from the beginning of life, *e.g.* some studies suggest that ATP serves as a key sperm-to-egg signal in the process of fertilisation (Foresta *et al.*, 1992). Developmentally regulated expression of P2 receptors has been demonstrated in a variety of tissues including chick muscle, retina and gastrointestinal tract, pointing to a critical role for P2 receptors in maturation and acquisition of highly specialised functions. As an example, we have recently shown that in developing rat skeletal muscle, timing of $P2X_2$ receptor expression was closely related to the re-distribution of acetylcholine receptors to the motor end-plate (see appendix II: Ryten *et al.*, 2001). In many cases, once specialised tissue functions have been obtained, the receptors are not required anymore and likely to be desensitised. However, receptors and key signals might be expressed again, if necessary, under specific pathophysiological conditions, such as regeneration following trauma and injury. As an example, chick muscle regains its previously lost response to ATP after denervation followed by reinnervation (Wells *et al.*, 1995). Other studies suggest a role for P2Y receptors in the early formation of the nervous system (Bogdanov *et al.*, 1997).

Nucleotides can both stimulate cell cycle progression and inhibit cell growth, depending on their concentration, the physiological state of target cells and receptor expression (Harada *et al.*, 2000). Stimulation of DNA synthesis and cell proliferation by extracellular nucleotides has been demonstrated in fibroblasts, thymocytes, keratinocytes, smooth muscle cells and haematopoietic cells amongst other cell types (Abbracchio and Burnstock, 1998). These effects seem to be mostly regulated by low ATP concentrations (micromolar range) acting via P2Y receptors linked to stimulation of PLC and subsequent $[Ca^{2+}]_i$ increase. ATP can also act synergistically with various growth factors or hormones. The modulation of cell growth and proliferation may play crucial roles following trauma, stress and hypoxia. At sites of damage, nucleotide concentrations are massively increased and may thus contribute to the initiation of healing mechanisms, ultimately leading to tissue repair and regeneration.

In contrast to its mitogenic actions, ATP at higher (millimolar) concentrations can induce apoptosis in a number of cell types, including thymocytes, osteoblasts and osteoclasts, probably by acting via the cytolytic $P2X_7$ receptor (Morrison *et al.*, 1998; Gartland *et al.*, 2001). ATP also exhibits anti-cancer activity, probably due to direct actions of ATP on the tumour tissue, as demonstrated by studies both *in vivo* and on cultured tumour cell lines (for a review, see Abbracchio and Burnstock, 1998).

Signalling via ATP has also been implicated in wound healing, inflammation and ischemia, mostly to assist in damage repair, but pro-inflammatory actions have also been reported (for a complete review on pathophysiology, see Abbracchio and Burnstock, 1998).

P2 RECEPTORS IN BONE

There is now growing evidence that extracellular nucleotides, signalling through P2 receptors, play an important role in bone remodelling. I will give a very brief overview on the most important findings here and discuss more details in the experimental chapters that follow.

The first evidence that osteoclasts respond to nucleotides came from observations that ATP elevated $[Ca^{2+}]_i$ in rabbit osteoclasts, and that these elevations were at least partly due to activation of G proteins, consistent with the involvement of P2Y receptors (Yu and Ferrier, 1993a; Yu and Ferrier, 1994). Experiments about four years ago provided the first electrophysiological evidence for the co-existence of both P2X and P2Y receptors on bone resorbing cells (Weidema *et al.*, 1997); however, nucleotide-induced elevation of $[Ca^{2+}]_i$ has now been shown to arise primarily through activation of P2Y receptors (Weidema *et al.*, 2001). There is *in situ* hybridisation evidence for the expression of the $P2Y_2$ receptor on osteoclasts derived from human osteoclastoma (Bowler *et al.*, 1995). However, the same group later reported that the effects of ATP on resorption pit formation by these osteoclast-like cells are not mediated via the $P2Y_2$ receptor (Bowler *et al.*, 1998a), suggesting that this action is mediated by a P2 receptor other than the $P2Y_2$ subtype. Functional studies from our lab showed that ATP, at low concentrations, stimulates bone resorption in synergism with low pH, suggesting an involvement of the $P2X_2$ receptor (Morrison *et al.*, 1998).

A number of early studies have shown that ATP and other nucleotides signal through P2 receptors to induce $[Ca^{2+}]_i$ rises and subsequent formation of IP_3 in various transformed osteoblast-like cell lines and human bone derived osteoblasts (Kumagai *et al.*, 1991; Schöfl *et al.*, 1992). Later studies suggested that both rat UMR-106 osteosarcoma cells and human osteoblasts express mixed populations of P2 receptors (probably P2Y with a proportion of receptor subtypes that varies between individual cells) (Yu and Ferrier, 1993b; Dixon *et al.*, 1997). The existence of multiple P2Y receptors in bone and osteoblasts was confirmed by molecular studies (Bowler *et al.*,

1995; Maier *et al.*, 1997). Additionally, osteoblasts have been shown to release ATP under shear stress (Bowler *et al.*, 1998b).

Similarly to osteoblasts and osteoclasts, chondrocytes respond to extracellular nucleotides with large elevations of $[Ca^{2+}]_i$, mostly by release from intracellular stores, consistent with the presence of P2Y receptors (D'Andrea and Vittur, 1996; Kaplan *et al.*, 1996). Nucleotides may play a role in regulating chondrocyte activity under both physiological and pathophysiological conditions, as will be discussed later.

The functions of extracellular nucleotides in regulating osteoblast and osteoclast activity are not well understood: roles of ATP in osteoclastogenesis, osteoclastic resorption and osteoblastic proliferation, as well as inhibition of appositional bone formation, have been suggested. However, the role of individual receptor subtypes in bone remodelling has not been clearly identified.

CHAPTER 2

EXPRESSION OF P2 RECEPTORS IN BONE AND CULTURED BONE CELLS

INTRODUCTION

There is growing evidence that ATP may function as an important local signalling molecule in bone and cartilage. Cross-inhibition studies with different agonists suggested that a mixed population of P2 receptors exists on rat osteoblast-like cells (Yu and Ferrier, 1993b). Heterogeneity of receptor expression was also demonstrated within a population of human osteoblasts, probably reflecting differences in the differentiation status of individual cells (Dixon *et al.*, 1997). The existence of P2 receptors on chondrocytes was demonstrated by the work of Russell and colleagues (Caswell *et al.*, 1991; Leong *et al.*, 1994). Cultured osteoblasts, as well as chondrocytes, are capable of releasing ATP constitutively at concentrations that may activate P2 receptors in the local microenvironment (Hatori *et al.*, 1995; Bowler *et al.*, 1998b; Lloyd *et al.*, 1999).

Initial studies using cultured rabbit osteoclasts indicated that exogenous ATP was able to elicit an intracellular Ca^{2+} pulse via two different pathways: Ca^{2+} influx across the cell membrane via Ca^{2+} channels, and G protein-coupled internal Ca^{2+} release (Yu and Ferrier, 1993a; Yu and Ferrier, 1994). In addition, ATP induced a transient intracellular pH decrease in osteoclasts that was Ca^{2+}-independent (Yu and Ferrier, 1995). Electrophysiological experiments provided evidence for the co-expression of P2X and P2Y receptors on rat osteoclasts, ATP activating both non-selective cation channels and Ca^{2+}-dependent K^+ channels (Weidema *et al.*, 1997). However, nucleotide-induced elevation of $[Ca^{2+}]_i$ has now been suggested to arise primarily through activation of P2Y

receptors (Weidema *et al.*, 2001). Using *in situ* hybridisation, the work of Bowler and colleagues showed that the $P2Y_2$ receptor was expressed on osteoclasts derived from human giant cell tumours (Bowler *et al.*, 1995). The same group also reported that ATP exerted a small stimulatory effect on resorption pit formation by giant cell tumour osteoclasts, but that this effect was not mediated by the $P2Y_2$ receptor (Bowler *et al.*, 1998a). Recent functional studies showed the potent stimulatory effect of ATP on the formation and resorptive activity of normal mammalian osteoclasts (Morrison *et al.*, 1998). The stimulatory effect on resorption was amplified greatly when osteoclasts were activated by culture in acidified medium, which may be consistent with the involvement of the $P2X_2$ receptor subtype, the only receptor of this family that requires extracellular acidification to show its full sensitivity to ATP (King *et al.*, 1996).

However, despite the identification of numerous P2Y receptors on bone and osteoblasts by molecular studies (*in situ* hybridisation for $P2Y_2$ and RT-PCR for all mammalian P2Y receptors (Bowler *et al.*, 1995; Maier *et al.*, 1997; Bowler *et al.*, 1998a)), the exact distribution of P2 receptors in bone, especially the presence or absence of P2X receptors, remains to be characterised.

In this chapter, a novel set of polyclonal antibodies against $P2X_{1\text{-}7}$ receptors supplied by Roche Bioscience (Palo Alto, CA, USA), an anti-$P2Y_1$ antibody donated by Dr Matute (Leioa, Spain), new anti-$P2Y_{1,2,4}$ antibodies that have only recently become commerciably available from Alomone Labs (Jerusalem, Israel), and oligonucleotide probes corresponding to $P2X_{2,4}$ and $P2Y_{1,2,4,6}$ mRNAs were used to investigate the expression of P2 receptors in sections of neonatal rat bone and in cultured rat bone cells by immunohistochemistry and *in situ* hybridisation.

MATERIALS AND METHODS

Tissue Preparation

2-day-old neonatal Sprague-Dawley rats were killed by cervical dislocation. Long bones and calvariae were removed immediately, transferred to Hanks Balanced Salt Solution (HBSS), placed onto a cork block, embedded in Tissue-Tek® (Sakura Finetek, Netherlands), rapidly frozen in liquid nitrogen-cooled isopentane and stored in liquid nitrogen. Cryostat sections (10 μm) of undecalcified, unfixed bone were prepared and collected on gelatin-coated or polysine-coated slides (BDH/Merck, UK) for immunocytochemistry or *in situ* hybridisation, respectively. Sections were kept frozen until used, and air-dried at room temperature prior to use. Unless otherwise specified, all reagents were purchased from Sigma (UK).

Cell Culture

Primary rat osteoblastic cells were obtained by sequential enzyme digestion of excised calvarial bones from 2-day-old neonatal Sprague-Dawley rats using a 3-step process (1% trypsin for 10 minutes (min); 0.2 % collagenase type II for 30 min; 0.2 % collagenase type II for 60 min, all at 37°C), rejecting the first two digests. The cells were resuspended in Dulbecco's Modified Eagle Medium (DMEM) with Glutamax® + 10% foetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (GIBCO, UK), seeded at a concentration of $2x10^4$ cells/chamber on LabTek 8 chamber slides (Nunc Life Technologies, UK) and incubated in a humidified atmosphere of 5% CO_2 – 95% air for two to four days (d) until confluence. At confluence, these cells were mainly strongly positive for alkaline phosphatase, as assessed by cytochemical staining (Sigma Kit 86C).

Mixed cell populations containing osteoclasts were obtained by rapidly mincing the excised long bones of 2-day-old neonatal Sprague-Dawley rats (killed by cervical dislocation), in Minimum Essential Medium (MEM) + 10% FBS (GIBCO, UK), followed by vortexing. The resulting cell suspension was allowed to sediment for 60 min

onto LabTek 8 chamber slides. Chambers were rinsed twice with phosphate buffered saline (PBS) before incubation with MEM for 4 hours (h) in a humidified atmosphere of 5% CO_2 – 95% air. Cultures were fixed in 4% paraformaldehyde in 0.1 M PBS and processed for *in situ* hybridisation or immunocytochemistry.

Immunocytochemistry

The immunogens used for production of polyclonal P2X antibodies were synthetic peptides corresponding to 15 AA in the intracellular C-termini of the cloned rat P2X receptors, covalently linked to keyhole limpet haemocyanin. The peptide sequences are as follows:

$P2X_1$: AA 385-399, ATSSTLGLQENMRTS
$P2X_2$: AA 458-472, QQDSTSTDPKGLAQL
$P2X_3$: AA 383-397, VEKQSTDSGAYSIGH
$P2X_4$: AA 374-388, YVEDYEQGLSGEMNQ
$P2X_5$: AA 437-451, RENAIVNVKQSQILH
$P2X_6$: AA 357-371, EAGFYWRTKYEEARA
$P2X_7$: AA 555-569, TWRFVSQDMADFAIL

The polyclonal antibodies were raised by multiple monthly injections of New Zealand rabbits with the peptides (performed by Research Genetics, USA). The specificity of the antisera was verified by immunoblotting with membrane preparations from CHO K1 cells expressing the cloned $P2X_{1-7}$ receptors. Antibodies recognised only one protein of the expected size in the heterologous expression systems, and were shown to be receptor-subtype-specific (Oglesby *et al.*, 1999). Antibodies were kept frozen at a stock concentration of 1 mg/ml.

Polyclonal anti-$P2Y_1$, $P2Y_2$, and $P2Y_4$ antibodies were obtained from Alomone Labs (Israel), and corresponded to the third extracellular loop of the $P2Y_1$ (AA 242-258) and $P2Y_2$ receptor (AA 227-244), and to the intracellular C-terminus of the $P2Y_4$ receptor

(AA 337-350). Antibodies were kept frozen at a stock concentration of 0.6 mg/ml ($P2Y_1$, $P2Y_2$) or 0.3 mg/ml ($P2Y_4$).

For immunostaining of cryostat sections, the avidin-biotin technique was employed according to the protocol developed by Llewellyn-Smith *et al.* (1992, 1993). Air-dried serial sections of bones were fixed in 4% formaldehyde and 0.02% picric acid in 0.1 M phosphate buffer (pH 7.4) for 2 min. After washing in PBS for 20 min, endogenous peroxidase activity was blocked by treating the sections with 0.5% H_2O_2 and 50% methanol for 10 min. Non-specific binding sites were blocked by 20 min pre-incubation in 10% normal horse serum (NHS; Harlan Sera-Lab, UK) in PBS containing 0.05% merthiolate, followed by incubation with the primary antibodies diluted 1:100 or 1:200 in antibody diluent (10% NHS in PBS + 2.5% sodium chloride (NaCl)) at 4°C overnight. Subsequently, the sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch Lab, USA) diluted 1:500 in 1% NHS in PBS for 1 h, and then with ExtrAvidin peroxidase diluted 1:1000 in PBS for 1 h at room temperature. For colour reaction, a solution containing 0.05% 3,3'-diaminobenzidine 0.04% nickel ammonium sulfate, 0.2% β-D-glucose, 0.004% ammonium nitrate, and 1.2U/ml glucose oxidase in 0.01 M PBS was applied for 6 min. Contrast Green (Kirkegaard & Perry Laboratories, USA) was used as a counterstain. Sections were washed three times with PBS after each of the above steps except after the pre-incubation with 10% NHS. After the last wash, sections were dehydrated twice in isopropanol and mounted with Eukitt (BDH, UK). Control experiments were carried out with primary antibody, secondary or tertiary stages omitted from the staining procedure, and the primary antibodies pre-absorbed with the corresponding peptides. For the latter, 1.5 µl of anti-P2X antibodies (at 1 mg/ml) were incubated overnight at 4°C with 24 µl of the respective peptide at 5 mg/ml. 275 µl of NHS was added to give 300 µl of a 5 µg/ml concentration of the antibodies. This solution was centrifuged for 15 min at 13,000 rpm and the supernatant used for immunohistochemistry.

For immunofluorescent staining of the cells with the $P2X_{1\text{-}7}$ and $P2Y_{1, 2, 4}$ antibodies, fixed cells were treated with methanol at -20°C for 7 min. The cells were pre-incubated

in 10% NHS in PBS for 30 min at room temperature, followed by incubation with the primary antibodies diluted 1:100 to 1:200 in 10% NHS in PBS at 4°C overnight. Biotinylated donkey anti-rabbit IgG, diluted 1:500 in 1% NHS in PBS, was applied for 1 h followed by fluorescein- or Texas Red-labelled streptavidin (Amersham International, UK) diluted 1:200 in PBS for 1 h at room temperature.

The affinity purified anti-$P2Y_1$ antibody was a kind donation of Dr Carlos Matute, Leioa, Spain (Moran-Jimenez and Matute, 2000). For immunostaining with this antibody, fixed cultured osteoclasts were pre-incubated in 10% NHS and 0.1 % Triton X-100 in PBS for 30 min at room temperature, followed by overnight incubation at 4°C with 2 μg/ml anti-$P2Y_1$ in the same solution. Biotinylated donkey anti-rabbit IgG, diluted 1:500 in 1 % NHS in PBS and 0.1 % Triton X-100, was applied for 1 h, followed by fluoresceinated streptavidin, diluted 1:200 in PBS for 1 h at room temperature. Control experiments were carried out with antiserum which had been preabsorbed overnight at 4°C with the immunogenic peptide (15 μM = 31 μg/ml) (Moran-Jimenez and Matute, 2000).

Western blot

Specificity of the $P2X_2$ antibody was tested by immunoblotting with rat calvarial osteoblastic cell extracts. Cells were grown to confluence as described above, rinsed with PBS and lifted from the culture plates with PBS containing 0.2% ethylen-diamine tetraacetic acid (EDTA), and spun down; the cell pellet was resuspended in 1ml of ice cold RIPA buffer (PBS containing 1% Nonidet, 0.5% deoxycholate, 0.1% sodium dodecylsulphate (SDS), 0.1mg/ml phenylmethylsulfonylfluoride, 30μl/ml aprotinin and 1mM sodium orthovanadate). These lysates were passed through a 21-gauge needle to sheer DNA and were then spun for 10 min, at 4°C. The supernatants were used for Western blotting in a Mini-Protean 2 Electrophoresis and Trans-Blotting Cell (Bio-Rad, USA), according to the manufacturer's instructions.

Proteins were loaded on Tris-HCl Ready Gels (10% gel) (Bio-Rad) and run under reducing conditions (10% SDS and 26 mM dithiothreitol). Biotinylated molecular

weight markers were obtained from Sigma (B2787). Proteins were transferred onto a Hybond ECL-nitrocellulose membrane (Amersham International, UK). The nitrocellulose was blocked at room temperature in PBS containing 3% milk powder and 0.05% Tween 20, and then incubated in the same solution containing 2.5μg/ml $P2X_2$ antibody overnight at 4°C. For detection, the ECL chemiluminescence method was performed, using a biotinylated donkey anti rabbit IgG, peroxidase-linked streptavidin and ECL Western Blotting reagents (all purchased from Amersham International, UK). The signal was visualised on a Hyperfilm ECL and scanned with an Umax Powerlook 2 flatbed scanner.

The Western blot was kindly performed by Rainer Glass at the ANI.

In situ hybridisation

Anti-sense oligonucleotides (45-mer) directed against receptor subtype-specific sequences were designed for use in *in situ* hybridisation experiments. These sequences correspond to the C-terminal 15 AA of the rat $P2X_2$ and $P2X_4$ subtypes, the peptidic sequences used for the generation of the anti-peptide antibodies, and against the third extracellular domain of the rat $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ subtypes. The oligonucleotide sequences are as follows:

$P2X_2$: 5'-AGTTGGGCCAAACCTTTGGGGTCCGTGGATGTGGAGTCCTGTTG-3'
$P2X_4$: 5'-CTGGTTCATCTCCCCCGAAAGACCCTGCTCGTAGTCTTCCACATA-3'
$P2Y_1$: 5'-AGGTGGCATAAACCCTGTCGTTGAAATCACACATTTCTGGGGTCT-3'
$P2Y_2$: 5'-GATGGCGTTGAGGGTGTGGCAACTGAGGTCAAGTGATCGGAAGGA-3'
$P2Y_4$: 5'-GACAATGTTCAGCACATGACAGTCAGCTTGCAACAGTCTTGCCTG-3'
$P2Y_6$: 5 –TGCCTTTGTAGGCTGCAGCGAAGGTCTCCAGTACCGGGCAAGAGA-3'

The above primers were labelled at the 3' end with digoxigenin dUTP using an oligonucleotide tailing kit (Roche Diagnostics, UK). Digoxigenin, a naturally occurring plant steroid, is not found in animal tissues, so cytoplasmic localisation of the immunoproduct is considered to be specific.

Following fixation in 4% formaldehyde in PBS for 10 min, slides containing sections or cultured cells (LabTek chamber slides) were dehydrated in graded ethanol and air-dried. The hybridisation buffer contained 2x SSC buffer (GIBCO, UK), 0.1mg/ml sheared and denatured salmon sperm DNA, 0.1mg/ml tRNA, 50% deionized formamide, 1x Denhardt's solution and 1 ng/μl digoxigenin-labelled probe. Before hybridisation, pre-hybridisation was done at 37°C for 2 h in a humidified chamber in hybridisation buffer without digoxigenin-labelled probe. The slides were then incubated at the same temperature for 16 h with digoxigenin-labelled probe.

After washing with decreasing salt solutions (twice with 2x SSC for 5 min at room temperature, twice with 2x SSC for 15 min at 37°C, twice with 1x SSC for 15 min at 37°C, twice with 0.5x SSC for 30 min at 37°C), slides were blocked in 2% normal sheep serum (NSS) in wash buffer (0.1 M Tris-HCl, 0.15 M NaCl; pH 7.4) for 2 h at room temperature. They were then incubated with anti-digoxigenin antibody (diluted 1:1000 to 1 μg/ml in 2% NSS in wash buffer) conjugated with alkaline phosphatase for 2 h. The colour reaction was made with 45μl 4-nitro blue tetrazolium salt, 35μl 5-bromo-4-chloro-3-indolyl-phosphate solution in 10ml detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M $MgCl_2$; pH 9.5) in the dark for up to 16 h. Negative controls were performed by hybridising in the presence of 100-fold excess of unlabelled probe, and by hybridising without adding the labelled probe.

RESULTS

Immunocytochemical localisation of $P2X_{1-7}$ receptors

Sections of neonatal rat long bones and calvariae showed different staining patterns with each P2X receptor antibody. In long bones and calvariae, osteoblasts on bone surfaces and chondrocytes showed specific immunostaining for $P2X_2$ and $P2X_5$ receptors, whereas in control preparations pre-treated with the relevant P2X-peptide, immunostaining was abolished or greatly reduced (**Figs. 2.1 F,G and 2.4 A,B,C,D**).

Specific immunostaining for the $P2X_1$ receptor was only observed in smooth muscle cells of blood vessels. No specific immunostaining for either $P2X_3$, $P2X_4$ or $P2X_6$ receptors was seen in sections of long bones and of calvariae; however, adjacent skeletal muscle tissue was strongly positive for $P2X_6$. Antibodies against $P2X_7$ produced no staining in bone, but strong staining in the adjacent keratinising and exfoliating layers of skin.

Fluorescence techniques revealed immunostaining for P2X receptors in osteoclasts isolated from neonatal rat long bones, and in primary rat calvarial osteoblastic cells. In osteoclasts, the most prominent immunostaining was observed with the $P2X_2$ antibody (**Fig. 2.1 A,B**); in controls pre-treated with the $P2X_2$ peptide, staining was completely abolished (**Fig. 2.1 D**). Cytoplasmic staining was observed using the $P2X_4$ antibody in osteoclasts (**Fig. 2.3 D**). A small proportion of cultured osteoclasts also showed nuclear staining with anti-$P2X_4$ antibody. However, in control preparations, the cytoplasmic staining was abolished, whereas nuclear staining remained, indicating that this was non-specific (**Fig. 2.3 E**). Strong nuclear staining was seen with the $P2X_7$ antibody (**Fig. 2.5 A**); in control preparations the reaction was greatly reduced (**Fig. 2.5 B**). No specific immunostaining was observed for $P2X_1$, $P2X_3$, $P2X_5$ or $P2X_6$ receptors in cultured osteoclasts.

Rat osteoblastic cells cultured from neonatal rat calvariae showed strong staining with anti-$P2X_2$ and anti-$P2X_5$ antibodies (**Fig. 2.4 E**); in controls pre-treated with the

corresponding peptides, staining was completely abolished or reduced, respectively (**Fig. 2.4 F**). No specific immunostaining was observed for $P2X_1$, $P2X_3$, $P2X_4$, $P2X_6$ or $P2X_7$ receptors in cultured osteoblasts.

To verify the novel finding of abundant $P2X_2$ expression in bone, immunoblotting was performed with rat calvarial osteoblastic cells. A single $P2X_2$-reactive band at approximately 70-80 kDa was detected (**Fig. 2.2**). This band was in the expected weight range, as determined by immunoblotting with membrane fractions of cell lines expressing recombinant $P2X_2$ receptors (Oglesby *et al.*, 1999).

In situ hybridisation

The expression of $P2X_2$, $P2X_4$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ mRNAs was investigated in sections of rat long bone and calvariae, and in cultured rat osteoclasts and osteoblastic cells. *In situ* hybridisation on long bone and calvarial sections, using $P2X_2$, $P2Y_1$ and $P2Y_2$ receptor probes, revealed intense, specific localisation over osteoblasts on bone surfaces and over chondrocytes, both in the growth plate in long bones and in cartilage in the skull (**Figs. 2.1 H, 2.6 E and 2.7 A**). Use of the $P2X_4$, $P2Y_4$ and $P2Y_6$ receptor mRNA probes resulted only in weak signals that were not consistently detectable in bone sections (**Fig. 2.3 A**). Negative controls of serial sections performed by hybridising in the presence of 100-fold excess of unlabelled probe significantly reduced the signals for all probes (**Figs. 2.1 K, 2.6 F and 2.7 B**).

On rat osteoclasts, *in situ* hybridisation revealed intense specific localisation of $P2X_2$, $P2X_4$, $P2Y_1$ and $P2Y_2$ receptor mRNAs (**Figs. 2.1 C, 2.3 B, 2.6 A,B and 2.7 C,)**, whereas the $P2Y_4$ and $P2Y_6$ receptor probes and negative controls showed significantly less staining (**Figs. 2.1 E, 2.3 C, 2.6 C and 2.7 D**). $P2X_2$, $P2Y_1$ and $P2Y_2$ receptor mRNAs were also highly expressed on primary rat calvarial osteoblastic cells (**Fig. 2.7 E**), in contrast with $P2X_4$, $P2Y_4$ and $P2Y_6$ receptor mRNAs, which were undetectable. Negative controls showed reduced staining of cultured osteoblasts (**Fig. 2.7 F**).

Immunocytochemical localisation of $P2Y_{1,2,4}$ receptors

Due to the limited availibility of the anti-$P2Y_1$ antibody donated from Dr Matute (Leioa, Spain), the expression of the $P2Y_1$ receptor protein was originally only studied in cultured rat osteoclasts. Immunocytochemistry revealed intense specific localisation of $P2Y_1$ receptor protein in cultured osteoclasts (**Fig. 2.6 D**).

However, recently, anti-$P2Y_{1,2,4}$ antibodies have become commercially available and thus been tested on bone sections and cultured osteoblasts. They confirm the findings of the *in situ* hybridisation results: osteoblasts, both in culture and in sections, and chondrocytes show positive staining for $P2Y_1$ (**Fig. 2.6 G,H**) and $P2Y_2$ (**Fig. 2.7 G,H**) receptors, whereas no staining was observed with the $P2Y_4$ antibody. Negative controls showed greatly reduced staining.

The results of this chapter are summarised in Table 2.1.

Cell type	Receptor expression
Osteoclasts	$P2X_2$ $P2X_4$ $P2X_7$ $P2Y_1$ $P2Y_2$
Osteoblasts	$P2X_2$ $P2X_5$ $P2Y_1$ $P2Y_2$
Chondrocytes	$P2X_2$ $P2X_5$ $P2Y_1$ $P2Y_2$

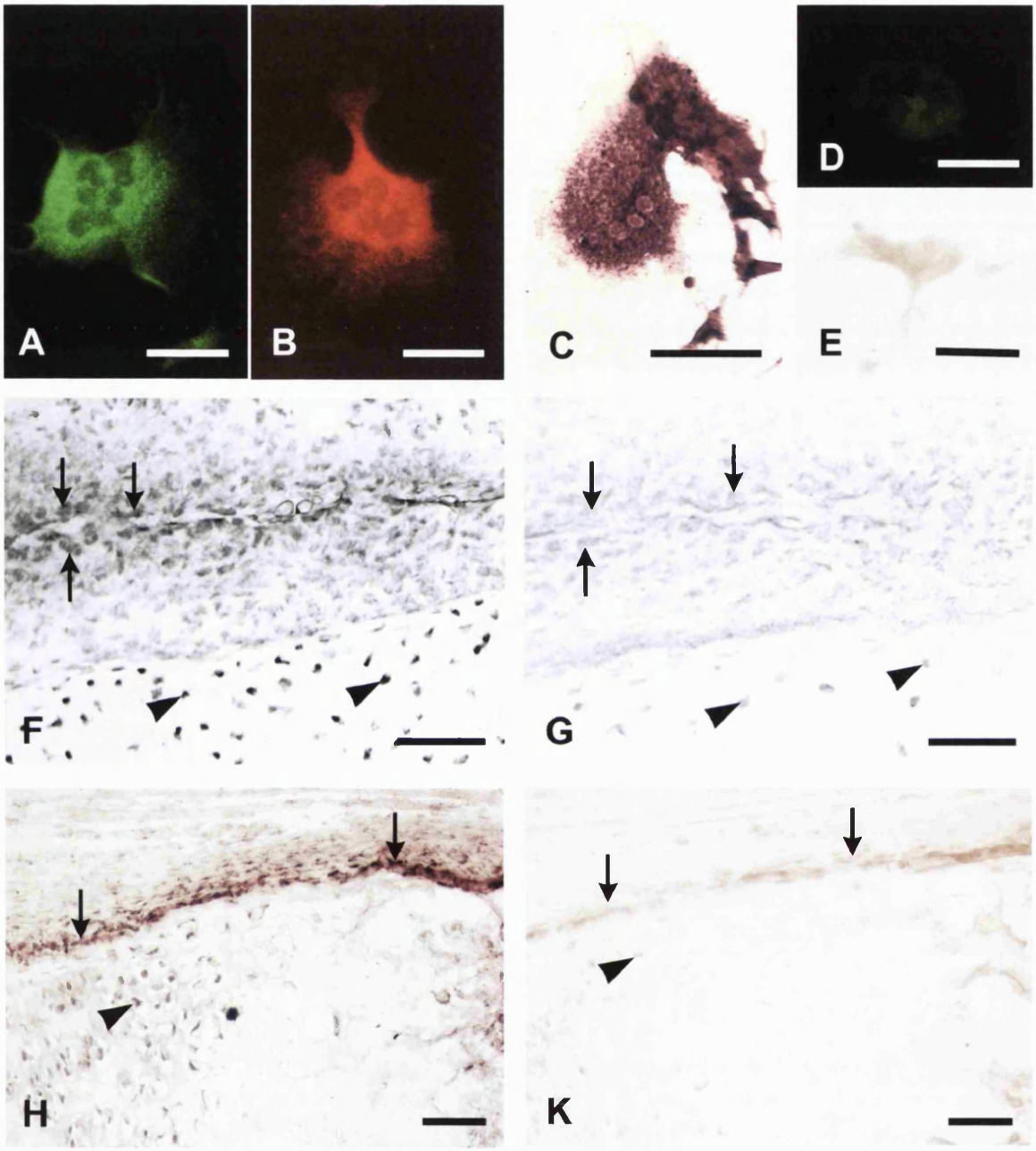
Table 2.1

Expression of P2X and P2Y receptors on bone cells

Figure 2.1

Localisation of $P2X_2$ receptor subtype on bone sections and bone cells.

(A, B, D) Specific immunostaining of cultured rat osteoclasts with anti-$P2X_2$ antibody, visualised with fluorescein (A) or Texas Red (B), which was abolished after pre-absorption of anti-$P2X_2$ antibody with $P2X_2$ peptide (D). **(C, E)** Specific *in situ* localisation of $P2X_2$ receptor probe on cultured rat osteoclast (C) was almost abolished in control preparations hybridised in the presence of excess unlabelled probe (E). **(F, G)** Serial sections of rat calvaria showing immunostaining of osteoblasts (arrows) and chondrocytes (arrowheads) with anti-$P2X_2$ antibody (F); immunostaining of osteoblasts (arrows) and chondrocytes (arrowheads) in control rat calvarial section was almost abolished after pre-absorption of anti-$P2X_2$ antibody with $P2X_2$ peptide (G). **(H, K)** Specific *in situ* localisation of $P2X_2$ receptor probe on osteoblasts (arrows) and growth plate chondrocytes (arrowheads) in rat long bone section (H); localisation was greatly reduced in control preparations hybridised in the presence of excess unlabelled probe (K). Scale bars = 25 μm (A,B,D,E) and 50 μm (C,F,G,H,K).


A
B
C
D
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H
K

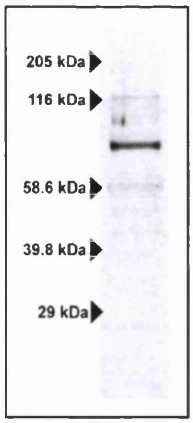


Figure 2.2

Western blotting of rat calvarial osteoblastic cell lysates.

The blot was probed with polyclonal anti-rat $P2X_2$ antibody. A single $P2X_2$-reactive band of approximately 70-80 kDa was detected. Molecular weight markers are indicated on the left side.

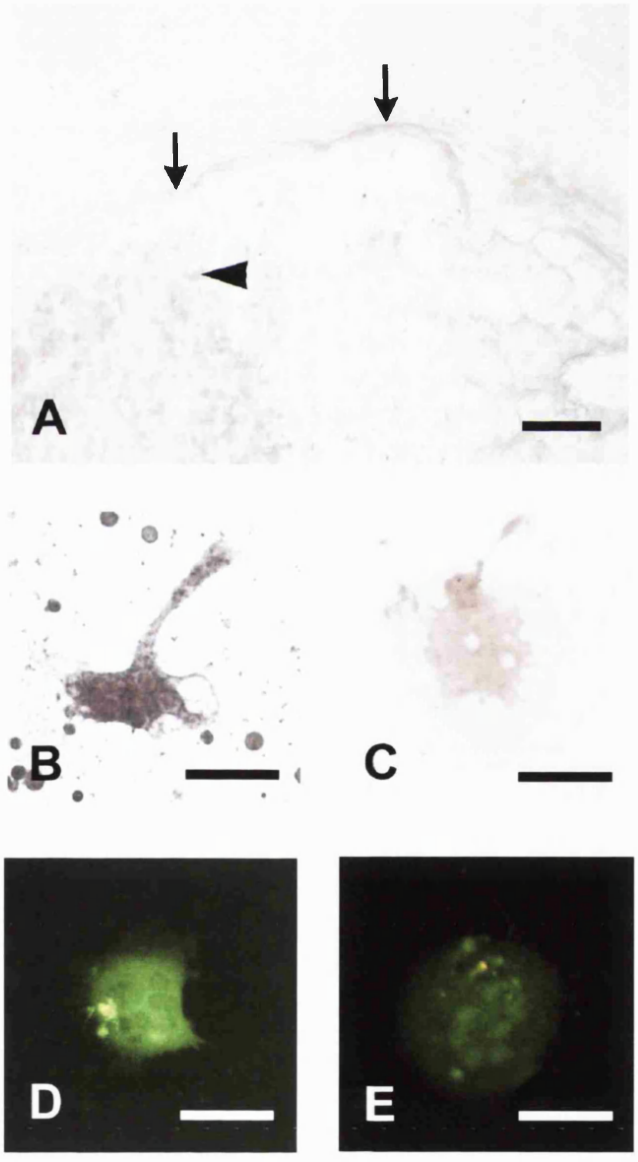


Figure 2.3

Localisation of $P2X_4$ receptor subtype on rat osteoclasts.

(**A**) *In situ* hybridisation on rat long bone sections revealed no localisation of $P2X_4$ receptor mRNA on osteoblasts (arrow) or chondrocytes (arrowhead). (**B**) Specific *in situ* localisation of $P2X_4$ receptor probe on cultured rat osteoclast. (**C**) Control rat osteoclast hybridised in the presence of excess unlabelled probe showed greatly reduced localisation of $P2X_4$ receptor probe. (**D**) Strong cytoplasmic immunostaining on cultured rat osteoclast with anti-$P2X_4$ antibody, visualised with fluorescein. (**E**) Reduced anti-$P2X_4$ immunostaining in control rat osteoclast preparations pre-absorbed with $P2X_4$ peptide. Scale bars = 50μm (A) and 25μm (B-E).

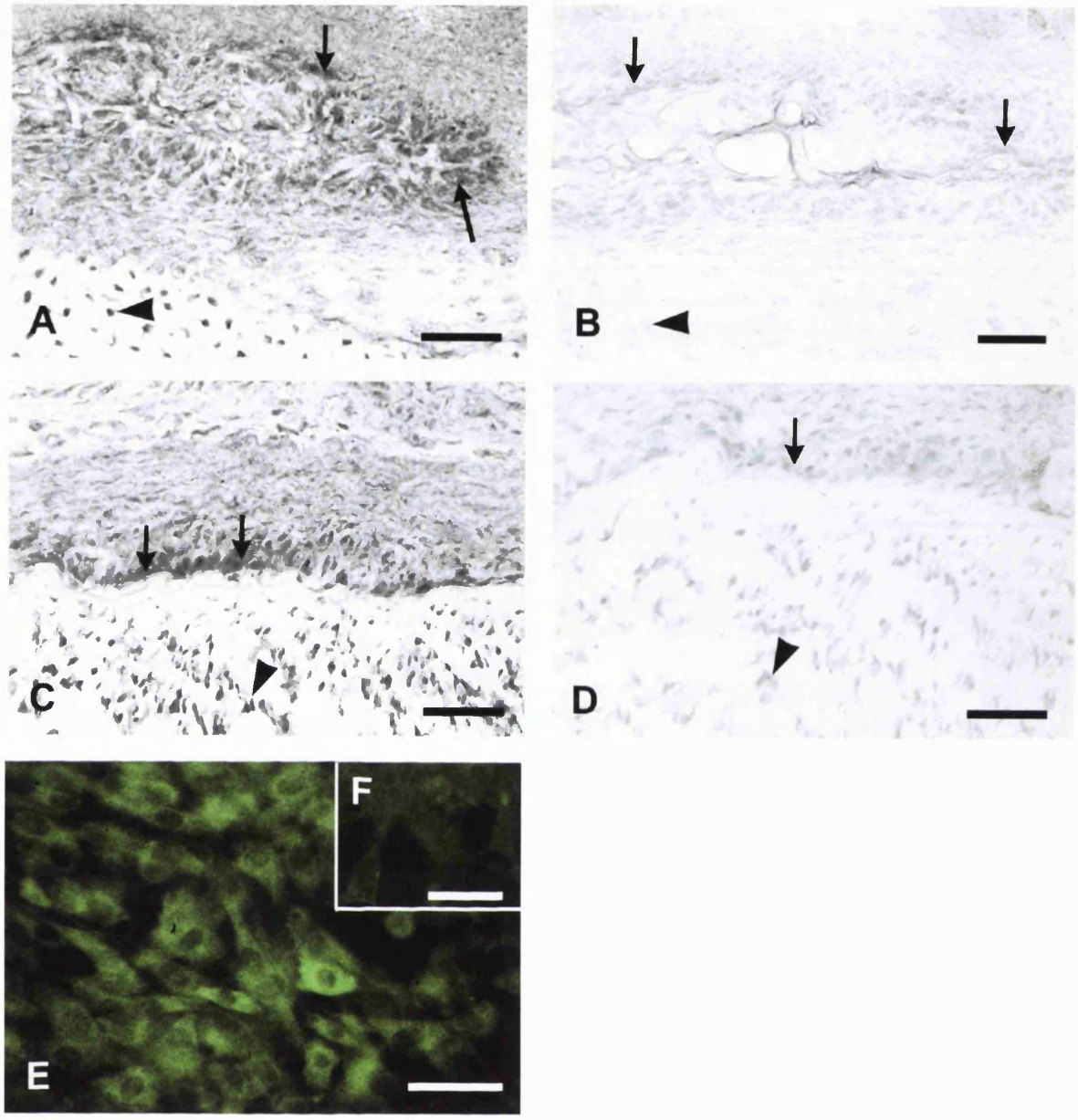


Figure 2.4

Localisation of $P2X_5$ receptor subtype on rat bone sections and cultured rat osteoblasts.

(**A, B, C, D**) Immunostaining with anti-$P2X_5$ antibody on serial sections of rat calvariae (A, B) and rat long bone (C, D). (A, C) Localisation of immunostaining on osteoblasts (arrows) lining the bone surface, and on chondrocytes (arrowheads), which was almost abolished in control sections (B, D) pre-absorbed with $P2X_5$-peptide. (**E**) Strong immunostaining in cultured rat calvarial osteoblastic cells with anti-$P2X_5$ antibody, visualised with fluorescein. (**F**) Immunostaining on cultured osteoblastic cells was greatly reduced in control preparation pre-absorbed with $P2X_5$-peptide. Scale bars = 50μm (A-F).

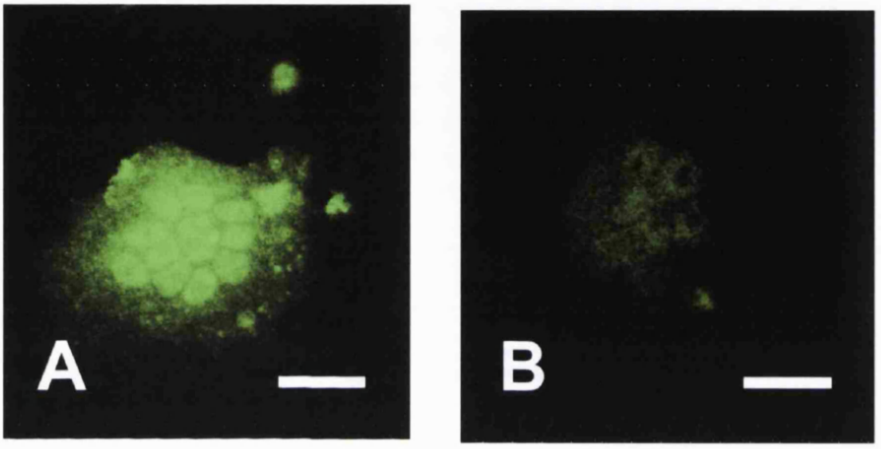


Figure 2.5

Localisation of $P2X_7$ receptor subtype on cultured rat osteoclast, visualised with fluorescein.

(**A**) Strong nuclear immunostaining of rat osteoclasts with anti-$P2X_7$ antibody, which was almost abolished in control cell (**B**) pre-absorbed with $P2X_7$-peptide. Scale bars = 25μm (A, B).

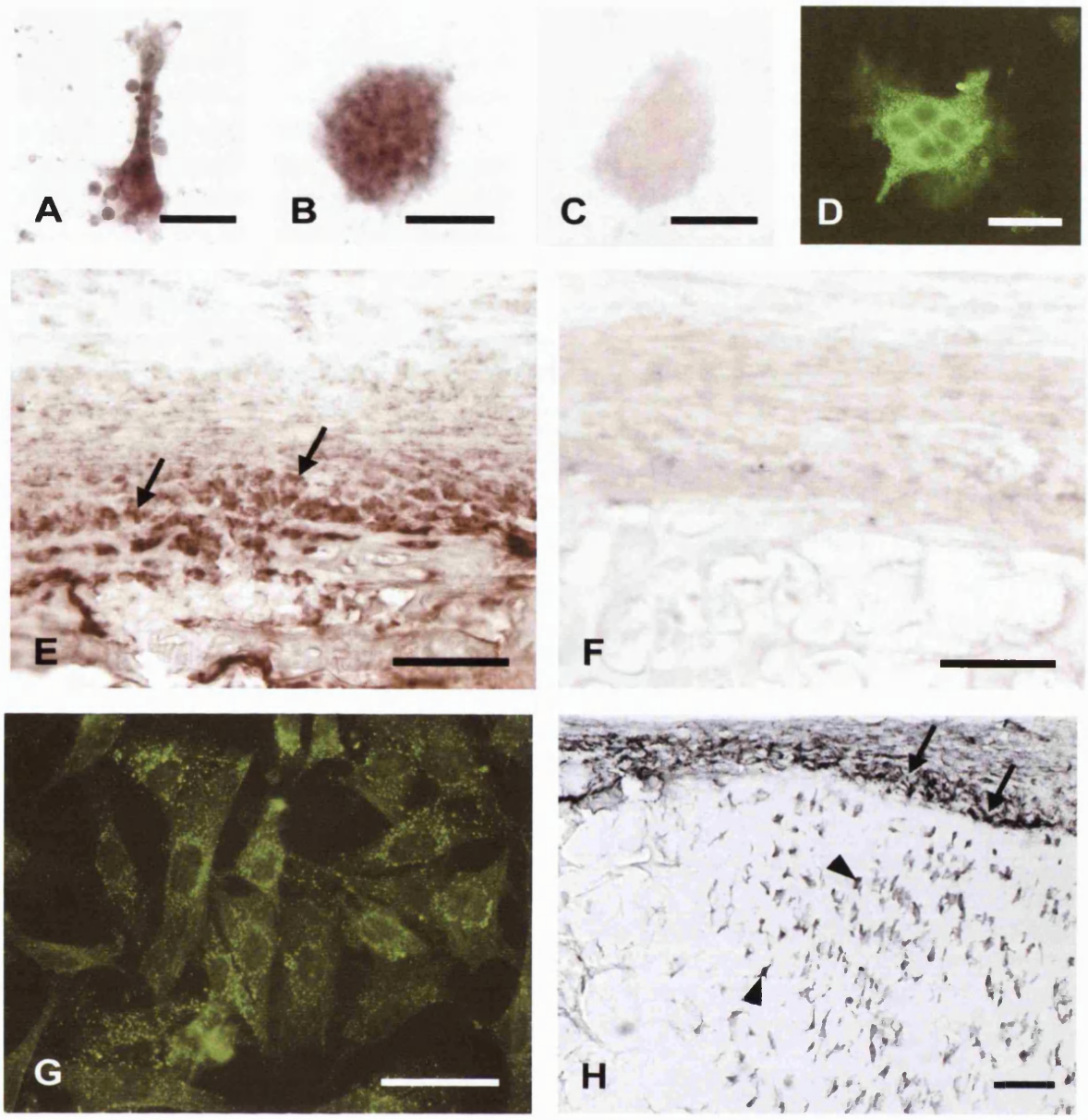


Figure 2.6

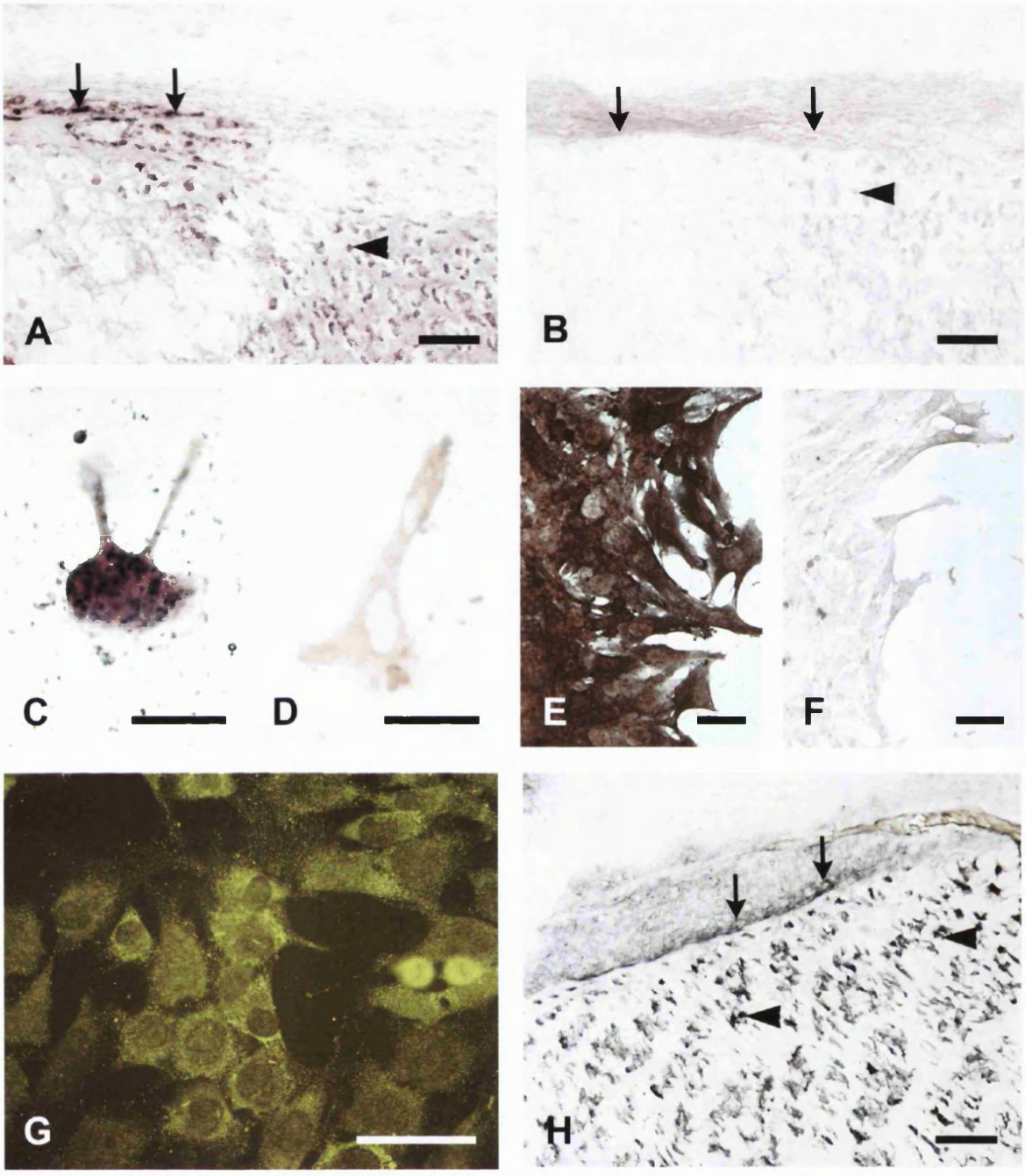
Localisation of $P2Y_1$ receptor subtype in rat long bone sections and on cultured bone cells.

(**A, B, E**) Specific *in situ* localisation of $P2Y_1$ receptor probe on cultured rat osteoclasts (A, B) and on osteoblasts lining the bone surface (arrows) in frozen rat long bone section (E). (**C, F**) Localisation on cultured rat osteoclast (C) and on osteoblasts in long bone section (F) was reduced greatly in control preparations hybridised in the presence of excess unlabelled probe. (**D**) Immunocytochemistry with $P2Y_1$ antibody (donation from Dr Matute, Spain) revealed specific staining in cultured rat osteoclast. (**G, H**) Specific immunostaining in rat osteoblasts with anti-$P2Y_1$ antibody (Alomone Labs), both in culture of primary rat calvarial osteoblasts, visualised with fluorescein (G), and on rat long bone sections (arrows) (H). Chondrocytes in long bone sections showed positive immunostaining as well (arrowheads) (H). Scale bars = 20 μm (A,B,C,D) and 50 μm (E, F, G, H).

Figure 2.7

Localisation of $P2Y_2$ receptor subtype in rat long bone sections of and on cultured bone cells.

(**A**) Specific *in situ* localisation of $P2Y_2$ receptor probe on osteoblasts lining the bone surface (arrows) and on chondrocytes in the growth plate (arrowhead). (**B**) Signal was greatly reduced in osteoblasts (arrows) and chondrocytes (arrowhead) in control preparation hybridised in the presence of excess unlabelled $P2Y_2$ probe. (**C, D**) Specific *in situ* localisation of $P2Y_2$ receptor probe on cultured rat osteoclast (C), which was almost abolished in control (D) hybridised in the presence of excess unlabelled $P2Y_2$ probe. (**E, F**) Specific *in situ* localisation of $P2Y_2$ receptor probe on cultured primary osteoblastic cells from rat calvaria (E), which was almost abolished in control (F) hybridised in the presence of excess unlabelled $P2Y_2$ probe. (**G, H**) Strong immunostaining in rat calvarial osteoblastic cells with anti-$P2Y_2$ antibody, both in culture, visualised with fluorescein (G), and in rat long bone sections (arrows) (H). Chondrocytes in long bone sections were immunopositive as well (arrowheads) (H). Scale bars = 50 μm (A,B,G,H) and 25μm (C,D,E,F).


A
B
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DISCUSSION

In this study the first direct evidence for the expression of the $P2X_2$ receptor in bone cells was obtained, using immunocytochemistry and *in situ* hybridisation techniques. Earlier studies on the general distribution of P2X subunits suggested that the $P2X_2$ subunit is located primarily in neural tissues in the central and peripheral nervous systems, but it has now been found to be present in other tissues, including vascular smooth muscle (King, 1998). The present study demonstrates immunostaining and *in situ* hybridisation signals for the $P2X_2$ receptor on bone sections and on cultured bone cells, including osteoclasts and primary cultures of osteoblastic rat calvarial cells. The finding of the $P2X_2$ receptor subtype on osteoclasts, shown by two independent methods, is of particular interest and will be discussed further in Chapter 5.

In addition to $P2X_2$, the expression of the $P2X_4$ receptor subtype was also detected in osteoclasts by immunocytochemistry and *in situ* hybridisation, in agreement with a recent report that detected $P2X_4$ receptor mRNA in rabbit osteoclasts by PCR (Naemsch *et al.*, 1999). This study also reported electrophysiological evidence for the $P2X_4$ receptor: ATP caused a rapidly activated, non-selective inward cation current, which was insensitive to suramin and potentiated by Zn^{2+}. These characteristics are consistent with the $P2X_4$ receptor, and the authors thus concluded that $P2X_4$ might mediate the stimulatory effect of ATP on bone resorption. However, their results were not entirely conclusive because ADP, α,β-meATP and β,γ-meATP, which are not agonists at the $P2X_4$ receptor, also evoked inward currents in a subpopulation of osteoclasts (Naemsch *et al.*, 1999), pointing to the involvement of other receptor subtypes, and Zn^{2+} has also been shown to potentiate responses of ATP at the $P2X_2$ receptor (Wildman *et al.*, 1998).

My immunocytochemical studies also revealed positive immunostaining for the $P2X_5$ receptor subtype on osteoblasts, both in culture and in bone sections. This agrees with a recent study demonstrating $P2X_5$ expression in human osteoblast-like cells by PCR; however, in contrast to my results, $P2X_4$, $P2X_6$ and $P2X_7$ expression were also reported, but no evidence for $P2X_1$, $P2X_2$ and $P2X_3$ was found. Based on additional experiments,

the authours suggested that the $P2X_5$ receptor might be responsible for the mitogenic effects of ATP on osteoblasts (Nakamura *et al.*, 2000). Earlier studies of stratified epithelia showed that $P2X_5$ immunoreactivity was indeed restricted to the metabolically active, differentiating cell layers in epithelia and hair follicles, while $P2X_7$ receptors were associated with keratinising cells undergoing cell death (Gröschel-Stewart *et al.*, 1999). Thus, $P2X_5$ receptors may also participate in the regulation of osteoblastic differentiation and proliferation. The effects of ATP on osteoblastic function will be further explored in Chapter 4.

Cultured osteoclasts showed nuclear immunostaining for the $P2X_7$ receptor subtype. $P2X_7$ (formerly P2Z) is a bifunctional receptor which, in the absence of divalent cations, mediates the formation of large cytolytic membrane pores. The function of this receptor has thus been associated with lytic and apoptotic events (Surprenant *et al.*, 1996; Burnstock, 1997). Osteoclasts, and recently also human osteoblasts, have been reported to die in a manner reminiscent of apoptosis when exposed to high concentrations of ATP (1-2 mM) (Morrison *et al.*, 1998; Gartland *et al.*, 2001b). The latter study also described the expression of $P2X_7$ at the mRNA and protein level on primary human osteoblasts and SaOS-2 cells (human osteosarcoma cell line). This contrasts with my results, finding no evidence for $P2X_7$ expression on rat osteoblasts, and with an earlier study reporting that high ATP concentrations caused formation of pores in murine osteoclasts and macrophages, but not in osteogenic or chondrogenic cells (Modderman *et al.*, 1994).

However, apart from programmed cell death, $P2X_7$ receptors could also be involved in a different, crucial process in osteoclast biology: the receptor has been implicated in the formation of multinucleated giant cells by mediating the fusion of murine macrophage-like cells (Chiozzi *et al.*, 1997). It is therefore conceivable that fusion of osteoclast (and/or macrophage-) precursors is initiated by $P2X_7$-mediated pore formation in the membranes of adjacent cells, which could lead to the development of cytoplasmic bridges and cell fusion. Preliminary results on osteoclasts do indeed support this hypothesis (Gartland *et al.*, 2001a). Similar to my findings, a nuclear localisation of the $P2X_7$ receptor subtype has recently been reported for epithelial cells using the same

polyclonal antibody (Gröschel-Stewart *et al.*, 1999). The functional significance of this observation is still under investigation, but nuclear or perinuclear expression might indicate that the receptor remains internalised until intracellular signals are received for its functional expression in the membrane. This could prevent apoptosis of fully functional cells.

There is electrophysiological evidence for the co-expression of both P2X and P2Y receptors on rat osteoclasts (Weidema *et al.*, 1997). The results presented here agree with these findings. Localisation of $P2Y_2$ receptor mRNA in bone sections and its expression not only by osteoblasts, but also by osteoclasts and chondrocytes, is demonstrated. These findings are consistent with earlier work which detected $P2Y_2$ mRNA in osteoclastoma giant cells by *in situ* hybridisation, and in human bone-derived cells by PCR (Bowler *et al.*, 1995). They are also in agreement with studies on the effects of extracellular nucleotides on intracellular Ca^{2+} levels in osteoblast-like cells, both of human and rat origin, that suggested the existence of two different P2 receptor subtypes (including $P2Y_2$), linked to Ca^{2+} mobilisation (Reimer and Dixon, 1992; Schöfl *et al.*, 1992).

The finding that $P2X_2$, $P2X_5$, $P2Y_1$ and $P2Y_2$ receptors were strongly expressed by osteoblasts, both in bone sections and *in vitro*, implies that exogenous ATP could therefore stimulate resorption via direct effects on mature osteoclasts, or via indirect effects on other bone cells such as osteoblasts, or both. Osteoblasts have been shown to release ATP under shear stress conditions via a non-lytic mechanism, which could be inhibited by glibenclamide (Bowler *et al.*, 1998b). The failure to detect $P2Y_4$ expression in rat osteoblastic cells contrasts with the recent identification of $P2Y_4$ transcripts in human osteoblastic cells by PCR (Maier *et al.*, 1997); this may reflect a species difference or changes in cellular differentiation states.

The localisation studies also showed the presence of P2 receptors in chondrocytes, both P2X ($P2X_2$, $P2X_5$) and P2Y ($P2Y_1$, $P2Y_2$). This is the first evidence for the existence of P2X receptors in chondrocytes, and their functional role will need to be explored. However, the finding of both $P2Y_1$ and $P2Y_2$ is in agreement with suggestions

made some time ago. First studies on the role of P2 receptors in chondrocytes showed that ATP and ADP, and less strongly UTP, stimulate the production of PGE by cultured human chondrocytes (Caswell *et al.*, 1991). This ATP-induced PGE release was enhanced by the pro-inflammatory cytokines IL-1β and TNF-α (Caswell *et al.*, 1992; Leong *et al.*, 1993), and as shown more recently, also by IL-1α (Koolpe *et al.*, 1999). This might provide a mechanism by which nucleotides, in synergism with pro-inflammatory cytokines, could contribute to the pathophysiology of arthritic conditions. Additionally, extracellular ATP and UTP, but not ADP, have been shown to stimulate cartilage resorption, as monitored by proteoglycan breakdown from cultures of bovine nasal cartilage (Leong *et al.*, 1990; Leong *et al.*, 1994; Brown *et al.*, 1997); again, this was enhanced by simultaneous application of IL-1β and TNF-α.

Later studies confirmed the synergistic actions of nucleotides on chondrocytes: extracellular nucleotides enhance FGF-induced proliferation of sheep growth plate chondrocytes. ATP and UTP were more effective than ADP and 2-meSATP, which is consistent with the involvement of the $P2Y_2$ or $P2Y_4$ receptor subtypes (Kaplan *et al.*, 1996). Most of the ATP effects appear to be mediated by transient elevation of $[Ca^{2+}]_i$ (Kaplan *et al.*, 1996; Koolpe *et al.*, 1997); oscillations of $[Ca^{2+}]_i$ in response to ATP have also been reported for human chondrocytes (D'Andrea and Vittur, 1996). Further evidence for P2Y receptors was observed in both studies: responses to ATP were still seen upon removeal of extracellular Ca^{2+}, and were abolished by thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, thus causing depletion of intracellular Ca^{2+} stores.

As for osteoblasts, cultured chondrocytes are also capable of constitutively releasing ATP at concentrations in the micromolar range which may activate P2 receptors in the local microenvironment (Hatori *et al.*, 1995; Lloyd *et al.*, 1999). P-glycoprotein, a member of the ABC protein family, is expressed in hypertrophic chondrocytes (Mangham *et al.*, 1996) and could play a role in releasing ATP. However, exogenous ATP has been shown to be rapidly hydrolysed to pyrophosphate in resting chondrocyte cultures due to the presence of ecto-nucleotidases, especially of E-NPPs (Caswell and

Russell, 1985; Graff *et al.*, 2000). In contrast to osteoblasts, extracellular ATP has not yet been shown to exert a mitogenic response in chondrocytes on its own (Hatori *et al.*, 1995), but only to enhance FGF-induced proliferation (Kaplan *et al.*, 1996). Interestingly, the acquisition of purinergic receptors appears to be associated with expression of the mature, post-mitotic chondrocyte phenotype because immature chondrocytes, freshly isolated from the chick embryonic sternum, failed to respond to ATP (Hung *et al.*, 1997).

In summary, the immunohistochemical and *in situ* hybridisation results presented in this chapter are consistent with a wide range of earlier experimental findings and demonstrate for the first time the presence of $P2X_2$ receptors on osteoblasts and osteoclasts. The identification of $P2X_2$ receptors on osteoclasts provides a possible mechanism for the critical activation of osteoclasts at low pH (see Chapter 5). Evidence for a possible function of the $P2Y_1$ and $P2Y_2$ receptors on osteoclasts and osteoblasts will be provided in the next two chapters.

CHAPTER 3

EXTRACELLULAR ADP IS A POWERFUL OSTEOLYTIC AGENT: EVIDENCE FOR SIGNALLING THROUGH THE $P2Y_1$ RECEPTOR ON BONE CELLS

INTRODUCTION

As shown by immunocytochemistry and *in situ* hybridisation in Chapter 2, both osteoblasts and osteoclasts express several P2 receptors, both of the P2X and P2Y families. This confirmed earlier findings that both osteoclasts and osteoblasts respond to extracellular nucleotides with a rise in intracellular Ca^{2+} (Reimer and Dixon, 1992; Schöfl *et al.*, 1992; Yu and Ferrier, 1993b; Yu and Ferrier, 1994; Weidema *et al.*, 1997) and, in the case of osteoclasts, also with a decrease in intracellular pH (Yu and Ferrier, 1995).

It has previously been shown that ATP is capable of stimulating osteoclastic bone resorption (Morrison *et al.*, 1998), and this was suggested to be mediated via the pH-sensitive $P2X_2$ receptor. An earlier study proposed that the $P2Y_2$ receptor, which was found to be expressed on osteoclasts derived from a giant cell tumour, might mediate the pro-resorptive actions of ATP (Bowler *et al.*, 1995). However, this could not be confirmed in a follow-up study because the potent $P2Y_2$ agonist, UTP, in contrast to ATP, failed to stimulate bone resorption by giant cell tumour-derived human osteoclasts (Bowler *et al.*, 1998a). Most recently, the $P2X_4$ receptor was identified at the molecular level on rabbit osteoclasts, and proposed to be the receptor mediating the osteolytic

effects of ATP (Naemsch *et al.*, 1999). However, the latter report studied osteoclast function only by electrophysiology, but did not apply any resorption assay techniques. The two earlier investigations failed to investigate effects of nucleotides other than ATP and UTP. Thus, few studies to date have related the presence of P2 receptors to specific functions of bone cells. The aim of this study was to investigate the effects of a wider and more complete range of P2 receptor agonists on bone resorption, and to determine which receptors might be involved in mediating any effects.

MATERIALS AND METHODS

Materials and solutions

Culture media and buffers were purchased from Gibco (UK). All other reagents were purchased from Sigma (UK) unless stated otherwise. Stock solutions of nucleotides (10 mM) were prepared in PBS and stored at -80°C. Stock solutions of dexamethasone, $1,25(OH)_2D_3$, PGE_2 and indomethacin were prepared in ethanol and stored at -20°C. The final concentration of ethanol in cultures did not exceed 1 part in 500. Untreated elephant ivory was kindly provided by HM Customs and Excise (London Heathrow Airport, UK).

Resorption pit formation assay

The effects of extracellular nucleotides on resorption pit formation by mature rat osteoclasts were studied using modifications of an assay described previously (Arnett and Spowage, 1996). All experiments were performed using minimum essential medium supplemented with Earle's salts, 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (complete mixture abbreviated 'MEM'). In most experiments, MEM was acidified by the direct addition of small amounts of concentrated HCl (10 mEq/l H^+, equivalent to 85 μl of 11.5 M HCl per 100 ml MEM). This has the effect of reducing HCO_3^- concentration and producing an operating pH close to 6.95 in a 5% CO_2 environment (see **Fig. 3.1**), which is optimal for resorption pit formation (Murrills *et al.*, 1998). Elephant ivory (dentine) was prepared by cutting 250 μm thick transverse wafers using a low speed diamond saw (Buehler, UK). These dentine wafers were soaked for 2 h in distilled water to reduce brittleness; 5 mm diameter discs were cut from wet dentine wafers using a standard paper punch, washed extensively by sonication in distilled water and stored dry at room temperature. Before use, dentine discs were sterilised by brief immersion in ethanol, allowed to air-dry and then rinsed in sterile PBS. Sterile discs were graphite pencil-numbered, transferred to 96-well plates and pre-wetted with 50 μl MEM.

Mixed cell populations containing osteoclasts were obtained by mincing rapidly the pooled long bones of 2-day-old Sprague-Dawley rat pups, killed by cervical dislocation (n = 5), in 3 ml MEM in a non-tissue culture-treated plastic dish to prevent rapid adhesion of cells to the plastic. Minced bones were triturated 10 times through a wide mouth polyethylene transfer pipette, and the resulting suspension, including small bone fragments, was transferred to a 7ml 'bijou' tube, followed by vortexing for 30 seconds (sec). The mixture is allowed to settle for a few seconds and the supernatant is transferred to a second bijou tube, avoiding bone fragments, using a 1 ml-polyethylene pipette. To maximise cell recovery, the dish and remaining bone fragments are rinsed and vortexed with a further 2 ml of MEM, which is pooled with the first supernatant, resulting in approximately 4-5ml cell suspension. 100 µl cell suspension was added to each pre-wetted disc and allowed to sediment for 45 min at 37°C. Thus, 4 mls of cells suspension prepares around 40 discs. Discs were then rinsed 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 5 ml of MEM and 5 replicate dentine 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 clinical blood gas analyser (ABL 330 blood gas analyser, Radiometer, Denmark), with careful precautions to prevent CO_2 loss. This automated micro blood gas and acid-base balance analyser uses a three-electrode system to measure quantitatively pH, P_{CO_2} and P_{O_2} in the injected sample (200 µl). The ABL330 automatically equilibrates two buffer solutions (high and low pH) by means of air and pure CO_2. These equilibrated solutions are used for electrode calibration, which was set to occur every 2 h. In addition, known quality control standards were regularly used to maintain accuracy. The first medium measurement, taken immediately after removing the culture plates from the incubator, is assumed to provide a P_{CO_2} value that is the same for all wells and that reflects the actual P_{CO_2} during the 26 h incubation. Measured P_{CO_2} typically drops for each subsequent reading from wells in a multiwell plate, causing pH values to rise accordingly. The pH readings for each well are then back-corrected to the pH value associated with the

initially measured PCO_2 value, using pH – PCO_2 calibration curves previously recorded for the appropriate media (see **Fig. 3.1**).

After the 26 h incubation period, dentine discs were washed twice in PBS, fixed in 2% glutaraldehyde for 5 min, washed twice with PBS and stained for TRAP (Sigma Kit 387-A). The numbers of TRAP-positive multinucleated osteoclasts (three or more nuclei) were assessed 'blind' using transmitted light microscopy. After staining with 1% toluidine blue in 1% sodium borate, the number of stromal, mononuclear cells was estimated from three randomly selected fields at ×20. All cells were then removed from the discs by sonication in 0.25 M ammonium hydroxide. Discrete resorption pits were counted 'blind' by scanning the entire surface of each disc using reflected light microscopy (Nikon Labophot 2A, with 100W epi-illumination and metallurgical objectives, Nikon, UK) after restaining in 1% toluidine blue in 1% sodium borate for 2 min.

Mouse calvarial bone resorption assay

The method, which measures bone resorption as Ca^{2+} release from neonatal mouse calvariae, is similar to that described in detail by Meghji *et al.* (1998). 5-day-old MF1 mice (Harlan Ltd., UK) were killed by cervical dislocation. Fronto-parietal bones were removed and trimmed of any adhering connective tissue and interparietal bone, taking care not to damage the periosteum. Dissected calvariae were pooled, washed free of blood and adherent brain tissue in HBSS, and then divided along the sagittal suture. Half calvariae were cultured individually on 1 cm^2 stainless steel grids (Minimesh, FDP quality, Expanded Metal Co., UK) at the air-liquid interface in 6-well plates with 1.5 ml of Biggers, Gwatkin and Heyner medium (BGJb), 5% foetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, in a humidified 5% CO_2 incubator. After an initial 24 h pre-incubation period to enable pre-equilibration of bones with the medium, the medium was removed and replaced with control or test media. Each experimental group consisted of five individual cultures. The cultures were then incubated for 72 h without further medium changes, and without opening the incubator door, so as to ensure

constant CO_2 levels and to minimise pH fluctuations. Culture medium acidification was achieved by adding small amounts of concentrated HCl (5 mEq/l H^+) to the culture medium.

After 72 h, experiments were terminated by withdrawing culture medium, washing bones once with PBS, followed by fixation and decalcification in 95% ethanol / 5% glacial acetic acid for 10 min. Incubator P_{CO_2} was determined by immediately measuring a culture medium sample using the blood gas analyser. The mean final pH of each treatment group was determined by removing and pooling a 100 µl sample from each replicate; the pooled samples were then re-equilibrated with CO_2 in the incubator before measurement using the blood gas analyser; slight differences in CO_2 tension between groups were normalised to the initially measured value using pH - PCO_2 calibration curves constructed for BGJ medium, as previously described (Murrills *et al.*, 1998).

Resorption was assessed as the Ca^{2+} release into the culture medium over the 72h period. Ca^{2+} concentrations in culture medium at the end of experiments were measured colorimetrically by autoanalyser (Chem Lab Instruments, UK) using the following procedure: samples were acidified with excess 1 M HCl and subjected to continuous flow dialysis against the metal complexing agent, cresolphthalein complexone (CPC), to separate Ca^{2+} from proteins; 8-hydroxyquinoline (2.5 g/l) was added to samples to eliminate Mg^{2+} interference. Dialysed Ca^{2+} bound to CPC was then determined following reaction with 2-amino-2-methylpropano-1-ol; the absorbance of the resultant purple-coloured solution was measured at 570 nm. The basal Ca^{2+} concentration of the BGJb medium after addition of 5% FCS was 2 mM.

Following fixation and decalcification, calvariae were stained for TRAP and mounted whole in melted glycerol jelly for transmitted light microscopy.

Dissection of calvariae and Ca^{2+} measurements were performed by Dr Sajeda Meghji (Eastman Dental Institute, UCL).

Osteoclast formation assays

The effects of nucleotides on osteoclast formation were studied using two different mouse marrow cultures systems. For the first assay, long bones of two 8-week-old MF1 mice (Harlan Ltd., UK), killed by cervical dislocation, were fragmented in 5 ml unmodified MEM, triturated and vortexed for 1 min. The resulting cell suspension was allowed to sediment for 2 h onto sterile 5 mm diameter dentine discs, pre-wetted with 50 µl MEM, in 96-well plates (100 µl cell suspension/disc). Dentine discs were then carefully removed, without washing, and placed in pre-equilibrated test or control media in a 6-well plate. Each test or control well contained 5 ml of non-acidified MEM with 10 nM $1,25(OH)_2D_3$, 10 nM dexamethasone, 20 ng/ml human recombinant M-CSF, 1 ng/ml RANKL (a kind gift of Dr Colin Dunstan, Amgen, USA), 100 nM PGE_2 and 6 replicate dentine discs; cultures were incubated for 10 d in a humidified atmosphere of 5% CO_2 - 95% air, with medium changes every 2-3 d. Osteoclast formation has been shown to proceed better in the presence of dexamethasone (Lader and Flanagan, 1998), and PGE_2 is also generally considered to stimulate osteoclast formation.

For the first 7 d, NaOH was added to a running pH of ~ 7.35-7.4, which has been shown to be required for optimal osteoclast formation (Morrison and Arnett, 1998); for the last 3 d, MEM was acidified by addition of HCl (to pH ~ 6.9-7.0) to ensure resorptive activity. This is optimal to study formation and resorption at the same time. The ability of these multinucleated cells to form resorption pits is also an essential indicator that they are genuine osteoclasts. Medium pH and P_{CO_2} were monitored during and at the end of experiments using a blood gas analyser. After 10 d incubation, the discs were fixed in 2% glutaraldehyde, and stained for TRAP. A control group of dentine discs was also removed, fixed and stained after 3 d incubation to check for the presence of any mature osteoclasts that might have been released during the initial cell preparation. The total number of TRAP-positive multinucleated osteoclasts and of discrete resorption pits was assessed 'blind' by transmitted and reflected light microscopy.

As an alternative second procedure, bone marrow cells were isolated from 8-week-old MF1 mice, using a modification of a method described previously (Fuller *et al.*,

2000). The marrow cavity of the long bones was flushed into a dish by slowly injecting MEM at one end of the bone using a sterile 25-gauge needle. The resulting suspension was washed twice, resuspended in MEM and incubated overnight in a 75cm^2 flask at a density of 3 x 10^6 cells/ml MEM containing M-CSF at 5 ng/ml. During overnight incubation, stromal cells adhere to the flasks. After 24 h, non-adherent cells were harvested, washed and resuspended (10^6/ml) in MEM containing M-CSF at 30ng/ml and RANKL at 10ng/ml. This suspension was added to the wells of either 96-well plates containing pre-wetted dentine discs (100µl) or 48-well plates (800µl). After a 24 h preincubation period, dentine discs were transferred to 6-well plates (6 replicates/well) and test media were added. Cultures were fed every 3 d by replacing half the medium with fresh medium and reagents. The functional absence of contaminating stromal cells was confirmed in cultures in which M-CSF was omitted; such cultures showed no cell growth. After 10 d of treatment, 48-well plates and dentine discs were fixed with 2% glutaraldehyde and assessed for TRAP or bone resorption as described above.

In some assays, area measurements, rather than discrete pit counts, were necessary because these assays require high cell densities to function, resulting in groups of TRAP-positive osteoclasts associated with extensive, often conjoined areas of resorption. Area of resorption was assessed 'blind' by reflected light microscopy via output from a Sony CCD colour video camera (DXC-151A; Sony Corporation, Japan) using standard 'dot count' morphometry.

Statistics

Statistical comparisons were made by one-way analysis of variance (disaggregated rat osteoclast assay and mouse calvarial cultures) or Mann-Whitney test (mouse marrow cultures) using 'InStat' (Version 1.13, GraphPAD software); representative data are presented as means ± SEM for 5 or 6 replicates. Results are presented for representative experiments that were each repeated at least 2-3 times.

RESULTS

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

Figure 3.1 demonstrates the relationship between pH, P_{CO_2} and HCO_3^- in tissue culture media. Unmodified MEM with Earle's salts and 10% FBS 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 85 mmHg), while HCO_3^- remains relatively constant, acidifies the medium to give an operating pH close to 7.0 (respiratory acidosis). However, for osteoclast cultures, a reduced operating pH is more conveniently achieved by adding concentrated HCl directly to media containing Earle's salts and 10% FBS. Addition of 82 µl of 11.5 M HCl to 100 ml of MEM provides 10 mEq/l H^+, and results in an operating pH of about 7.0 in a 5% CO_2 atmosphere (metabolic acidosis) (**Fig. 3.1**).

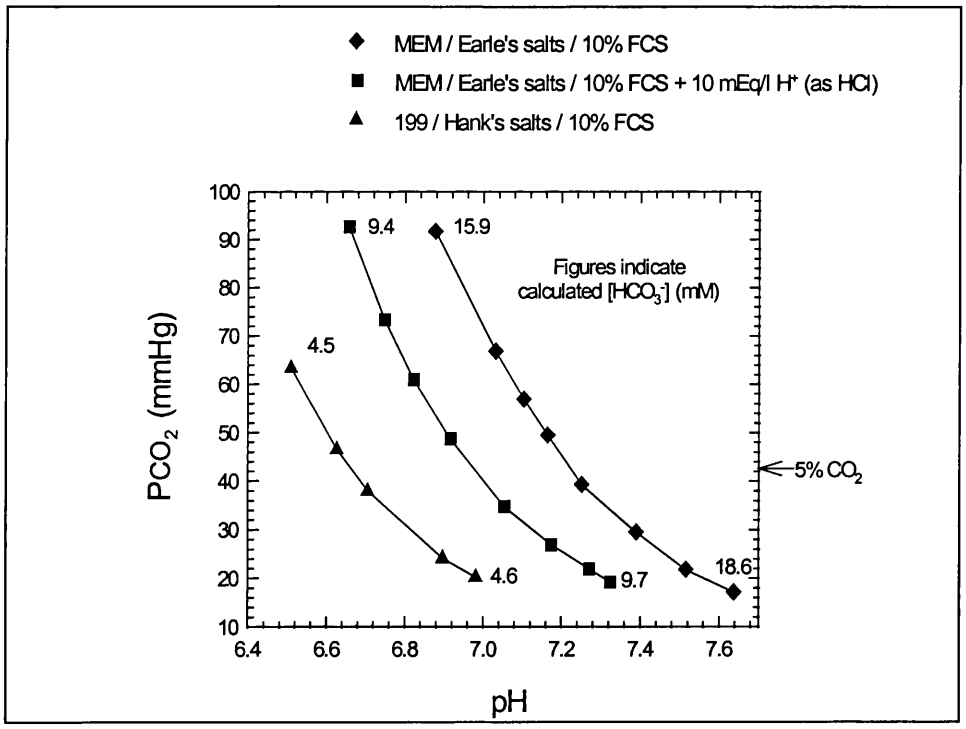


Figure 3.1

Relationship between pH, P_{CO_2} and HCO_3^- in tissue culture media.
Reproduced with permission of Dr Tim Arnett, UCL.

Effect of ADP on mature rat osteoclasts

While screening a range of P2 receptor agonists (adenosine, AMP, ADP, ATP, UDP, UTP), I found that extracellular ADP is a novel powerful stimulator of bone resorption and osteoclast formation. ADP exerted a reproducible, biphasic effect on resorption pit formation by rat osteoclasts in acid-activated 26 h cultures. Figure 3.2 shows a typical, biphasic concentration curve for the effects of ADP: in this experiment, high stimulatory effects were evident in the low nanomolar range (20 – 200 nM) to low micromolar range (2 μM), with up to 2-fold increases in pit formation (**Fig. 3.2**); in other experiments, ADP increased resorption pit formation up to 3-fold (compare **Fig. 3.3**). At higher ADP concentrations of 20 μM to 200 μM, there was no stimulatory effect on resorption. Numbers of mononuclear cells (MNC) (*i.e.* cells of osteoblastic/ fibroblastic morphology) were unaltered by ADP treatment (**Table 3.1**). Although numbers of osteoclasts were lower at all concentrations of ADP than in the control group, this was not assumed to be of any significance because it was never observed in other experiments and probably reflects the high 'noise' in the rat osteoclast assay.

[ADP]	Number of osteoclasts (Oc) /	Number of MNC / disc
0	60.4 ± 4.1	2785 ± 198
2 nM	35.4 ± 6.1	2726 ± 159
20 nM	39.6 ± 3.6	2564 ± 241
200 nM	37.8 ± 3.8	2491 ± 181
2 μM	43.6 ± 2.2	2257 ± 281
20 μM	46.4 ± 4.8	2642 ± 289
200 μM	41.6 ± 6.7	2782 ± 439

Table 3.1

Lack of effect of ADP on the number of mononuclear cells / disc.

Values are means ± SEM (n = 5).

The stimulatory effect of ADP on rat osteoclast resorption pit formation was only observable clearly when culture medium was acidified to a running pH of ≤ 7.0 by addition of H^+ as HCl (**Fig. 3.3**). In the absence of ADP, acidification (pH reduction from 7.08 to 6.82) elicited a 4-fold increase in resorption; in non-acidified MEM (pH 7.11), ADP caused no significant stimulation. However, culturing osteoclasts in acidified MEM with addition of 1 μM ADP resulted in a 3-fold increase in number of pits formed per osteoclast compared to acidified control and a 13-fold increase compared to non-acidified control, suggesting a synergy between the stimulatory effects of acidification and ADP. Similar acidification dependency was observed with all active P2 receptor agonists tested, thus all further experiments were conducted at low pH. Numbers of osteoclasts and mononuclear cells were unaltered by acid and/or ADP treatment (**Table 3.2**).

Treatment	Number of Oc / disc	Number of MNC / disc
Control	42 ± 7.6	3291 ± 210
Acid control	57 ± 7.3	3473 ± 200
ADP 1 μM	77 ± 10	3491 ± 393
ADP 1 μM + acid	69.3 ± 8.4	3346 ± 303

Table 3.2

Lack of effect of ADP and/ or acid on the number of cells / disc.

Values are means ± SEM (n = 5).

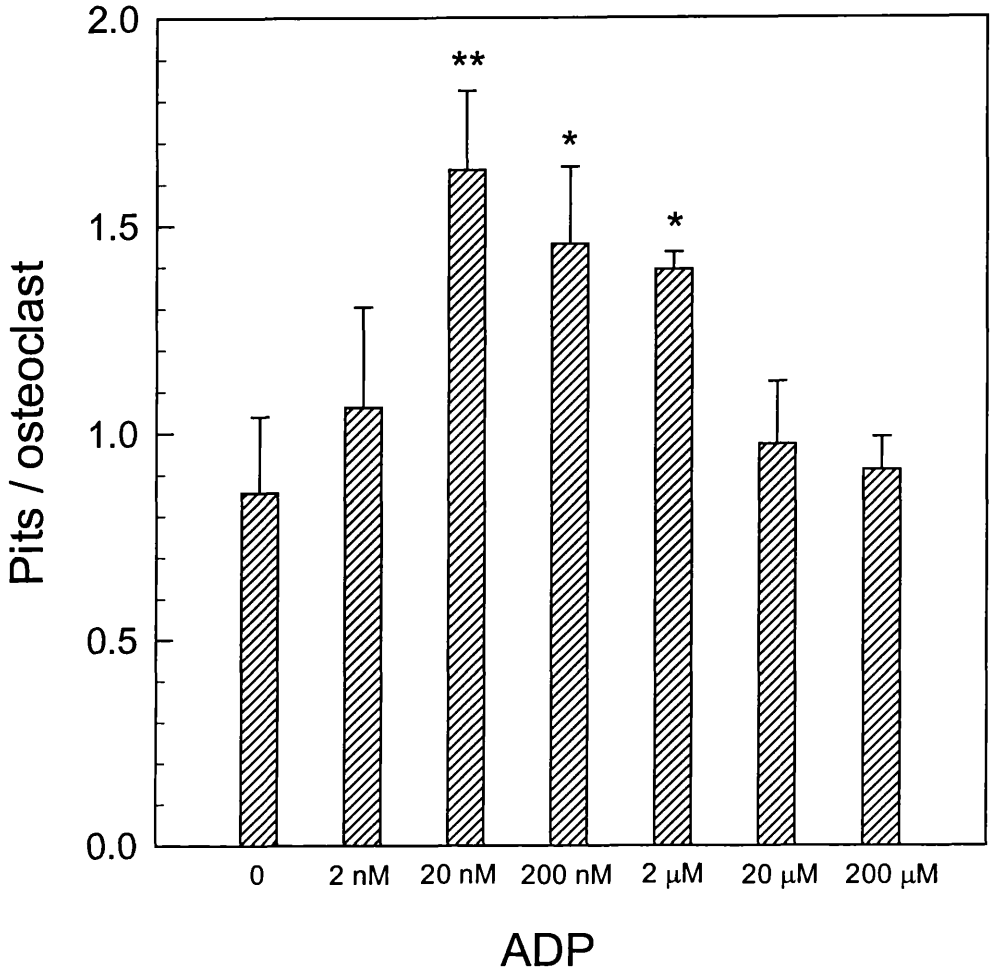


Figure 3.2

Effect of ADP on resorption pit formation by rat osteoclasts.

Osteoclasts were cultured on 5 mm dentine discs in acidified medium (pH ~ 6.9) for 26 h. Large stimulatory effects were evident in the nanomolar to low micromolar range (20 nM - 2 μM) where ADP caused up to 2-fold increases in pit formation. Values are means ± SEM (n = 5). Significantly different from control (0): * $p < 0.05$; ** $p < 0.01$.

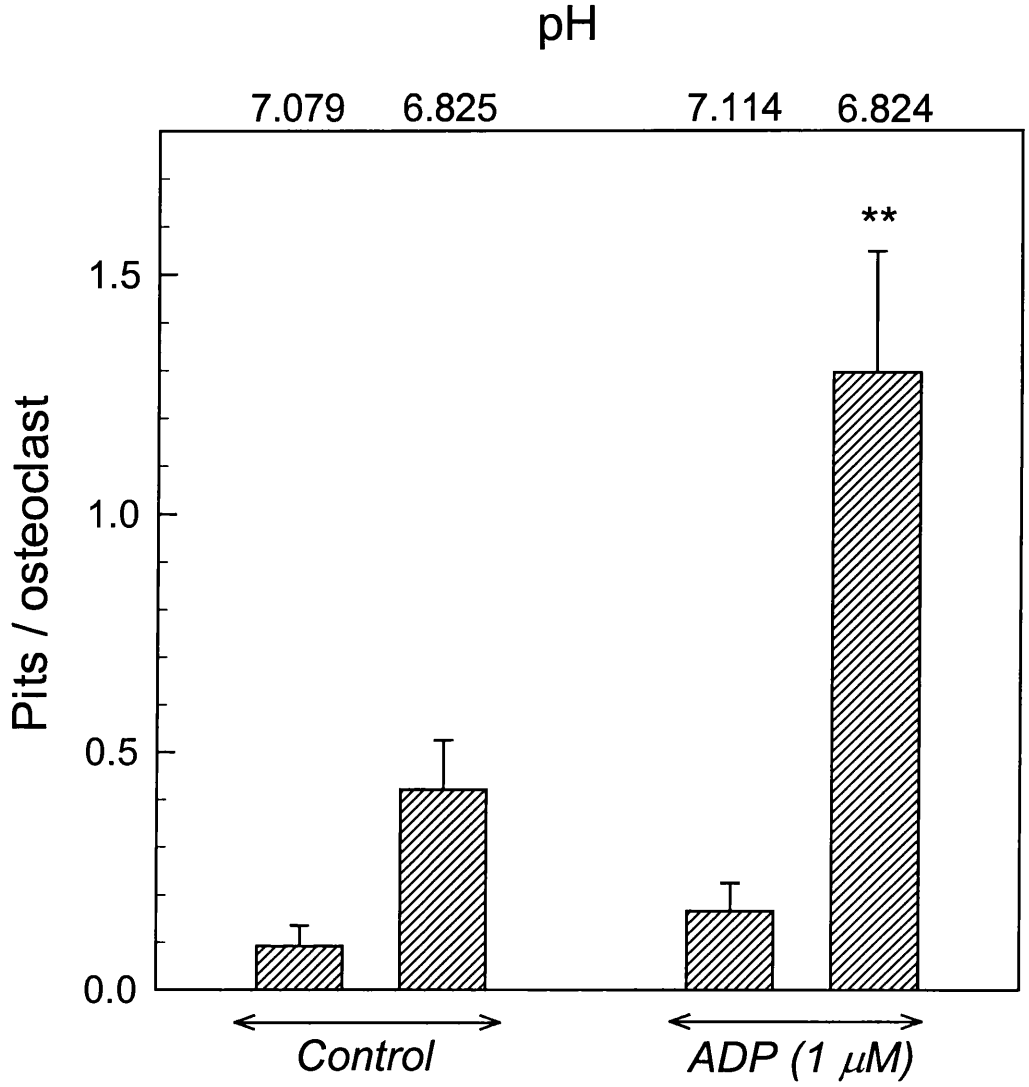


Figure 3.3

Comparison of the effects of ADP on resorption pit formation by rat osteoclasts cultured in unmodified MEM (pH ~7.1) or in acidified MEM (pH ~ 6.8) for 26 h.

The figure shows potentiation of ADP-stimulated resorption at low pH. Values are means ± SEM (n = 5). Significantly different from acidified control: ** $p < 0.01$.

2-methylthioADP (2-meSADP), a highly selective $P2Y_1$ receptor agonist, was able to mimic the action of ADP (**Fig. 3.4**). At 200 nM, the effect of 2-meSADP was somewhat greater than that of ADP, but the difference was not statistically significant. However, no stimulatory effect was observed with 1 μM 2-meSADP, suggesting that this analogue stimulates resorption effectively only within a very narrow concentration range. Numbers of osteoclasts and mononuclear cells were unaltered by 2-meSADP or ADP treatment (**Table 3.3**).

Treatment	Number of Oc / disc	Number of MNC / disc
Control	12.2 ± 1.2	3264 ± 89
ADP 0.04 μM	13.8 ± 1.6	3724 ± 227
ADP 0.2 μM	15.2 ± 3.4	3531 ± 221
ADP 1 μM	13.8 ± 1.7	3759 ± 44
2-meSADP 0.04 μM	17.2 ± 2.1	2836 ± 271
2-meSADP 0.2 μM	13.2 ± 1.8	3339 ± 58
2-meSADP 1 μM	17.5 ± 4.5	2494 ± 504

Table 3.3

Lack of effect of ADP and 2-meSADP on the number of cells / disc.

Values are means ± SEM (n = 5).

To determine whether ADP itself is the signalling molecule, further degradation products of ADP, namely AMP and adenosine, were tested. Adenosine and AMP are both potent agonists at P1 receptors. Neither adenosine nor AMP had a significant effect on bone resorption, compared to ADP at 0.2 μM, which doubled resorption (**Fig. 3.5**). Additionally, numbers of osteoclasts and mononuclear cells were unaltered by adenosine or AMP treatment (**Table 3.4**).

Treatment	Number of Oc / disc	Number of MNC / disc
Control	43.6 ± 8.9	3599 ± 290
Adenosine 0.2 μM	53.6 ± 4.7	3994 ± 323
Adenosine 1 μM	48.8 ± 6.7	2869 ± 311
AMP 0.2 μM	37.2 ± 5.3	3455 ± 59
AMP 1 μM	38.6 ± 3.7	3759 ± 168
ADP 0.2 μM	28.4 ± 3.2	3372 ± 33

Table 3.4

Lack of effect of adenosine, AMP and ADP on the number of cells / disc.

Values are means ± SEM (n = 5).

ADP is the major agonist at the $P2Y_1$ receptor, but is also a less potent agonist at the $P2X_1$ receptor. We therefore investigated the action of $P2X_1$ agonists, α,β-meATP (EC_{50} 1.5 μM) and β,γ-meATP (EC_{50} 2 μM) (King, 1998) on bone resorption (**Fig. 3.6**). Neither α,β-meATP nor β,γ-meATP at concentrations of 0.5 μM and 5 μM had a significant stimulatory effect on bone resorption, thus suggesting lack of involvement of the $P2X_1$ receptor. Numbers of osteoclasts and mononuclear cells were unaltered by any treatment (**Table 3.5**).

Treatment	Number of Oc / disc	Number of MNC / disc
Control	24.4 ± 4.2	3042 ± 212
ADP 0.2 μM	19.5 ± 1.3	2846 ± 304
ADP 1 μM	19.8 ± 3.6	3135 ± 145
α,β-meATP 0.5 μM	26.8 ± 1.2	2568 ± 313
α,β-meATP 5 μM	23.8 ± 2.2	2361 ± 397
β,γ-meATP 0.5 μM	27.2 ± 2.7	2212 ± 298
β,γ-meATP 5 μM	19.6 ± 2.5	2514 ± 158

Table 3.5

Lack of effect of ADP, α,β-meATP and β,γ-meATP on the number of cells / disc.

Values are means ± SEM (n = 5).

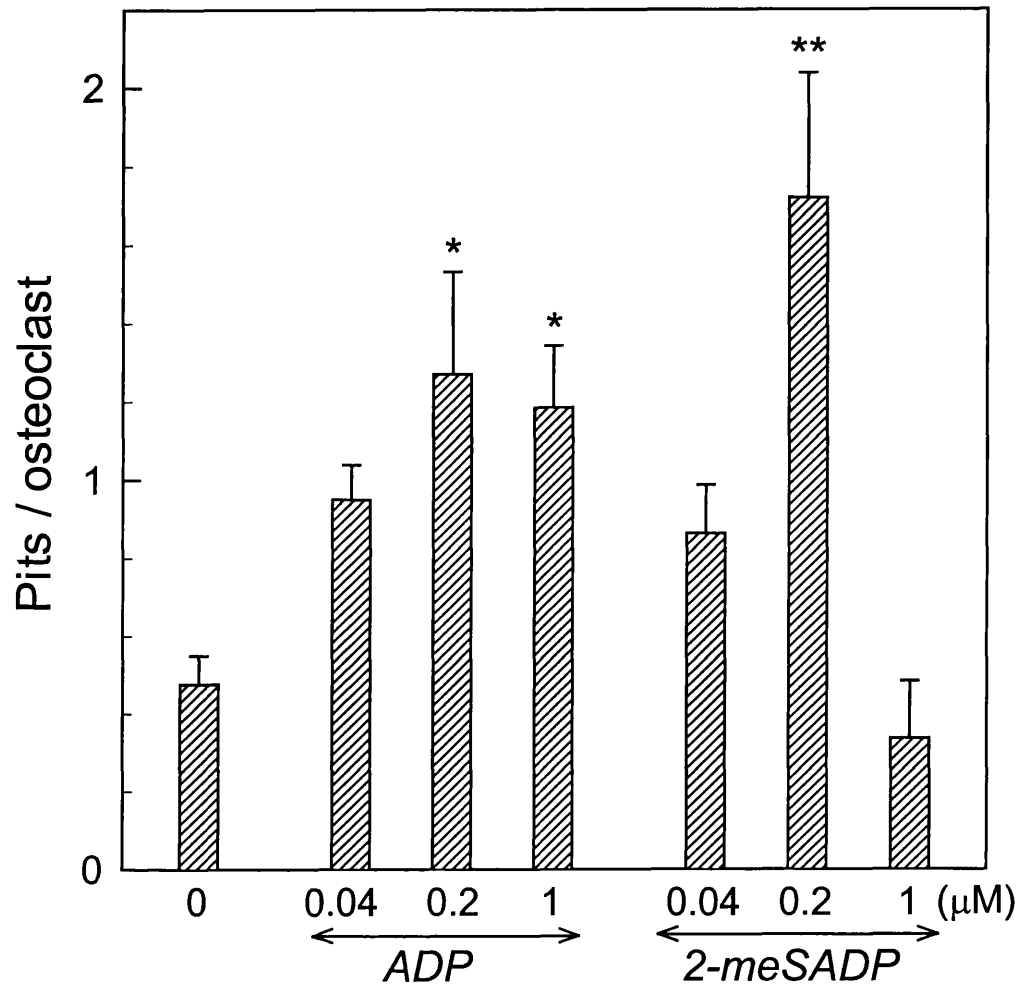


Figure 3.4

Effect of the selective $P2Y_1$ agonist 2-methylthioADP (2-meSADP) on resorption pit formation by rat osteoclasts.

Osteoclasts were cultured on 5 mm dentine discs in acidified medium (pH ~ 6.9) for 26 h. 2-meSADP was able to mimic the ADP effect with a peak effect at 0.2 μM, increasing resorption pit formation up to 3.5-fold. Values are means ± SEM (n = 5). Significantly different from control (0): * $p < 0.05$; ** $p < 0.01$.

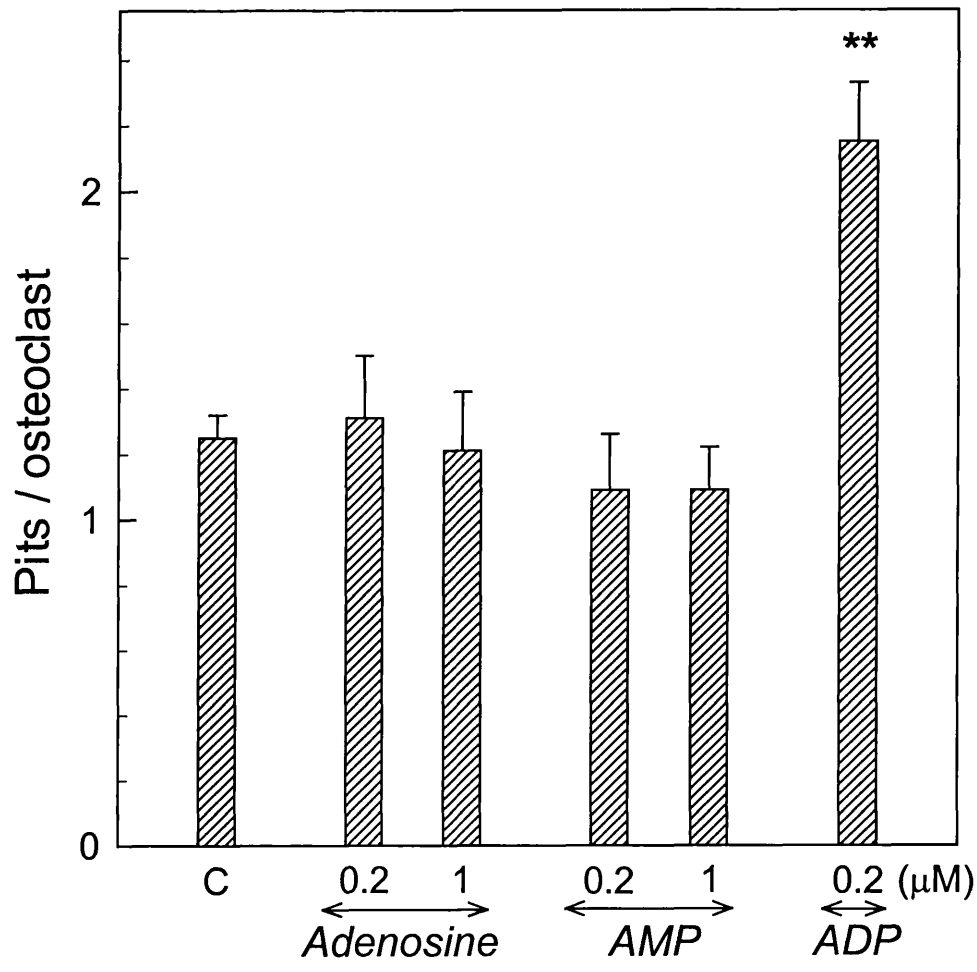


Figure 3.5

Lack of effect of further degradation products of ADP, namely AMP and adenosine, on resorption pit formation by rat osteoclasts.

Osteoclasts were cultured on 5 mm dentine discs in acidified medium (pH ~ 6.9) for 26 h. Adenosine and AMP had no effect on bone resorption compared to ADP at 0.2 μM, indicating that ADP itself is the signalling agent. Values are means ± SEM (n = 5). Significantly different from control (C): ** $p < 0.01$.

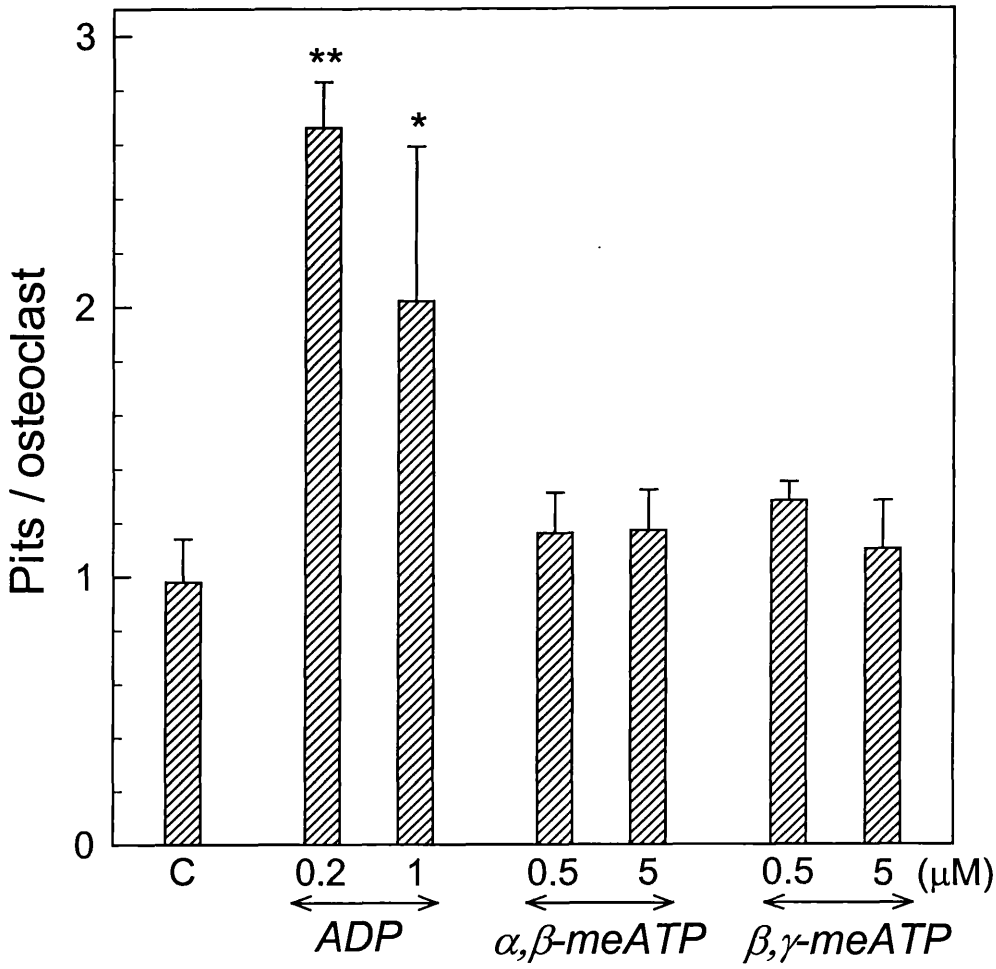


Figure 3.6

Failure of agonists for the $P2X_1$ receptor subtype (α,β-meATP and β,γ-meATP) to mimic the stimulatory effect of ADP on resorption pit formation.

These data exclude the involvement of the $P2X_1$ receptor in mediating the effect of ADP. Osteoclasts were cultured on 5 mm dentine discs in acidified medium (pH ~ 6.9) for 26 h. Values are means ± SEM (n = 5). Significantly different from control (C): * $p < 0.05$; ** $p < 0.01$.

To study further the involvement of the $P2Y_1$ receptor, the compound MRS 2179 (N6-methyl-2'-deoxy-adenosine-3',5'-bisphosphate, a kind gift from Dr. KA Jacobson, NIH, Bethesda, MD, USA) was tested, the most potent $P2Y_1$ receptor antagonist reported to date (Boyer *et al.*, 1998; Jacobson *et al.*, 1999). The 2-fold stimulatory effects of ADP at 0.2 μM was blocked in a non-toxic manner by MRS 2179 at 0.02 – 20 μM (**Fig. 3.7**), whereas the control values were unchanged by the antagonist. Numbers of osteoclasts and mononuclear cells were unaltered by any treatment (**Table 3.6**).

Treatment	Number of Oc / disc	Number of MNC / disc
Control	43 ± 6.8	4552 ± 572
MRS 0.2 μM	38 ± 3.5	5134 ± 352
MRS 2 μM	48 ± 6.7	4001 ± 439
ADP 0.2 μM	53 ± 2.8	4311 ± 453
ADP 0.2 μM + MRS 0.02 μM	44 ± 4.7	3329 ± 57
ADP 0.2 μM + MRS 0.2 μM	48 ± 4.2	4572 ± 264
ADP 0.2 μM + MRS 2 μM	49 ± 4.4	4429 ± 345
ADP 0.2 μM + MRS 20 μM	47 ± 7.3	4001 ± 413

Table 3.6

Lack of effect of ADP and MRS 2179 (MRS) on the number of cells / disc.
Values are means ± SEM (n = 5).

Note that although baseline levels of resorption pit formation and cell numbers vary somewhat between individual assays, as would be expected for primary cell cultures of this nature, relative treatment/control effects were highly reproducible.

UTP and UDP were not found to have reproducible effects on resorption pit formation (results not shown).

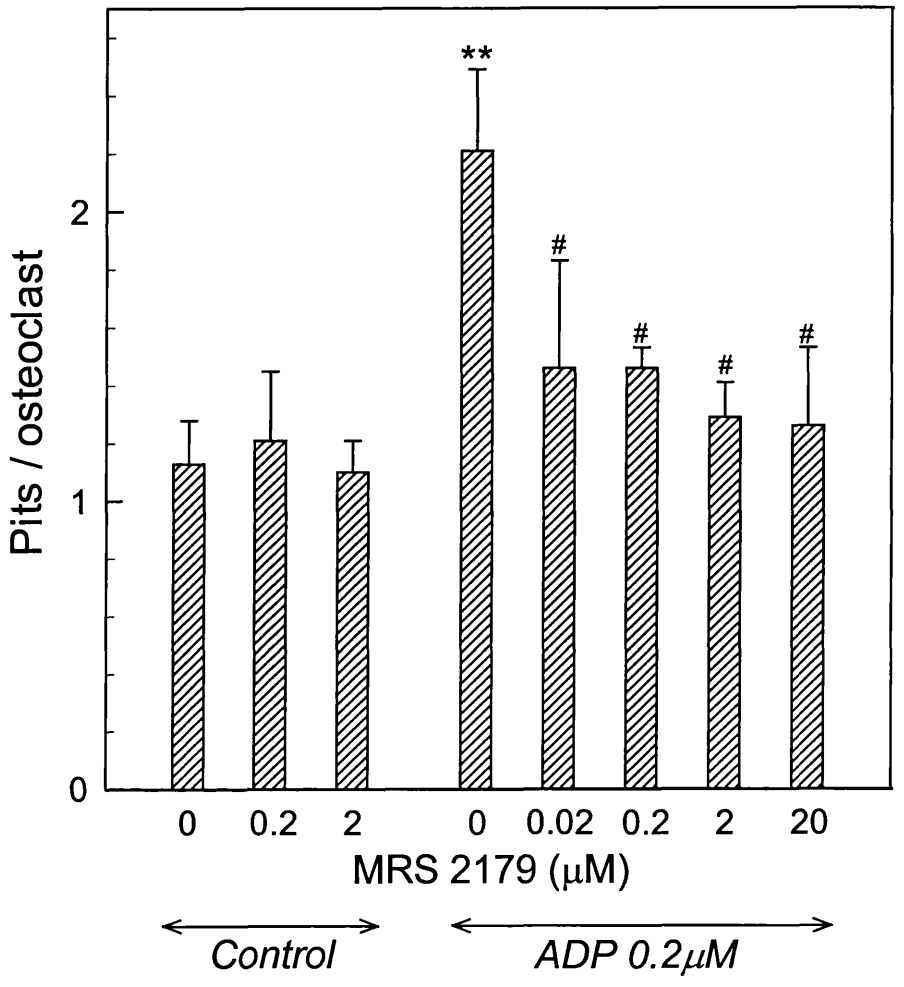


Figure 3.7

Inhibition of ADP-stimulated resorption pit formation by the $P2Y_1$ antagonist MRS 2179.

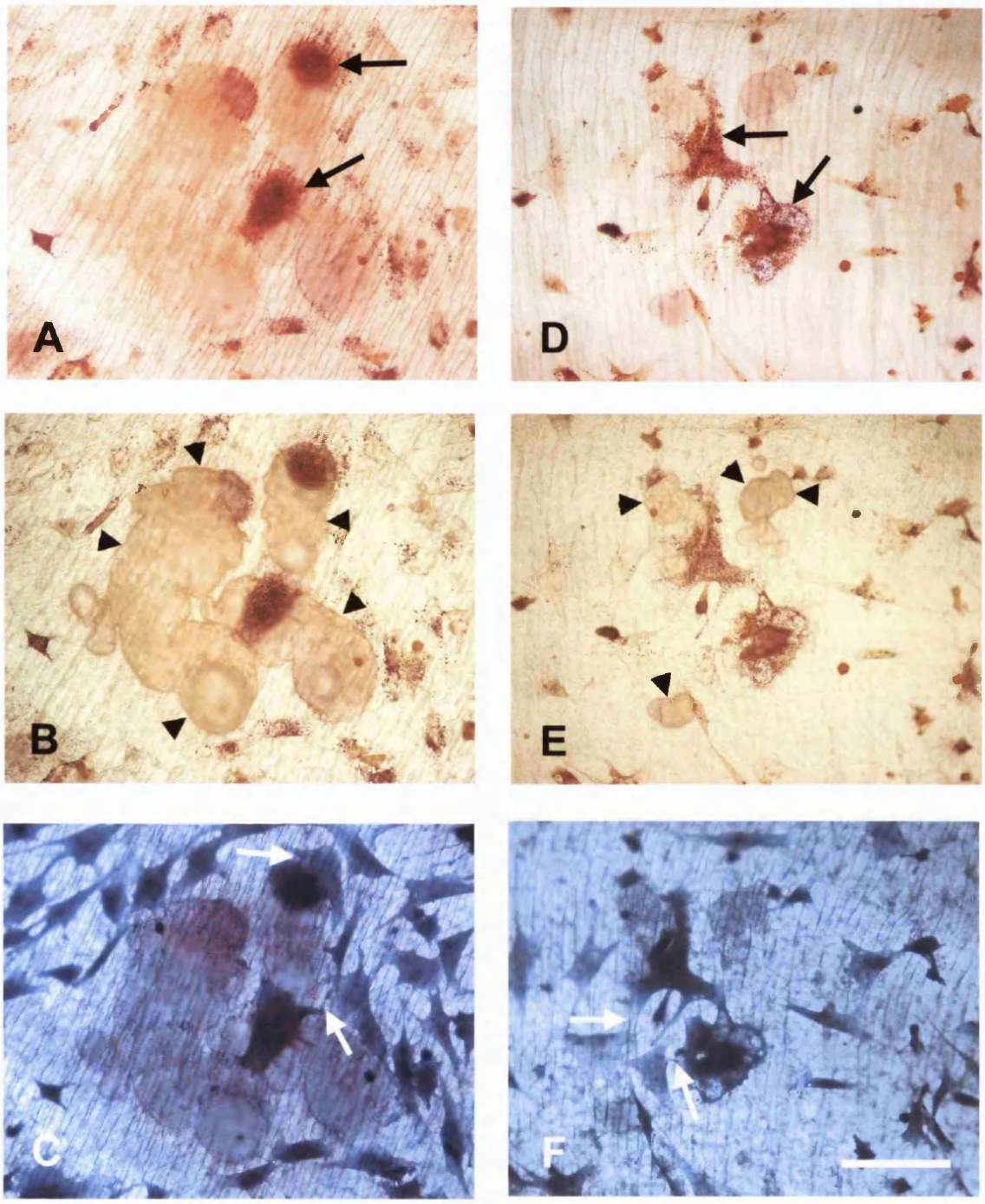
ADP-induced stimulation of resorption pit formation at 0.2 μM was inhibited by MRS 2179 (N^6-methyl-2'-deoxy-adenosine-3',5'-bisphosphate) in a non-toxic manner. Control values were unchanged upon addition of the antagonist. Values are means ± SEM (n = 5). Significantly different from control (0): ** $p < 0.01$. Significantly different from ADP at 0.2 μM: $^{\#}$ $p < 0.05$.

Figure 3.8

Typical appearance of a disaggregated rat osteoclast assay.

Cells isolated from rat long bones were cultured for 26 h on 5 mm dentine discs and fixed. (**A, D**) Discs stained with TRAP to visualise osteoclasts (arrows), viewed by transmitted light microscopy. (**B, E**) Same discs stained with TRAP, viewed by reflected light microscopy to visualise resorption pits (arrowheads). (**C, F**) Same discs stained with toluidine blue to visualise all cells present. Note the close contact between stromal cells and osteoclasts (white arrows). Scale bar = 40 μm.

Figure 3.8 demonstrates the typical appearance of a disaggregated rat osteoclast assay after staining with TRAP, viewed by light microscopy or reflective light microscopy, and after staining with toluidine blue, viewed by light microscopy (**Fig. 3.8**).



Effect of ADP on Ca^{2+} release from mouse calvariae

In 72 h cultures of mouse calvariae, extracellular ADP caused a dramatic increase in bone resorption. Figure 3.9 shows representative micrographs of whole-mount half mouse calvariae stained to demonstrate TRAP after 72 h culture stimulated with ADP at 5 μM and 50 μM (**Fig. 3.9 A,B**) or PGE_2 (**Fig. 3.9 C,D**). Note that these micrographs are shown to demonstrate the presence of large numbers of viable TRAP-positive osteoclasts in this type of culture, and not to demonstrate the higher degree of resorption in ADP- and PGE_2-treated cultures compared to control cultures. Differences in resorption were not always evident when examining stained calvariae by microscopy.

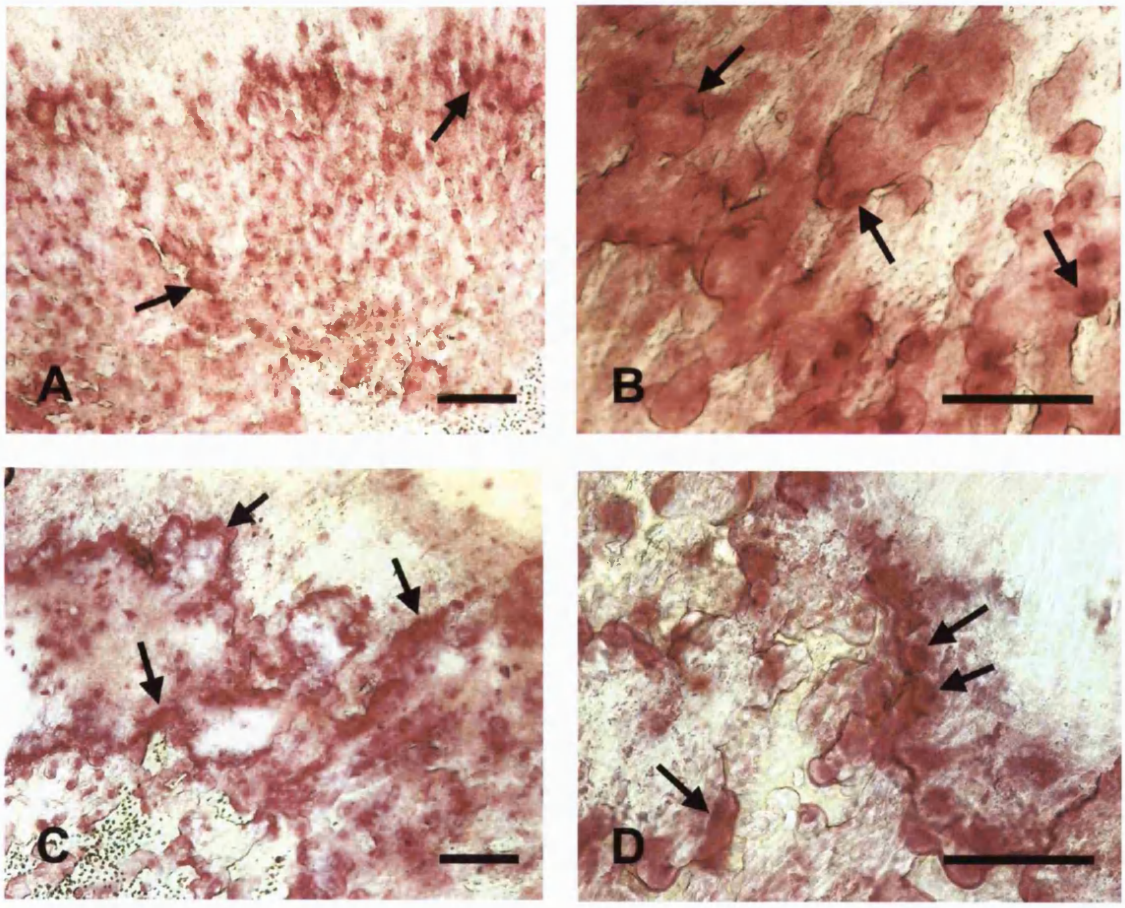


Figure 3.9

Whole-mount histology of cultured mouse calvarial bones.

(A, B, C, D) Representative micrographs of whole-mount half mouse calvariae, stained with TRAP, and mounted in melted glycerol jelly, after 72 h culture stimulated with ADP at 5 μM (A) and 50 μM (B) or PGE_2 (1 μM) (C, D); arrows point to examples of TRAP-positive osteoclasts lining resorption fronts. Scale bars = 200 μm.

Peak effects were observed in the range 5 - 50 μM ADP, but large stimulatory effects were observed at concentrations as low as 50 nM (= 0.05 μM) (**Fig. 3.10**). The peak effects of ADP in the mouse calvarial culture were observed at approximately 10-fold higher concentrations than in the disaggregated rat osteoclast system, reflecting the generally lower sensitivities exhibited by organ culture assays compared with isolated osteoclast assays. This is probably due to the fact that cells are directly exposed to the nucleotides or other agents in the disaggregated rat osteoclast assay, in contrast to calvarial cultures, where the osteoclasts are mainly located in cavities within the bone, and are thus not as directly exposed. The wider concentration range in bone organ culture may reflect partial degradation of the nucleotide agonists before they reach their target receptors, so that higher initial doses are needed.

In the presence of 5 - 50 μM extracellular ADP, osteoclast-mediated Ca^{2+} release was increased up to 6-fold compared to controls (**Figs. 3.10 and 3.11**). The peak stimulatory effects of ADP were equivalent to the maximal effects of PGE_2 at 1 μM (**Fig. 3.11**). Extracellular ADP appeared to be somewhat more effective than extracellular ATP which stimulated Ca^{2+} release up to 4-fold at 5 μM compared to control (**Fig. 3.10**). Similarly to the stimulatory effects on mature rat osteoclasts, the $P2Y_1$ selective agonist 2-meSADP mimicked the ADP effect and increased Ca^{2+} release 4-fold compared to control, with a peak effect at 5 μM (**Fig. 3.10**). At 50 μM, 2-meSADP increased Ca^{2+} release only 2-fold, again suggesting that 2-meSADP effectively stimulates resorption in a relatively narrow concentration range.

The selective $P2Y_1$ antagonist, MRS 2179, inhibited ADP-induced bone resorption (**Fig. 3.11**). The stimulatory effect of ADP at 5 μM could be reduced 3.6-fold by MRS 2179 at 5 μM. In addition, ADP-stimulated Ca^{2+} release was completely blocked by the cyclooxygenase inhibitor indomethacin at 0.1 and 1 μM (**Fig. 3.11**), suggesting that the effect may be mediated by prostaglandins, as for other established resorption stimulators in this system.

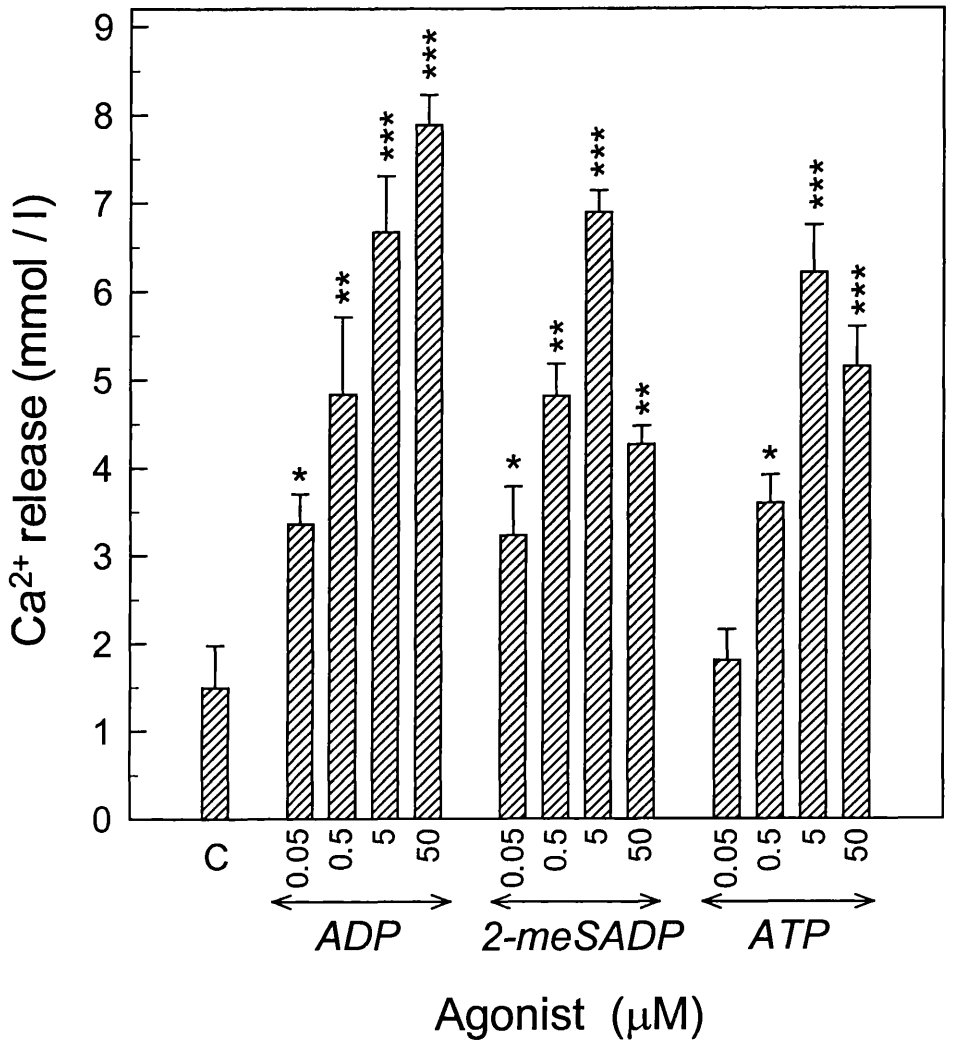


Figure 3.10

Stimulatory effect of ADP, 2-meSADP and ATP on Ca^{2+} release from mouse half-calvaria.

Bones were cultured for 3 d in acidified medium. ADP increased osteoclast-mediated Ca^{2+} release up to 6-fold, with peak effects close to 5 – 50 μM. The $P2Y_1$ selective agonist 2-meSADP was able to mimic the ADP effect. ADP appeared to be more potent than ATP. Values are means ± SEM (n = 5). Significantly different from control (C): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

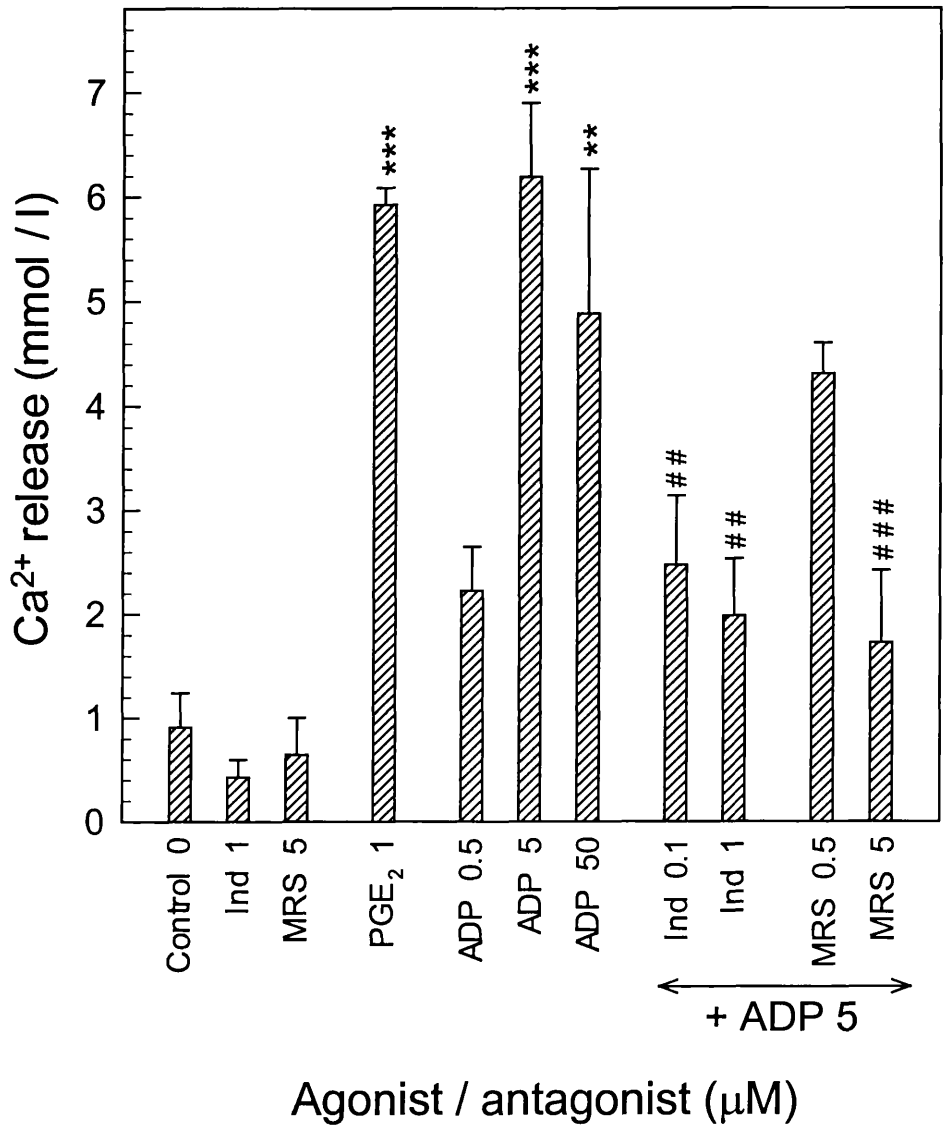


Figure 3.11

Inhibition of the stimulatory action of ADP on Ca^{2+} release from mouse half-calvariae by MRS 2179 (MRS) and indomethacin (Ind).

The stimulatory action of ADP was equivalent to the maximal effects of prostaglandin E_2 (PGE_2) at 1 μM. Values are means ± SEM (n = 5). Significantly different from control: ** $p < 0.01$; *** $p < 0.001$. Significantly different from ADP at 5 μM: $^{\#\#}$ $p < 0.01$; $^{\#\#\#}$ $p < 0.001$.

Effect of ADP in mouse marrow cultures (mixed cell population)

In 10 d mouse marrow cultures on dentine discs, extracellular ADP stimulated the formation of TRAP-positive osteoclasts and resorption pits reproducibly (**Fig. 3.12**). Effects were observed in the range 0.2 – 2 μM ADP. Lower concentrations were without effect (not shown). In the presence of 2 μM ADP, osteoclast formation was increased 2-fold, but number of pits was increased up to 5-fold compared with control. ATP at 2 μM was slightly more effective than ADP in stimulating osteoclast formation (2.4-fold increase), and showed a similar 5-fold stimulation of resorption. The concentration of RANKL (1 ng/ml) in these experiments was carefully chosen to permit relatively low-level osteoclast formation without masking potential stimulatory effects of other agents. Earlier experiments (not shown) demonstrated that there was no stimulatory effect of ADP when RANKL concentration was increased to 10 or 20 ng/ml in these whole marrow cultures.

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

Figure 3.13 demonstrates the typical appearance of a murine marrow assay after a 10 d culture (**Fig. 3.13**). Large numbers of TRAP-positive, resorbing osteoclasts have formed, in addition to high numbers of mononuclear stromal cells.

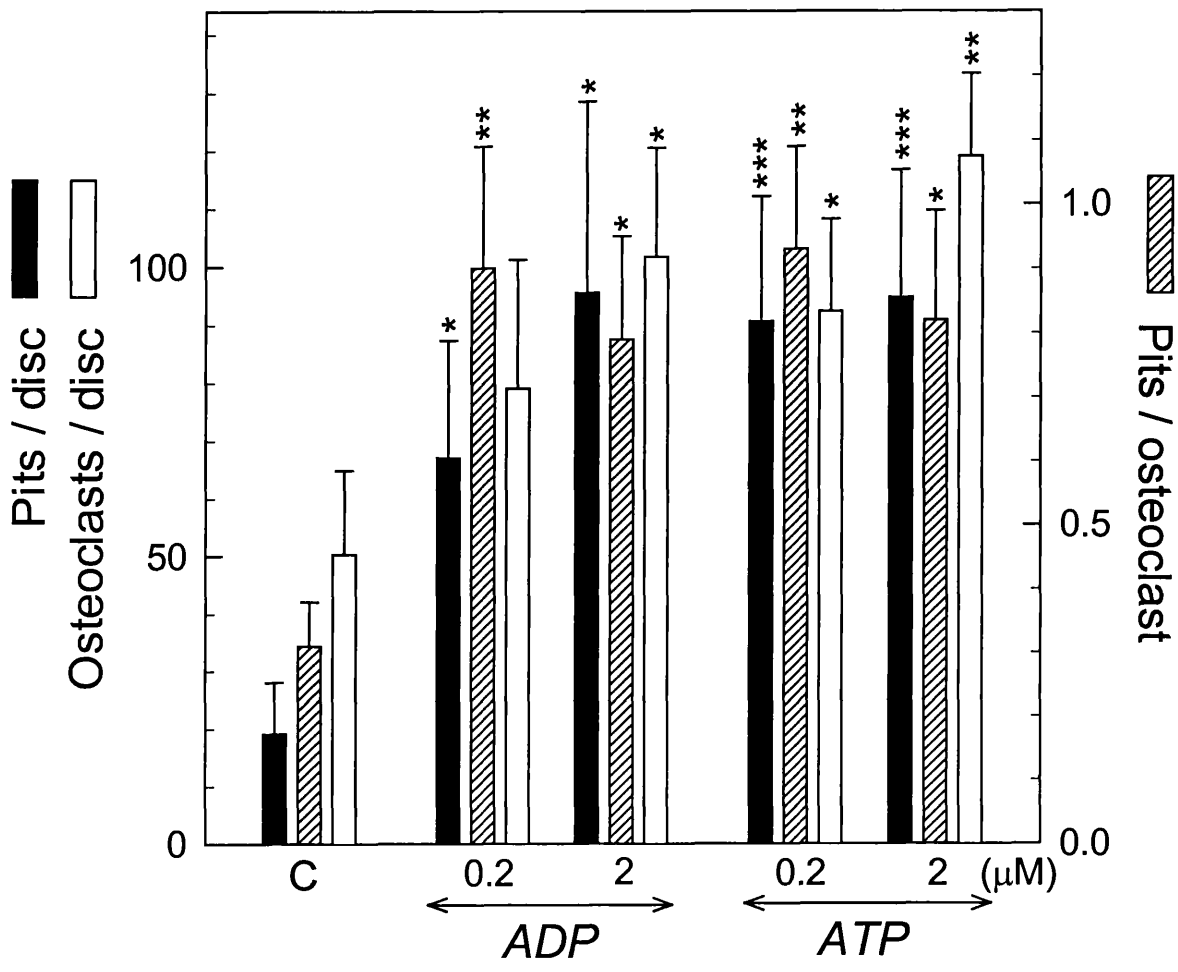


Figure 3.12

Effect of ADP and ATP on osteoclast formation and excavation of resorption pits in mouse marrow cultures maintained for 10 d on 5 mm dentine discs.

Values are means ± SEM (n = 6). Significantly different from control (C): * $p < 0.05$; ** $p < 0.01$.

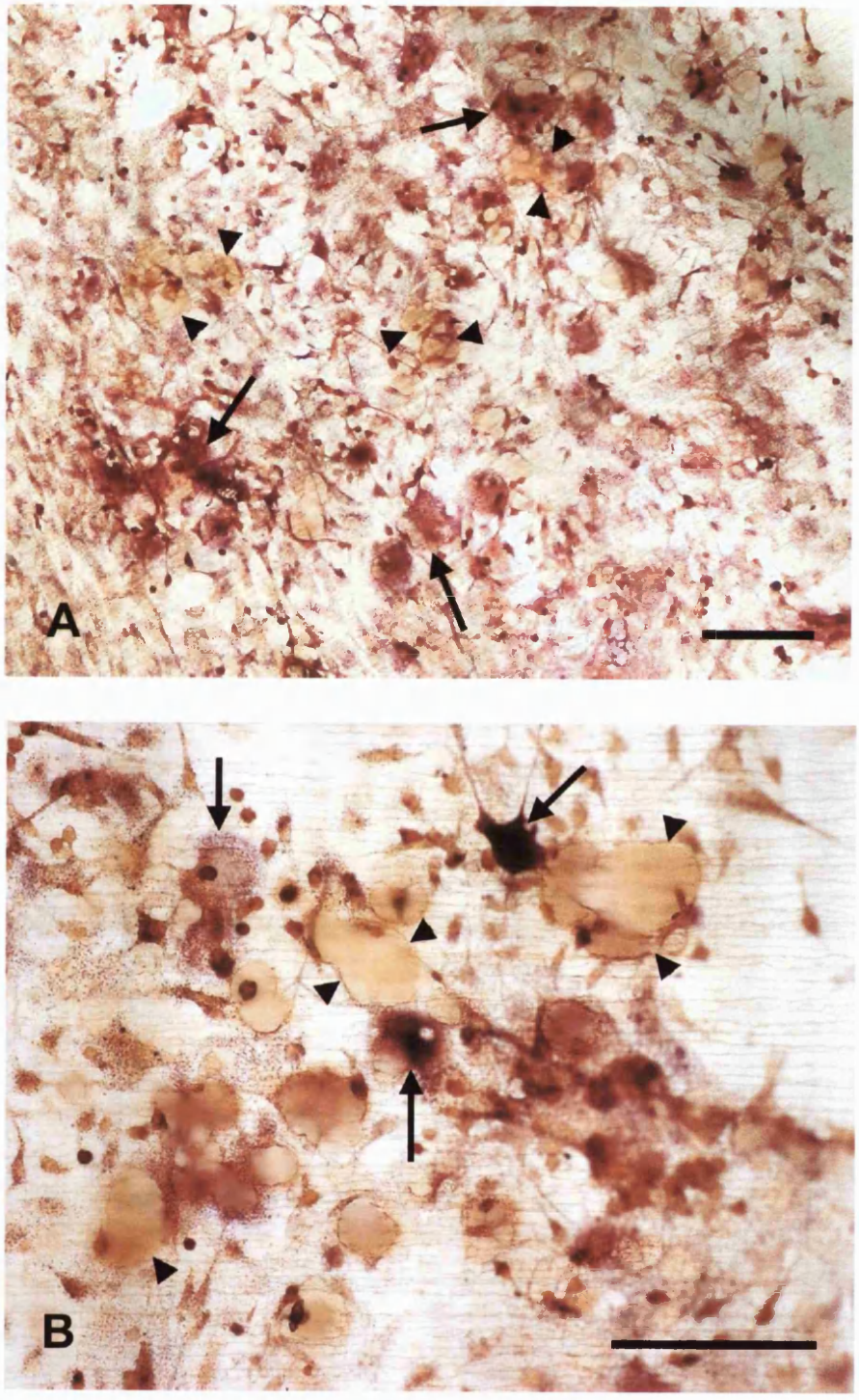


Figure 3.13

Typical appearance of a murine marrow assay cultured for 10 d on 5 mm dentine discs.

(**A, B**) Large numbers of TRAP-positive osteoclasts (arrows) have formed, in addition to high numbers of mononuclear stromal cells (unstained cells). Osteoclasts have excavated resorption pits (arrowheads). Scale bars = 100 μm.

Effect of ADP in stromal cell-free mouse marrow cultures

In experiments using stromal cell-free marrow cultures derived from marrow cells that were initially non-adherent, ADP at concentrations of 0.2 - 2 μM stimulated the formation of TRAP-positive multinuclear cells on dentine discs up to 2.7-fold (**Fig. 3.14**). ADP at 20 μM was without a significant effect. Total area of resorption was also increased 4.2-fold by ADP at 0.2 μM. However, this does probably only reflect the higher numbers of osteoclasts in the ADP-treated samples, resulting in generally increased resorption. When resorption is expressed as area resorbed/osteoclast, ADP at any concentration did not significantly enhance resorption. Numbers of mononuclear haematopoietic cells were unchanged by ADP or ATP treatment (**Table 3.7**). A typical appearance of stromal cell-free marrow cultures on dentine discs is shown in Figure 3.16 (**Fig. 3.16 A, B, C**).

Treatment	Number of mononuclear haematopoietic cells / disc
Control	1879 ± 266
ADP 0.2 μM	2075 ± 216
ADP 2 μM	2269 ± 335
ADP 20 μM	2269 ± 300
ATP 0.2 μM	2653 ± 309
ATP 2 μM	2218 ± 312

Table 3.7

Lack of effect of ADP and ATP on the number of mononuclear haematopoietic cells / disc.

Values are means ± SEM (n = 6).

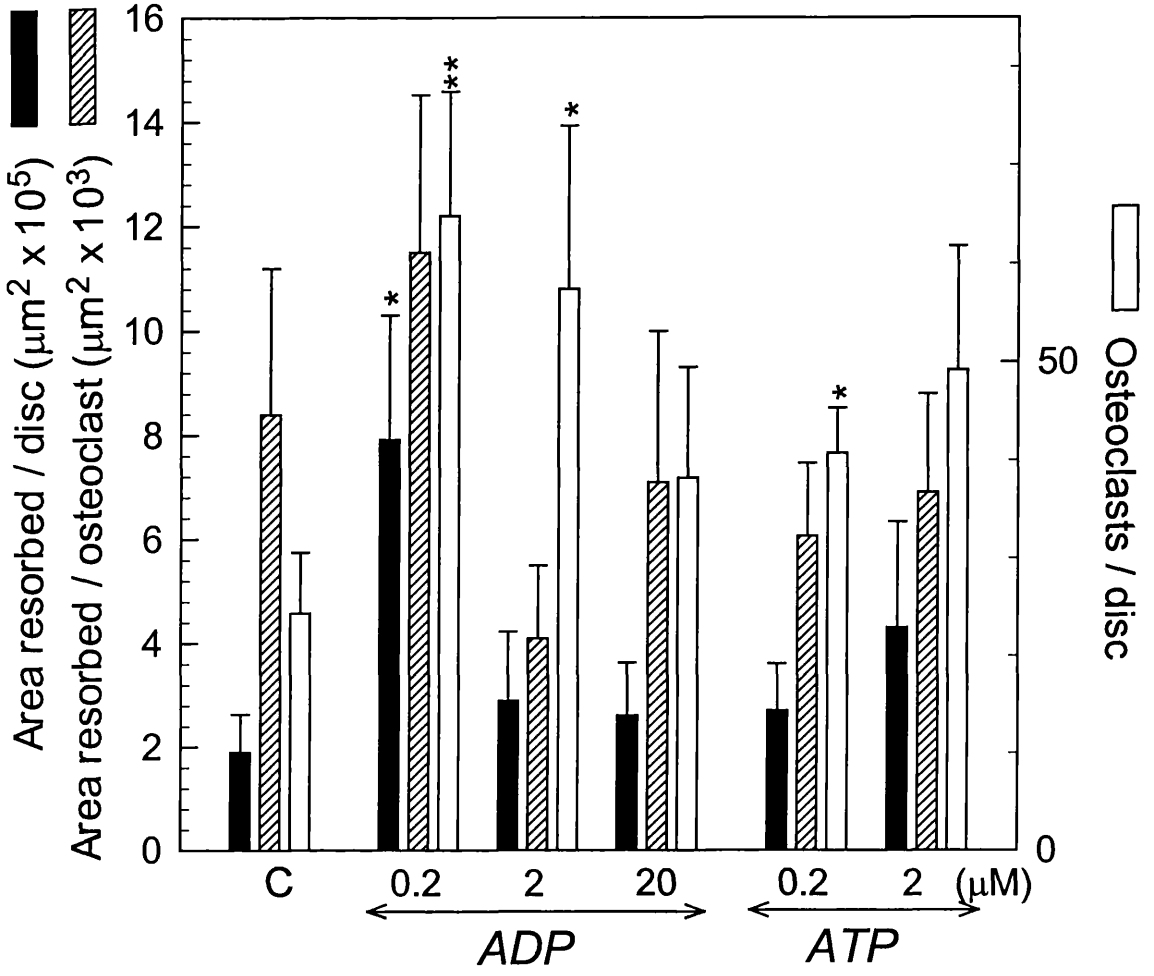


Figure 3.14

Effect of ADP and ATP on osteoclast formation and resorption in stromal cell-free mouse marrow cultures maintained for 10 d on 5 mm dentine discs.

Values are means ± SEM (n = 6). Significantly different from control (C): * $p < 0.05$, ** $p < 0.01$.

In cultures on 48-well plastic dishes, ADP and ATP at 2 μM almost doubled the number of TRAP-positive osteoclasts (**Fig. 3.15**). However, under these culture conditions on plastic, quite large numbers of giant, flattened polykaryons developed that do not resemble resorptive cells (**Fig. 3.16 D**). These polykaryons were not included in counting of TRAP-positive osteoclasts, as they were never seen on the more natural dentine substrate and might not represent *bona fide* osteoclasts.

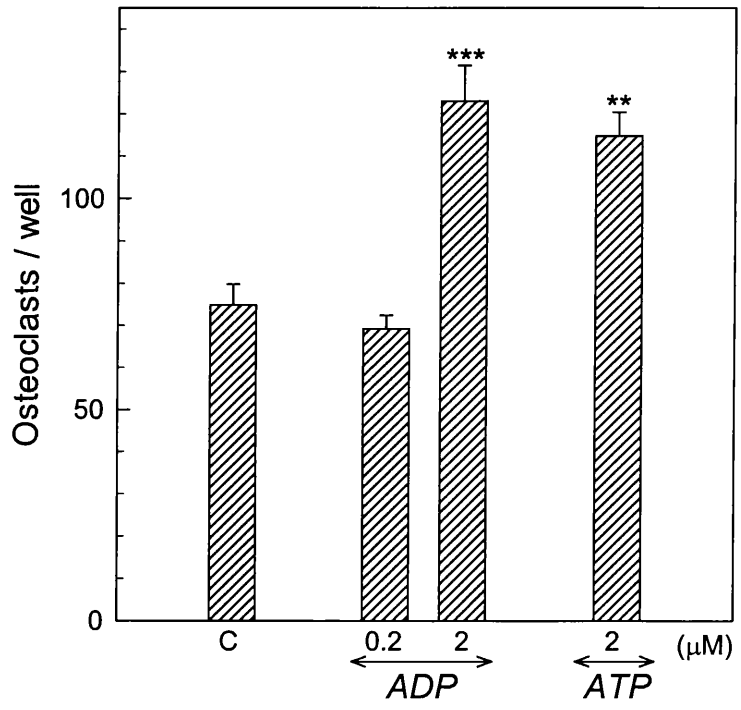


Figure 3.15

Stimulatory effect of ADP and ATP on osteoclast formation in stromal cell-free mouse marrow cultures maintained for 10 d in 48-well culture plates.

Values are means ± SEM (n = 5). Significantly different from control (C): ** $p < 0.01$, *** $p < 0.001$.

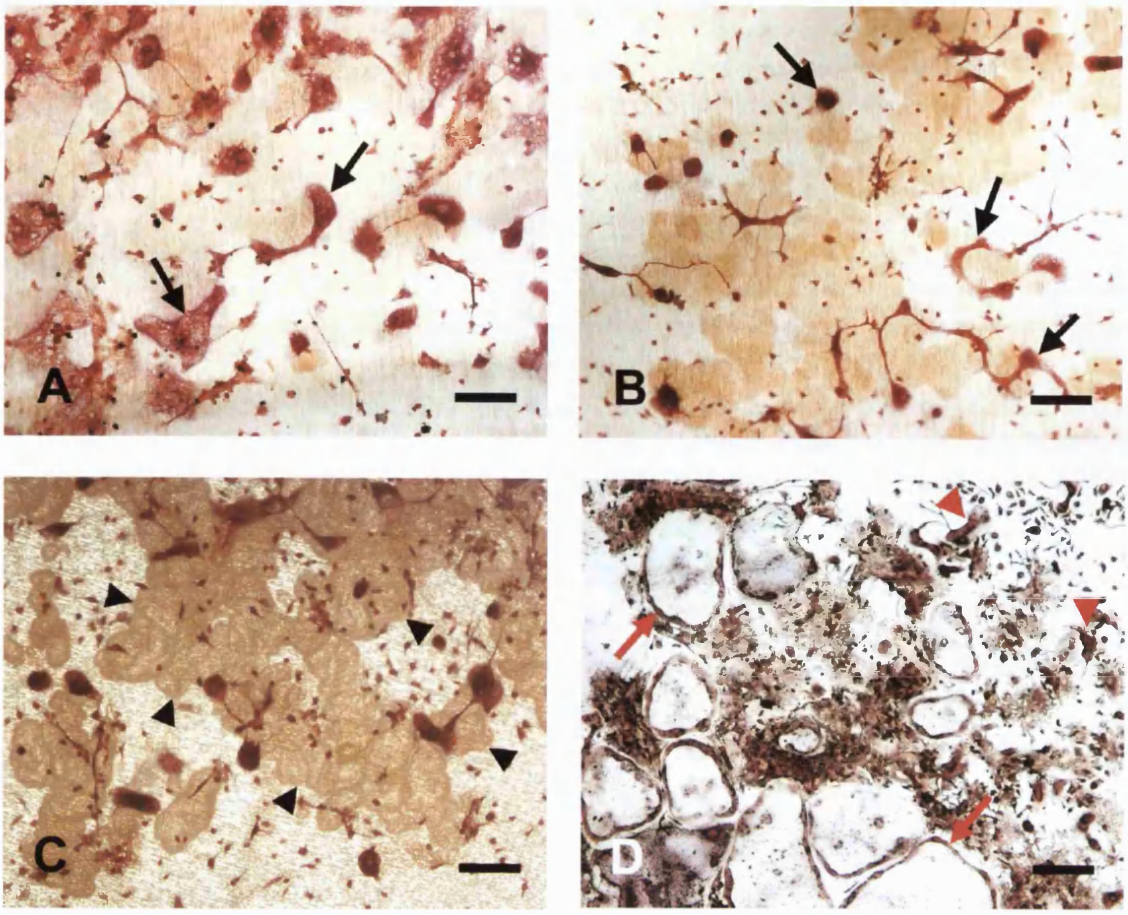


Figure 3.16

Typical appearance of stromal cell-free marrow cultures

(**A, B, C**) Typical appearance of TRAP-stained stromal cell-free marrow cultures on dentine discs, viewed by transmitted light microscopy to visualise large, TRAP-positive osteoclasts (arrows) (A, B), or by reflective light microscopy to visualise large contiguous areas of resorption (arrowheads) (C). (**D**) Typical appearance of stromal cell-free marrow cultures on plastic 48-well plates, demonstrating that under these culture conditions, quite large numbers of giant, flattened polykaryons develop (red arrows), in addition to 'normal' osteoclasts (red arrowheads). Scale bars = 50 μm.

DISCUSSION

This is the first study that links a specific P2 receptor to a key functional action of an extracellular nucleotide on bone. The results show that extracellular ADP, a potent agonist at the G protein-coupled $P2Y_1$ receptor, and 2-meSADP, a selective $P2Y_1$ receptor agonist, are potent stimulators of bone resorption at nanomolar to low micromolar concentrations, assessed by three independent methods in two different species (rat and mouse). To date, most studies on the $P2Y_1$ receptor, which was cloned from rat and mouse in 1995 as the '$P2_y$' receptor (Tokuyama *et al.*, 1995), have focused on its role in ADP-induced platelet shape change and activation. In the 1960s, ADP was recognised as a platelet-activating agent and implicated as a significant mediator in haemostasis and thrombosis (Born, 1962). This role was confirmed by the generation of $P2Y_1$-deficient mice, where platelet aggregation in response to ADP is impaired and bleeding time increased (Fabre *et al.*, 1999; Leon *et al.*, 1999).

In the present study, further degradation products of ADP, namely AMP and adenosine, had no significant effect on bone resorption. This indicates that ADP itself is the signalling agent, and excludes the involvement of P1 receptors for adenosine and AMP. In addition, the stimulatory ADP effect could be blocked in a non-toxic manner by the compound MRS 2179 (N6-methyl-2'-deoxy-adenosine-3',5'-bisphosphate), the most potent $P2Y_1$ receptor antagonist reported to date (Boyer *et al.*, 1998; Jacobson *et al.*, 1999). However, there is evidence that ADP can also act as a weak agonist at the $P2X_1$ receptor (Evans *et al.*, 1995; Bianchi *et al.*, 1999). To discriminate between the two receptor subtypes, subtype-selective agonists and histochemistry (see Chapter 2) were used, providing evidence that the stimulatory ADP effect is mediated via the $P2Y_1$ receptor, rather than via the $P2X_1$ receptor. As discussed in Chapter 2, $P2Y_1$ receptor mRNA and protein were found to be expressed on both osteoclasts and osteoblasts, whereas there was no evidence for the expression of the $P2X_1$ receptor on osteoclasts or osteoblasts. Extracellular ADP could therefore stimulate resorption directly, while signalling through the $P2Y_1$ receptor expressed on mature osteoclasts, or indirectly via

receptors expressed on osteoblasts, which in turn release pro-resorptive local factors, or both.

The osteolytic effects of ADP and 2-meSADP were observed at concentrations as low as 20 nM; effects of nucleotides on osteoclasts at such low concentrations have not been reported before, but similarly low concentrations of 0.3 – 3 μM induce platelet aggregation (Jarvis *et al.*, 2000). Two earlier studies investigated the actions of ADP at much higher concentrations on osteoclasts: ADP at 50 μM increased intracellular Ca^{2+} levels (Yu and Ferrier, 1994) and ADP at 100 μM induced an intracellular pH decrease in rabbit osteoclasts, probably by enhancing the Cl^-/HCO_3 exchange across the osteoclast cell membrane (Yu and Ferrier, 1995). However, the present results indicate that ADP exerts its major functional action on osteoclasts at concentrations ~1000-fold lower than in these previous studies and that ADP at concentrations between 20 μM and 200 μM is without effect. This agrees with a recent study reporting an unusual bell-shaped dose-response curve for both ADP at the $P2Y_1$ and UDP at the $P2Y_6$ receptor, pointing to a 'dual nature' of agonists at these receptors: low concentrations between 0.1 and 100 μM show agonist properties, but higher concentrations > 100 μM result in an antagonistic behaviour. The reason for this dual agonist activity is still unclear, but a 'two-site' receptor model has been proposed, with two distinct ligand-binding sites on one receptor molecule (Sak *et al.*, 2000).

Electrophysiological data have previously suggested the presence of functional P2Y receptors on osteoclasts: 10 - 50 μM ATP, which is a partial agonist at the $P2Y_1$ receptor, activated a K^+-selective outward current in rat osteoclasts that is dependent on P2Y receptor-mediated Ca^{2+} release from intracellular stores (Weidema *et al.*, 1997). More recent studies from the same group reported that the ADP analogue ADPβS, at 100 μM, elicited a Ca^{2+}-dependent K^+ current in rabbit osteoclasts, and at 1 μM, also raises $[Ca^{2+}]_i$ in rat osteoclasts, consistent with the presence of the $P2Y_1$ receptor on osteoclasts (Naemsch *et al.*, 1999; Weidema *et al.*, 2001).

Several studies have described effects of extracellular ADP on osteoblasts, in accordance with the histochemical evidence of $P2Y_1$ receptor expression on osteoblasts shown in Chapter 2. Two groups reported that ADP (at 0.1 μM and 0.4 μM, respectively) and 2-meSADP at 0.04 μM cause a transient increase in $[Ca^{2+}]_i$ in rat osteoblast-like UMR-106 cells (Kumagai *et al.*, 1991; Sistare *et al.*, 1994). This is consistent with my findings that ADP and 2-meSADP can act at nanomolar to submicromolar concentrations. However, as with osteoclasts, the majority of studies on osteoblasts investigated the actions of ADP at concentrations of 10 μM and higher (Reimer and Dixon, 1992; Dixon *et al.*, 1997; Bowler *et al.*, 1999).

Bone resorption requires both the formation of mature osteoclasts from haematopoietic precursors and their subsequent activation to form resorption pits. The present data suggest that ADP stimulates both osteoclast formation and activation, resulting in striking increases in total resorption observed in the 10 d mouse whole-marrow cultures. However, the observed stimulatory effect of ADP on osteoclast formation in mouse marrow cultures could also be due to a prolongation of osteoclast life span, in addition to enhanced recruitment of osteoclasts from progenitors. In the presence of stromal cells in whole-marrow cultures, 2 μM ADP increased osteoclast formation 2-fold, whereas total area of resorption was increased up to 5-fold, presumably reflecting stimulation of newly-formed mature osteoclasts to resorb, and consistent with my findings for rat osteoclasts in short-term cultures. However, in experiments using stromal cell-free marrow cultures, the increase in total area of resorption only reflected the parallel increase in osteoclast numbers, and neither ADP nor ATP increased the area resorbed/osteoclast ratio significantly. This might suggest that the presence of stromal cells is required for the stimulatory effect of nucleotides on resorption to occur, and that ADP exerts its pro-resorptive effect through the $P2Y_1$ receptor on osteoblasts/stromal cells, whereas ADP enhances osteoclast formation both in the absence and presence of stromal cells, probably mediated via receptors on osteoclasts and/or osteoclast precursors. Although this model is highly speculative, it could explain the presence of $P2Y_1$ receptors on both osteoclasts and osteoblasts. Stromal cells are present in the bone microenvironment *in vivo*, so data generated in co-culture systems may have a greater

degree of biological relevance. In this context, a recent preliminary study reports that ATP stimulation of human osteoblast-like osteosarcoma cells resulted in elevated RANKL mRNA expression 4 – 12 h post-stimulation, thus suggesting a potential mechanism by which ATP indirectly stimulates osteoclasts; similar to my results, they have also shown that ATP was ineffective at stimulating resorption by an osteoclast population alone (Buckley *et al.*, 2001a).

Perhaps the most striking effects of ADP, 2-meSADP, and ATP were observed in mouse calvarial bone organ cultures. Viability of the cultures was confirmed by whole-mount histology with TRAP staining, which demonstrated clearly that there are viable osteoclasts lining areas of resorption. Additionally, a recent paper has also shown that dead bones are characterised by Ca^{2+} influx, rather than -efflux (Meghji *et al.*, 2001). In the calvarial system, ADP was roughly as potent as PGE_2, a reference osteolytic agent for this system, in activating osteoclastic resorption. However, resorption stimulated by ADP was blocked by the cyclooxygenase inhibitor indomethacin, suggesting a requirement for endogenous prostaglandin synthesis in this system, as is the case for other osteolytic agents such as protons (Goldhaber and Rabadjija, 1987; Rabadjija *et al.*, 1990). This suggests that a wide range of osteolytic stimuli in the calvarial culture system signal through a common pathway to up-regulate prostaglandin biosynthesis and release, which in turn promotes resorption. It has long been known that adenine nucleotides are able to induce prostaglandin biosynthesis (Needleman *et al.*, 1974), and that purinergic nerve stimulation leads to release of prostaglandins involved in the physiological regulation of gut motility, with the two stimuli forming a functional link (Burnstock *et al.*, 1975). As discussed before, ATP, ADP and UTP all stimulate PGE production in chondrocytes (Caswell *et al.*, 1991; Koolpe *et al.*, 1999). Similarly to the results shown here, this ATP response was also abolished by indomethacin, suggesting that extracellular ATP was promoting *de novo* synthesis of PGE, possibly through PLA_2, which is activated by elevated $[Ca^{2+}]_i$.

A previous study showed that ATP at low concentrations (0.2 – 2 μM) is a potent stimulator of the activation and formation of rodent osteoclasts, but this effect was not

related to a specific P2 receptor subtype (Morrison *et al.*, 1998). The stimulatory effect on mature osteoclasts was only evident at low pH (~6.9), suggesting the possible involvement of the $P2X_2$ receptor, the only P2 receptor subtype that needs extracellular acidification to show its full sensitivity to ATP (King *et al.*, 1996). However, as shown here, a similarly low pH is also required for ADP to show its full stimulatory effects on osteoclastic resorption. This is also consistent with earlier studies showing that pro-resorptive effects of agents such as $1,25\text{-}(OH)_2D_3$ and PTH are acid-dependent (Arnett *et al.*, 1986; Murrills *et al.*, 1998). These findings point to a general dependency of osteolytic agents on slight local acidification. This will be explored further in Chapter 5.

To date the majority of studies on the effects of extracellular nucleotides on bone cells have focused on the actions of ATP, which is the nucleotide with the widest spectrum of biological activity and an agonist at all P2 receptor subtypes. Based on my findings that ADP appears to be somewhat more potent than ATP in stimulating mature osteoclasts, it can be suggested that, for several reasons, the $P2Y_1$ receptor may also be responsible for at least part of the stimulatory effects observed with ATP. Firstly, ATP has long been considered to be a potent agonist of the $P2Y_1$ receptor, based on early studies using the cloned chick receptor (Webb *et al.*, 1993). However, later studies suggested that pure ATP is in contrast a weak competitive antagonist at the mammalian $P2Y_1$ receptor and that ATP actions were only apparent due to ADP contamination present or newly formed by ecto-ATPases (Leon *et al.*, 1997; Hechler *et al.*, 1998; Vigne *et al.*, 1998). This issue remains highly controversial; for example potent ATP agonism of $P2Y_1$ has now been demonstrated on mammalian neurons (Filippov *et al.*, 2000) and on human astrocytoma cells (Palmer *et al.*, 1998) and, in the case of 2-meSATP, an ATP analogue, also on rat hepatocytes (Dixon, 2000). Secondly, ATP could be rapidly hydrolysed to ADP via ecto-nucleotidases present in the bone environment. For example, Sistare *et al.* showed that within 2 min about 25% of a 10 μM ATP-solution is metabolised into ADP by rat osteoblast-like cells (Sistare *et al.*, 1994). Similarly, white blood cells are able to quickly metabolise 10 μM ATP into ADP and into further degradation products, including AMP (Coade and Pearson, 1989), and apyrase, an enzyme with both ATPase and ADPase activity, can generate 20 μM ADP from 100 μM

ATP within 15 seconds (Vigne *et al.*, 1998). The presence of ecto-nucleotidases has also recently been demonstrated in bone marrow (Ogilvie *et al.*, 2000). Thirdly, commercial ATP samples are often contaminated with traces of around 1-5 % ADP; thus an ATP concentration of 2 μM would result in up to 0.02 – 0.1 μM ADP. As reported here, these concentrations are sufficient to exert a peak stimulatory effect on bone resorption.

There are a number of potential sources for extracellular nucleotides in the bone environment: ATP is an ubiquitous intracellular constituent at ~ 5 mM, thus a significant proportion can be released without loss of cell viability and any cell could potentially serve as a source of extracellular ATP. ATP can be released into the extracellular space from intact cells by vesicular release or a channel-like pathway, but also from damaged cells and during tissue injury. Platelets and red blood cells are also able to release ADP itself in the low micromolar range (Alkhamis *et al.*, 1990). The granules in platelets contain up to 40 nM of ATP and ADP per mg protein, and plasma concentrations of ATP/ADP of 20 – 100 μM have been measured following platelet activation (Gordon, 1986; Coade and Pearson, 1989). In addition, osteoblasts have also been shown to release ATP under shear stress conditions (Bowler *et al.*, 1998b).

The finding that ADP, probably signalling through the $P2Y_1$ receptor, is a powerful activator of osteoclasts and may also induce recruitment of osteoclasts, could potentially be of relevance to several pathophysiological conditions that lead to increased bone resorption. Firstly, inflammatory conditions such as rheumatoid arthritis lead to sustained systemic and localised bone loss. This has long been associated with suppressed bone formation, but recently there has been more evidence for increased osteoclast activity. To date, most studies suggest that this process is driven by pro-inflammatory and osteolytic cytokines, released from inflamed synovium, such as PGE, IL-6 and TNFs (Peel *et al.*, 1991; Gough *et al.*, 1998). Results from the mouse calvarial assay show that ADP is as powerfully pro-resorptive as PGE_2, or as discussed above, might even act through stimulating PGE release. In addition, release of nucleotides is increased under inflammatory conditions, suggesting an early role of extracellular nucleotides in the inflammatory process (Bodin and Burnstock, 1998); thus, ADP could mediate a

component of inflammatory bone loss. In addition, platelets also play a key role in inflammation by being induced to release their granule contents, including adenine nucleotides (Peerschke and Ghebrehiwet, 1998).

A second pathological condition where ADP-mediated bone resorption could play a major role is the bone loss associated with cancer metastases. Tumour cells are important sources of extracellular ATP (Burnstock, 1997; Pedersen *et al.*, 1999). Therefore, localised ATP/ADP release could recruit and stimulate osteoclasts. Most importantly, inflamed and cancerous tissues are also characterised by low extracellular pH (Steen *et al.*, 1992; Xu *et al.*, 1998), which would facilitate the osteolytic actions of ADP and ATP. Nucleotides may also be implicated in a wider spectrum of connective tissue destruction, *e.g.* net loss of the cartilage extracellular matrix occurs in all forms of arthritis, and ATP has been shown to stimulate cartilage resorption by acting on P2 receptors (Leong *et al.*, 1994; Brown *et al.*, 1997).

In conclusion, the results of this chapter point to a fundamental new mechanism for the local modulation of bone resorption by extracellular nucleotides at nanomolar to low micromolar concentrations.

CHAPTER 4

EFFECTS OF NUCLEOTIDES ON OSTEOBLASTIC FUNCTION

INTRODUCTION

P2 receptors and osteoblasts

A number of studies have shown that ATP and other nucleotides act through P2 receptors to transiently elevate $[Ca^{2+}]_i$ and induce formation of IP_3 in osteoblast-like cells and non-transformed human bone-derived cells (Kumagai *et al.*, 1989, 1991; Schöfl *et al.*, 1992). Studies on rat osteoblast-like UMR-106 cells demonstrated that extracellular nucleotides interact with at least two receptor subtypes coupled to internal $[Ca^{2+}]_i$ release and the pharmacological profiles were characteristic of $P2Y_1$- and $P2Y_2$-like receptors (Reimer and Dixon, 1992; Yu and Ferrier, 1993b; Sistare *et al.*, 1994). Studies on single cells and populations of human osteoblasts revealed a heterogeneity of receptor expression within one cell culture: ATP and UTP induced $[Ca^{2+}]_i$ rises in every cell tested, whereas only a subgroup of cells responded to ADP or 2-meSATP. This pattern of receptor expression might reflect changes in the osteoblast differentiation status (Dixon *et al.*, 1997). Activation of $P2Y_1$- and $P2Y_2$-like receptors might also result in diverse downstream signalling events (Gallinaro *et al.*, 1995).

The first molecular evidence for the expression of P2Y receptors by osteoblasts came with cloning of the human $P2Y_2$ receptor and its localisation in both osteoclast-like cells and in human osteoblasts derived from different sources (Bowler *et al.*, 1995). More recently, RT-PCR has been used to demonstrate the expression of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_7$ receptors in human bone and in osteoblastic cell lines (Maier *et al.*, 1997); however, $P2Y_7$ has now been shown to be a receptor for leukotriene B_4, and not a

true member of the P2Y receptor family. Consistent with my immunohistochemical results presented in Chapter 2, a new study reported the first evidence for the expression of P2X receptors in human osteoblasts, and implicated P2X receptors, especially the $P2X_5$ receptor, in the stimulation of DNA synthesis in osteoblasts (Nakamura *et al.*, 2000).

However, the functions of extracellular nucleotides in osteoblast biology are not yet well understood. Both ATP and adenosine are able to act as mitogens for osteoblastic cells, which might be mediated indirectly through enhancement of PGE synthesis by ATP application (Shimegi, 1996; Nakamura *et al.*, 2000). Additionally, several studies reported that nucleotides can act synergistically on osteoblasts with other factors, both with growth factors such as platelet-derived growth factor (PDGF) and IGF to induce proliferation (Suzuki *et al.*, 1993; Shimegi, 1996), but most notably with PTH to potentiate PTH-induced Ca^{2+} signalling and *c-fos* expression (Kaplan *et al.*, 1995; Sistare *et al.*, 1995; Bowler *et al.*, 1999; Buckley *et al.*, 2001b). Thus, localised release of nucleotides *in vivo* could create a highly targeted response to systemic factors.

Another possible function of P2 receptors on osteoblasts was suggested in a preliminary study: $P2Y_2$ receptor activation in osteoblasts induced rapid alterations in plasma membrane proton transport activity, resulting in an increase in the acidification rate by rat osteoblastic cells; however, no quantitative data or pH measurements were given (Kaplan and Dixon, 1996). Activation of $P2Y_2$ receptors can also propagate fast intercellular Ca^{2+} waves between rat osteoblastic cells, by a mechanism involving release of intracellular Ca^{2+} stores, in addition to gap junction-mediated intercellular communication (Jørgensen *et al.*, 1997).

Furthermore, extracellular nucleotides have 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). However, concentrations of ATP used were rather high (50 - 500 μM) and results for the effects of UTP were equivocal; thus, it is still unclear which receptor subtypes might be involved in the effects of nucleotides on bone formation and osteoblast proliferation, and how this might relate to the role of P2 receptors in bone

resorption. In this chapter, primary rat calvarial osteoblasts were used to investigate the effects of low-dose nucleotides on bone formation by osteoblasts (bone nodule assay) and cell proliferation.

Mineral formation in cell culture

In vitro, enzymatically isolated osteoblasts are able to synthesise an extracellular matrix (ECM) that mineralises in the presence of: (1) ascorbic acid; (2) an exogenous organic phosphate source, mostly β-glycerophosphate; and (3) a glucocorticoid, promoting bone formation (such as the synthetic glucocorticoid dexamethasone), based on a protocols first developed in the 1980s for rat calvarial osteoblasts (see Nefussi *et al.*, 1985). Ascorbic acid promotes collagen maturation, and hence ECM deposition. In its absence, hydroxylation of collagen proline residues fails to proceed, so that stable pro-collagen molecules cannot be formed. Treatment of post-confluent osteoblastic cells with ascorbic acid increases expression of differentiation markers such as alkaline phosphatase and osteocalcin, and is necessary for production of nodules and mineralised matrix in long-term cultures. Mineral deposition can only occur where high levels of alkaline phosphatase are present extracellularly to increase local inorganic phosphate concentrations (Hughes and Aubin, 1998). However, care must be taken concerning the concentration of β-glycerophosphate used: it was reported that medium supplementation with β-glycerophosphate should not exceed 2 mM, because higher concentrations promoted non-physiological, ectopic mineral deposition (Chung *et al.*, 1992). Characterisation of bone nodules has demonstrated that the process of nodule formation, matrix deposition and subsequent mineralisation follows a well-ordered, temporally defined pattern which appears analoguous to bone formation *in vivo*. At day 3 of culture, cells typically reach confluency; at day 5, cell clusters are formed; at day 7, nodules are built by a dense collagen type I fiber network surrounding the cell clusters; at day 11, little white mineralised nodules, with osteocyte-like cells embedded in the matrix, become visible to the naked eye; subseqently, these nodules increase in size, and

organised cell layers surrounding the nodules can be observed, resembling the *in vivo* situation.

With histochemical stains, mineralisation in form of bone nodules can be assessed: the von Kossa silver stain reacts positively with anions that complex silver and all phosphate-containing materials, whereas alizarin red stains complexed calcium.

METHODS

Bone nodule assay

Calvarial bones from 2-day-old neonatal Sprague-Dawley rats were excised and all fibrous tissue removed. Primary rat osteoblastic cells were obtained by sequential enzyme digestion using a 3-step process (1% trypsin in PBS for 10 min; 0.2% collagenase type II in HBSS for 30 min; 0.2% collagenase type II in HBSS for 60 min, all at 37°C), rejecting the first two digests. The cells were resuspended in Dulbecco's Modified Eagle Medium with Glutamax® + 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (complete mixture abbreviated 'DMEM'), and cultured in a humidified atmosphere of 5% CO_2 – 95% air in 75 cm^2 flasks until confluence (2-4 d). At confluence, cells were subcultured into 24-well plates at a density of 10^4/well in DMEM supplemented with ascorbic acid (50 μg/ml), β-glycerophosphate (2 mM) and dexamethasone (10^{-8} M). After a 24 h pre-incubation period, nucleotides (adenosine, ADP, ATP, UTP, 2-meSADP, at 0.2 – 125 μM) were added to the media. Half of the medium was replaced with fresh test media (with nucleotides double the desired concentration) or control medium every 3 d.

Cultures were examined daily using an Olympus IMT-2 inverted microscope with phase-contrast optics (Olympus Optical Company Ltd., Japan) and continued for 15-21 d, depending on the onset of cell cluster- and nodule formation, which varied from culture to culture. At the end of the experiment, cultures were washed three times with PBS, fixed in 2% glutaraldehyde for 5 min, washed again with PBS and, after three washes with 70% ethanol, left to air-dry for 30 min. Mineralised bone nodules were stained with alizarin red (1% solution w/v in water) for 5 min, and excessive stain rinsed three times with 50% ethanol, before plates were left to air-dry completely. Number of bone nodules and total area of bone nodules/well were analysed using the image analysis programme 'Scion Image' (Version Beta 4.02, downloaded from Scion Corporation at http://www.scioncorp.com). To that end, low magnification micrographs of each well, illuminated on a light-box, were taken with a Leica high-resolution digital camera (Leica

DC 200, software Version 2.51), converted to a greyscale image in 'Adobe Photoshop' (Version 5.0, Adobe Systems Inc), and then to a binary image using 'Scion Image' image processing software (with the same settings, *i.e.* threshold level and minimum particle size for every picture), and quantitated for both total number of nodules/well and total area covered by nodules/well. Data were analysed by 'Excel' and statistical differences assessed by one-way analysis of variance using 'InStat' (Version 1.13, GraphPAD Software).

Proliferation assays

Proliferation was assessed by two different approaches: (1) [^{3}H]thymidine incorporation into newly synthesised DNA of proliferating cells; and (2) measuring total nucleic acid content of cultured cells, using a protocol based on the 'FluoReporter® Blue Fluorometric dsDNA Quantification Kit (F-2962)' (Molecular Probes, Netherlands), which analyses total cellular DNA with the blue-fluorescent 'Hoechst 33258' nucleic acid stain. The bisbenzimidazole derivative 'Hoechst 33258' exhibits fluorescence enhancement upon binding to A-T rich regions of double stranded DNA, which is enhanced under high ionic strength conditions. The procedure is rapid, and all manipulations can be carried out in microplate wells on cells lysed by freezing in distilled water.

[^{3}H]thymidine incorporation

Primary rat osteoblastic cells were obtained and cultured till confluence as described above. At confluence, cells were subcultured into 24-well plates at a density of 10^4/ well in DMEM supplemented with 5% FBS. Cells were maintained for 24 h in this medium before replacement with the same medium containing nucleotides (adenosine, ADP, ATP, UTP 1-100 μM), and cultured for 2 or 5 d. Cells were labelled with 1μCi/ml of [3H]thymidine (Amersham International, UK) for the final 6 h of culture. Cell layers were then washed three times with PBS containing 1mM unlabelled thymidine, treated with 0.75% (w/v) trypsin and 0.03% EDTA (w/v) for 5 min at 37°C, and precipitated

twice at 4°C overnight with 7.5% trichloracetic acid in the presence of 0.2% (w/v) bovine serum albumin (all Sigma, UK), before final digestion with 0.2 M sodium hydroxide (NaOH) and liquid scintillation counting.

DNA quantification using Hoechst 33258

Primary rat osteoblastic cells were obtained and cultured till confluence as described above. At confluence, cells were subcultured into 48-well plates at a density of 10^4 or 2 x 10^4/well in DMEM, rendered quiescent by varying methods (see results section), and treated with nucleotides (adenosine, ADP, ATP, UTP, at 0.5 – 500 μM) for 2 - 4 d. To measure total DNA, cells were treated according to the manufacturer's protocol for 'FluoReporter® Blue Fluorometric dsDNA Quantification Kit (F-2962)' (Molecular Probes, Netherlands). Briefly, plates were emptied at the desired endpoints by overturning onto paper towels, stored at -80°C (up to 4 weeks), and thawed to room temperature. 450 μl distilled water was added per well, incubated at 37°C for 1 h, placed at -80°C until frozen, and then thawed to room temperature. 450 μl of aqueous Hoechst 33258 (stock solution 5mg/ml in 20% dimethyl sulfoxide (DMSO) (v/v)), diluted 1:500 in TNE buffer (10 mM Tris, 2 M NaCl, 1 mM EDTA, pH 7.4) were added. Fluorescence, using excitation and emission filters centered at 360 nm and 460 nm, respectively, was measured on a fluorescence plate reader. To obtain a standard curve, a titration for known amounts of calf thymus DNA (stock solution 10 μg/ml) (Sigma, UK), diluted in TE buffer (10 mM Tris base, 1 mM EDTA, pH 7.4) to a range of 0 - 2500 ng DNA, was performed using the same protocol. A linear regression curve was fit to the values (regression coefficient: 0.979) (using 'Sigma Plot' version 1.0, Jandel Scientific), and DNA concentrations for experimental fluorescence values were interpolated. The standard curve is shown in Figure 4.1 (**Fig. 4.1**).

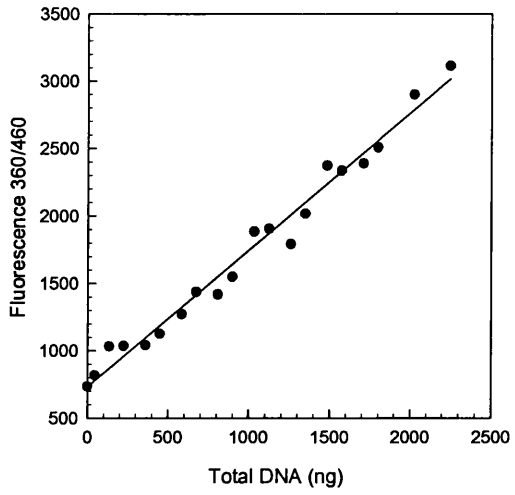


Figure 4.1

Standard curve for DNA quantification using Hoechst 33258 dye.

Fluorescence values were plotted against known amounts of calf thymus DNA (0-2500 ng), and a linear regression curve was fit to the values, using 'Sigma Plot'.

RESULTS

Adenosine, ADP, ATP, UTP and 2-meSADP, at concentrations between 0.1 and 500 μM, were tested for their effects on bone nodule formation and osteoblast proliferation.

Bone nodule assay

Cell clusters typically started to appear first at around day 3-6, and became denser and mineralised during the course of the culture, until the assay was usually stopped at around day 16-20. Examples of the appearance of bone nodules during culture and stained with alizarin red, at increasing magnifications, are shown in Figure 4.2 (**Fig. 4.2**).

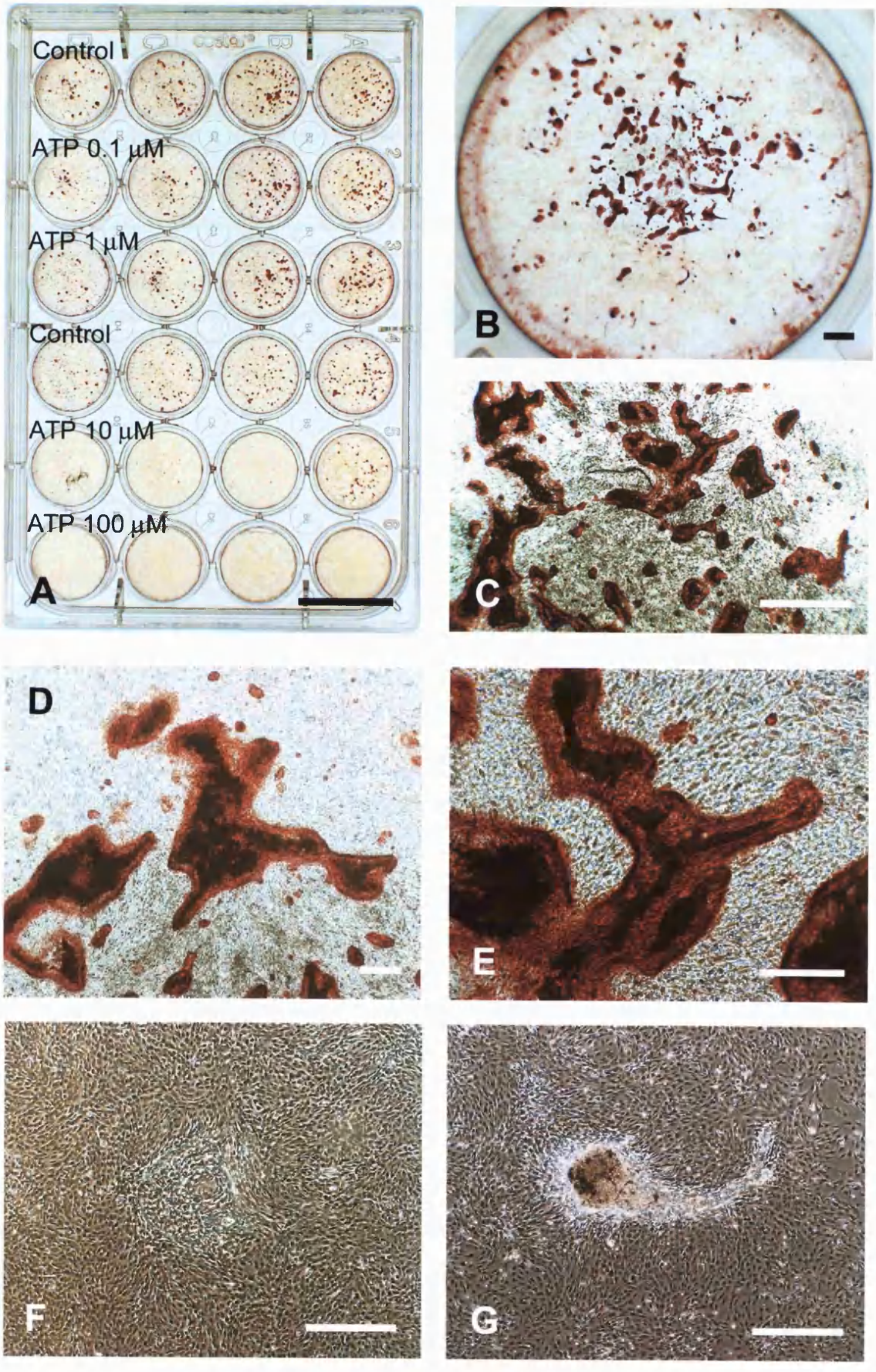
ATP and UTP had an inhibitory effect on bone nodule formation, both on the number of nodules and the total area of nodules/well. Strong inhibitory effects, as was the case for ATP and UTP, could be easily observed by naked eye (**Fig. 4.2 A**). As illustrated in Figure 4.3, the inhibitory effect of ATP started at 10 μM and was maximal at 100 μM (**Fig. 4.3**), whereas UTP started to inhibit bone nodule formation at concentrations as low as 1 μM, again with maximal inhibition at 100 μM (**Fig. 4.4**).

In contrast, neither adenosine (**Fig. 4.5**), nor ADP (**Fig. 4.6**), nor 2-meSADP were found to have an effect on bone nodule formation, suggesting that ATP, and no further degradation products, exerts the effect.

Figure 4.2

Appearance of bone nodules in culture, and stained with alizarin red.

(**A**) Picture of a 24-well plate, with two control rows and four rows treated with ATP from 0.1 to 100 μM (see labels); inhibitory effects of ATP on bone nodule formation were clearly visible by naked eye. **(B)** Appearance of a single well, as photographed before image analysis. **(C, D, E)** Appearance of bone nodules, formed by osteoblasts in culture and stained with alizarin red, at increasing magnification ×2 (C), ×5 (D), ×10 (E). **(F, G)** Osteoblasts showed first signs of bone nodule formation after ~5 d in culture by forming discrete multi-layered cell aggregations. Scale bars = 2 cm (A), 1 mm (B,C), 200 μM (D,E,F,G). (F,G) kindly provided by Siva Mahendran.


Control
ATP 0.1 μM
ATP 1 μM
Control
ATP 10 μM
ATP 100 μM
A
B
C
D
E
F
G

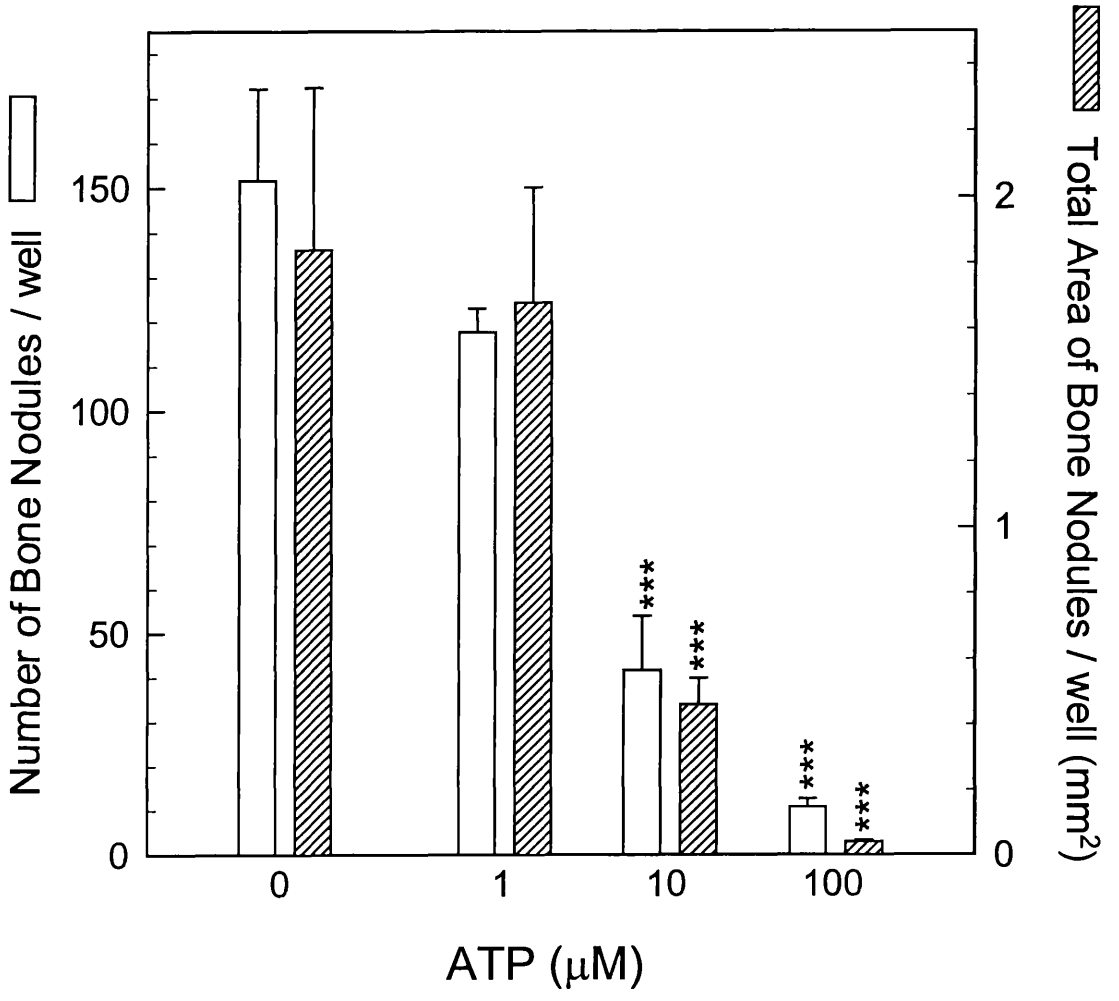


Figure 4.3

Inhibitory effect of ATP on bone nodule formation.

Concentrations of ATP ≥ 10 μM significantly inhibited number of bone nodules and total area of bone nodules/well. Values are means ± SEM (n = 6). Significantly different from control: *** $p < 0.001$.

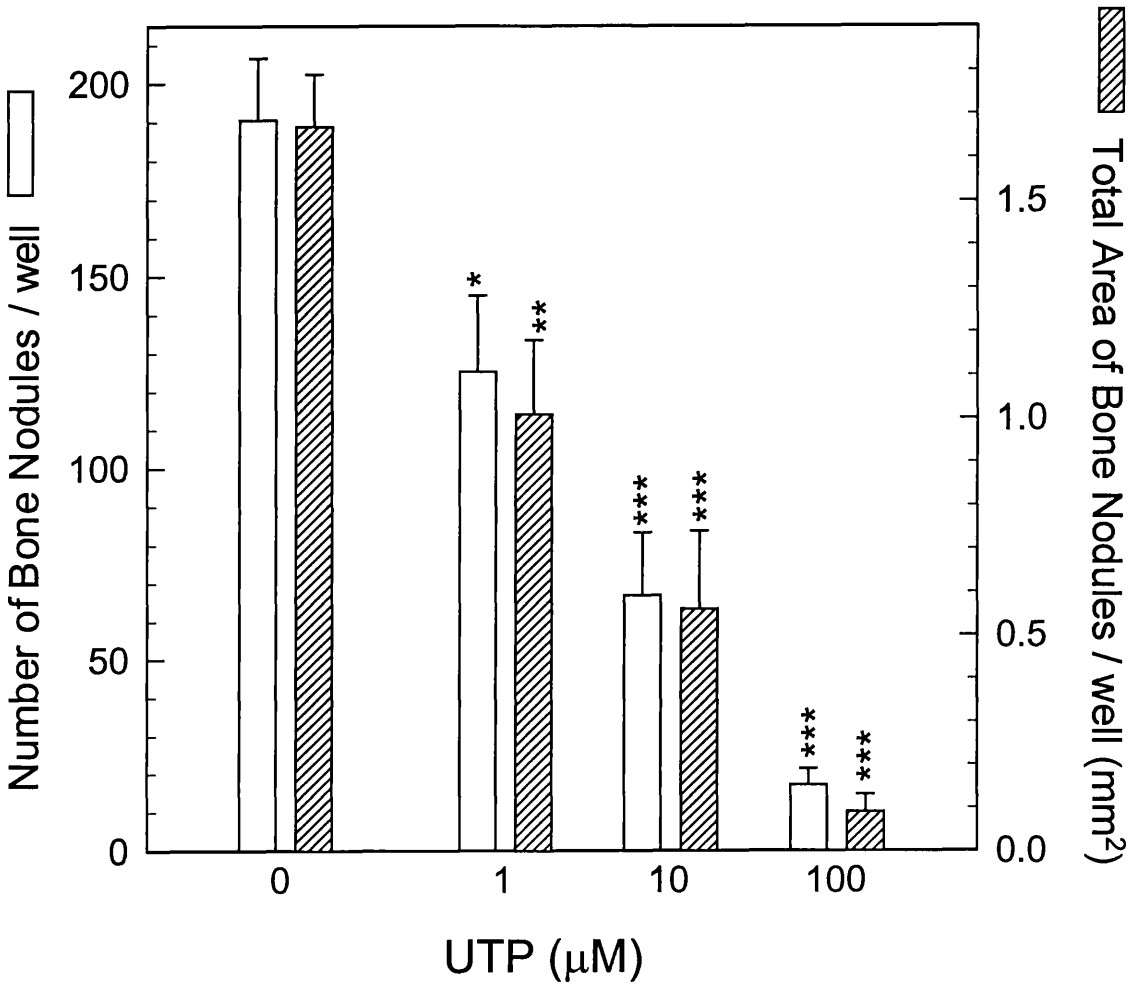


Figure 4.4

Inhibitory effect of UTP on bone nodule formation.

Concentrations of UTP ≥ 1 μM significantly inhibited number of bone nodules and total area of bone nodules/well. Values are means ± SEM (n = 6). Significantly different from control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

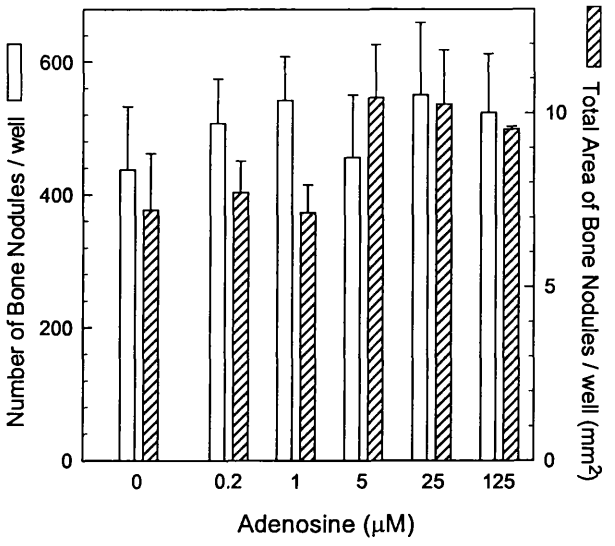


Figure 4.5

Lack of effect of adenosine on bone nodule formation.

Adenosine at concentrations between 0.2 – 125 μM had no effect on number of bone nodules and total area of bone nodules/well. Values are means ± SEM (n = 6).

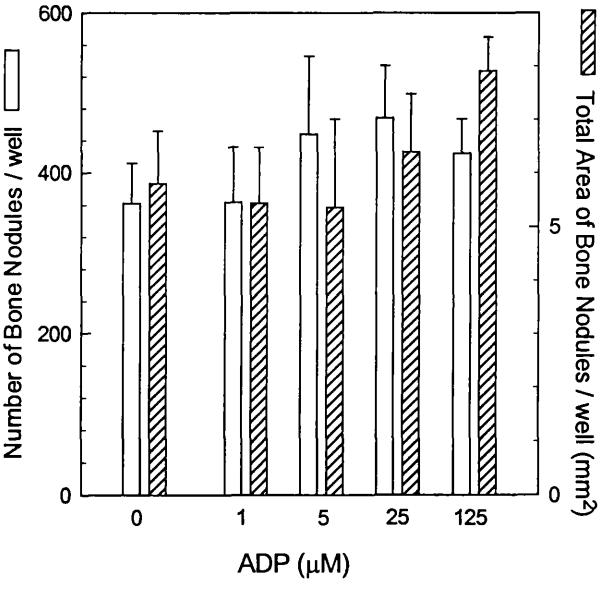


Figure 4.6

Lack of effect of ADP on bone nodule formation.

ADP at concentrations between 1 – 125 μM had no effect on number of bone nodules and total area of bone nodules/well. Values are means ± SEM (n = 6).

Proliferation assays

Effects of nucleotides on proliferation of primary rat calvarial osteoblasts were inconsistent and changed from culture to culture. Although a variety of cell culture conditions were tried by changing (1) the exposure time to nucleotides, (2) the total time in culture, (3) two independent methods to assess DNA synthesis and (4) different conditions to render the cells quiescent, no consistent effects were observed. Even when slight effects on proliferation (both inhibitory or stimulatory) were seen, they were only evident at relatively high concentrations (~ 100 μM). Thus, the biological significance of the proliferation studies is questionable in contrast to the clear-cut strong inhibitory effects of low-dose nucleotides on bone nodule formation. However, for completeness, some representative results are summarised below.

[^{3}H]thymidine incorporation

Using [^{3}H]thymidine incorporation methods to study osteoblast proliferation, UTP at 100 μM seemed to have a mitogenic effect when osteoblasts were cultured for 2 d (**Fig. 4.7 A**). However, after 5 d of culture, a different pattern was seen: UTP had no effect on the number of proliferating cells, whereas both adenosine and ATP, at 100 μM, exerted a weak inhibitory effect on cell proliferation, and ADP at 100 μM slightly stimulated proliferation (**Fig. 4.7 B**).

DNA quantification using Hoechst 33258

Examples of two sets of experiments using the DNA quantification assay with the fluorescent DNA stain 'Hoechst 33258' are summarised in the following two tables (**Tables 4.1, 4.2**). Table 4.1 shows results for one set of experiments where cells were subcultured at a density of 10^4/well into 48-well plates, rendered quiescent with DMEM/0.1% FBS for 48 h, before test media were added for a further 48 h. No treatment changed the total amount of DNA.

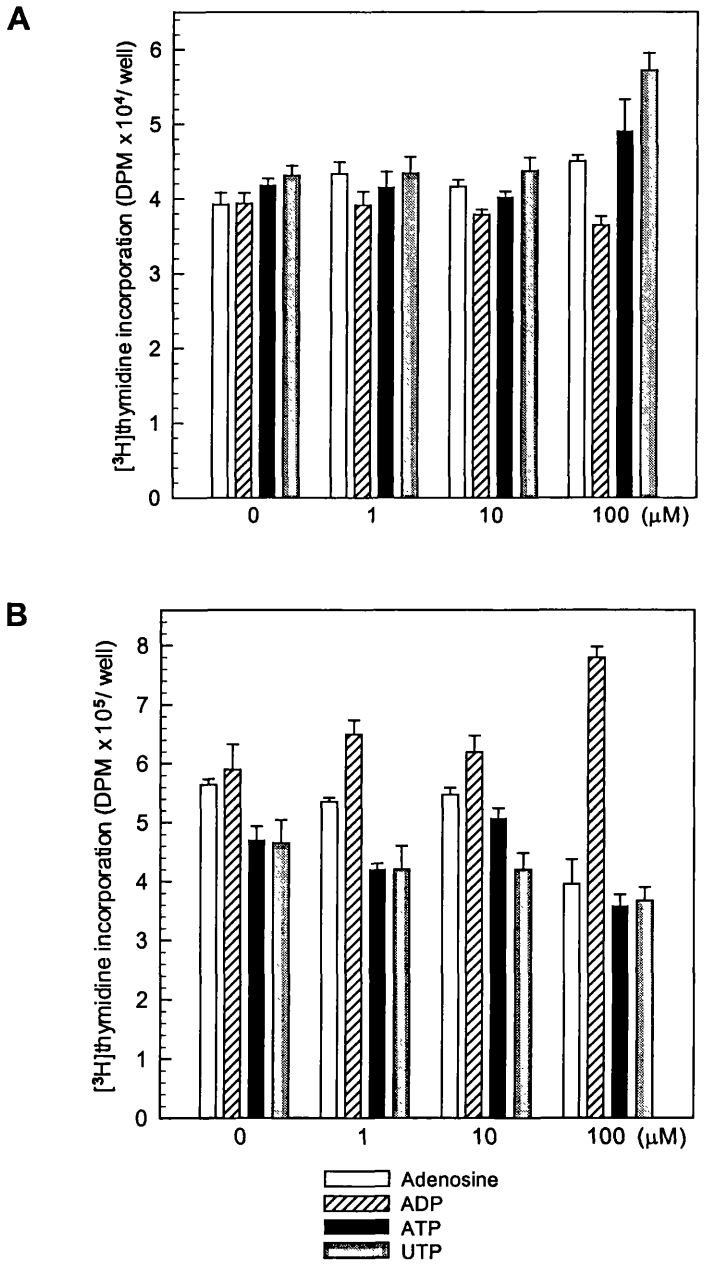


Figure 4.7

[^{3}H]thymidine incorporation

(A, B) Effects of nucleotides on cell proliferation of primary rat calvarial osteoblasts cultured for 2 days (A) or 5 days (B). After 2 days, UTP slightly stimulated proliferation (A), whereas after 5 days, adenosine and ATP exerted a slight inhibitory effect and ADP was slightly mitogenic (B) (all at 100 μM). Values are means ± SEM (n = 6).

Concentration (μM)	Total DNA/well (ng)			
	Adenosine *Mean ± SEM*	ADP *Mean ± SEM*	ATP *Mean ± SEM*	UTP *Mean ± SEM*
Control	1821 ± 43.5	2013 ± 51.8	1921 ± 25.1	1969 ± 30.8
0.5	1784 ± 33.6	1952 ± 53.6	1868 ± 34.0	1909 ± 40.0
5	1768 ± 78.5	1932 ± 42.3	1880 ± 51.8	1942 ± 28.0
Control	1818 ± 28.8	1956 ± 36.5	1881 ± 54.1	1942 ± 39.6
50	1787 ± 24.9	1916 ± 66.1	1831 ± 50.5	1903 ± 41.3
500	1713 ± 32.9	1830 ± 48.2	1752 ± 37.2	1879 ± 27.2

Table 4.1

Effects of nucleotides on rat osteoblast proliferation I.

Cells were cultured for 48 h (2 d) in test and control media (DMEM/0.1% FBS), after being rendered quiescent for 48 h in DMEM/0.1% FBS. Proliferation assessed by DNA quantification with Hoechst 33258. Values expressed as means ± SEM of total ng DNA/well (n = 8).

Table 4.2 shows results for a second set of experiments, where cells were subcultured at 10^4 cells/well, cultured in DMEM/5% FBS for 24 h, before nucleotides were added in DMEM/5% FBS. The assay was stopped after 48 h (2 d). The only slight effects were observed with 100 μM adenosine and ATP, which resulted in a reduction of cell number (**Table 4.2**).

Concentration (μM)	Total DNA/well (ng)			
	Adenosine *Mean ± SEM*	ADP *Mean ± SEM*	ATP *Mean ± SEM*	UTP *Mean ± SEM*
Control	2557 ± 40.3	2359 ± 37.2	2523 ± 11.5	2416 ± 22.8
1	2399 ± 64.1	2428 ± 48.5	2473 ± 20.1	2423 ± 23.6
10	2340 ± 51.8	2456 ± 31.5	2513 ± 44.4	2405 ± 9.4
100	2305 ± 64.3	2290 ± 69.9	2218 ± 25.7	2399 ± 25.9

Table 4.2

Effects of nucleotides on rat osteoblast proliferation II.

Cells were cultured for 48 h (2 d) in test and control media (DMEM/5% FBS), after being rendered quiescent for 24 h in DMEM/5% FBS. Proliferation assessed by DNA quantification with Hoechst 33258. Values expressed as means ± SEM of total ng DNA/well (n = 6).

DISCUSSION

Although studies on purinergic signalling in osteoblasts began some 12 years ago, the roles of nucleotides in regulating osteoblast functions were not entirely understood. This chapter aimed at investigating the effects of nucleotides on primary rat calvarial osteoblasts studying bone nodule formation, an *in vitro* model for bone formation by osteoblasts, and cell proliferation, using two different approaches: [^{3}H]thymidine incorporation, which assesses the number of cells undergoing active proliferation, and DNA quantification with the fluorescent dye Hoechst 33258, which determines the total cell number.

Both ATP and UTP, but not adenosine, ADP or 2-meSADP, significantly and consistently inhibited bone nodule formation, whereas no clear-cut major effects of nucleotides on cell proliferation were found. The potent inhibitory actions of ATP and UTP point to the involvement of either $P2Y_2$ or $P2Y_4$ receptors, since ATP and UTP are potent agonists at both receptor subtypes. However, since no evidence was found for the expression of the $P2Y_4$ receptor subtype on bone cells (Chapter 2), it can be suggested that the $P2Y_2$ receptor, expressed on osteoblasts, might be responsible for mediating these effects.

The inhibitory effects of UTP and ATP started at concentrations as low as 1 μM and 10 μM, respectively, which stands in sharp contrast with an earlier paper on the effects of nucleotides on appositional bone formation (Jones *et al.*, 1997). This earlier study employed a different *in vitro* model of bone formation, using the same type of primary rat calvarial cells, but seeding them on dentine slices with cut-in grooves, on which bone could be deposited. At the end of the experiment, number, length and area of bone loci were analysed. However, only when applying relatively high concentrations of 500 μM ATP, a significant inhibition of bone formation could be observed, and in one experiment, ATP at 50 μM even slightly stimulated bone formation. Lower inhibitory concentrations of 20 μM were reported for 2-meSATP and the non-hydrolysable ATP analogue ATPγS, whereas, similar to my studies, adenosine had no effect. ADP was not tested, and the effects of UTP were equivocal: in one experiment, 2 μM UTP stimulated

bone formation, whereas in another experiment, 2-20 μM of UTP inhibited bone formation. Thus, the results presented in this chapter provide a more conclusive result: both ATP and UTP, in the low micromolar range, are strong inhibitors of bone formation. The fact that in the earlier study ATPγS, a potent agonist at the $P2Y_2$ receptor, was inhibitory provides further evidence for the role of the $P2Y_2$ receptor in this process.

ATP at much higher, millimolar concentrations has long been known to play a role in the mineralisation process. ATP (> 5 mM) inhibits Ca^{2+} deposition because it is a potent stabiliser of amorphous calcium phosphate, thus inhibiting its conversion to hydroxyapatite *in vitro*. However, ATP at 1-5 mM can promote bone formation by providing pyrophosphate for the mineralisation process by matrix vesicles (Hsu and Anderson, 1977). This highlights the role for specific ecto-ATPases in the calcification process, which are responsible for the ATP-dependent Ca^{2+}-and P_i-depositing activity of bone or cartilage-derived matrix vesicles (Hsu and Anderson, 1996).

However, inhibitory effects of ATP and UTP on bone nodule formation in the present study were observed in the low micromolar range. Thus, an indirect, not receptor-mediated effect on mineralisation through the mechanism described above can probably be excluded. Additionally, negative effects on bone nodule formation, as observed here for ATP and UTP, are most likely due to inhibitory effects on cell aggregation and/or collagen/matrix deposition in the earlier stages of the culture, prior to mineralisation, and not to inhibiton of mineral deposition. From this work, it cannot be told how nucleotides signal intracellularly to inhibit bone formation. Nor was determined whether single and intermittent pulses of ATP or UTP would have the same consequence as continuous application.

In this context, it has recently been shown that induction of osteoblast differentiation by addition of ascorbic acid led to 3- to 4-fold increases in respiration and ATP production, resulting in a 5-fold increase in ATP content compared with that in immature, undifferentiated cells. This demonstrated that progressive osteoblast differentiation coincides with changes in cellular metabolism and mitochondrial activity, which are likely to play key roles in osteoblast function. An enlarged pool of ATP might

be stored in mature osteoblasts for later release in response to appropriate stimuli, so that ATP can act as a paracrine agonist at P2 receptors and, based on my results, potentially serve as a 'stopping signal' for bone formation (Komarova *et al.*, 2000).

Although only one paper has been published on nucleotides effects on osteoblastic bone formation, more studies have been performed on ECM formation in chondrocyte cultures. In contrast to the inhibitory effects of ATP and UTP on bone nodule formation, an increased accumulation of cartilage proteoglycan and collagen in the presence of relatively high-dose (500 μM) ATP and UTP, but not ADP, has been demonstrated in a chondrocyte pellet culture system, whereas no effect on cell number was observed. This illustrates that chondrocytes might undergo a long-term change to an anabolic, matrix-secreting phenotype in response to extracellular nucleotides. The anabolic response was only seen in the presence of serum, pointing to a cross-talk between growth factor receptor and purinergic signalling pathways (Croucher *et al.*, 2000).

The finding that ATP and UTP might play a role in osteoblastic activity agrees with a number of earlier studies. Kumagai *et al.* first reported in 1989 that ATP elevates cytosolic Ca^{2+} through mobilisation of $[Ca^{2+}]_i$, but not through Ca^{2+} influx, in rat osteoblastic UMR-106 cells (Kumagai *et al.*, 1989). In a follow-up study, a two-receptor model was suggested because ADP and UTP, in addition to ATP, also elicited a rapid transient increase in $[Ca^{2+}]_i$ at 1-100 μM, followed by an increase in IP_3 and IP_4 (Kumagai *et al.*, 1991). The presence of two functional P2Y receptors ($P2Y_1$ and $P2Y_2$–like) on osteoblasts, coupled to internal Ca^{2+} release, was confirmed by further experiments (Reimer and Dixon, 1992; Yu and Ferrier, 1993b; Sistare *et al.*, 1994; Sistare *et al.*, 1995), whereas adenosine, AMP, α,β-meATP and β,γ-meATP were not found to have an effect on $[Ca^{2+}]_i$. Reimer and Dixon reported a time-dependent inactivation of the Ca^{2+} signalling pathway with continued receptor occupation by ATP (1992). This may be physiologically important as a mechanism to limit the duration of the Ca^{2+} signal in the continued presence of extracellular nucleotides. Additionally, activation of PKC has been shown to selectively desensitise the $P2Y_1$, and not the $P2Y_2$ signalling pathway in osteoblastic cells, and may thus be implicated in the negative

feedback control of Ca^{2+} elevation (Gallinaro *et al.*, 1995). The avian $P2Y_1$ receptor has three consensus phosphorylation sites for PKC, which may play a role in receptor desensitisation (Webb *et al.*, 1993). *In vivo*, endocrine or paracrine factors, acting through PKC, may therefore regulate responsiveness of osteoblasts to extracellular nucleotides.

Kumagai *et al.* also observed a potentiated Ca^{2+} response to nucleotides in the presence of PTH (1991). This is consistent with later studies showing that activation of $P2Y_1$ and $P2Y_2$ receptors potentiates subsequent PTH receptor-mediated Ca^{2+} signalling (Kaplan *et al.*, 1995; Sistare *et al.*, 1995; Bowler *et al.*, 1999; Buckley *et al.*, 2001b). It has been suggested that PTH receptors are capable of activating adenylate cyclase through G_S proteins, but might be unable to activate PLC until cells receive a signal as a consequence of P2 receptor activation (Sistare *et al.*, 1995).

Signalling events involved in the synergistic effect of PTH (PTH 1-31, but not PTH 3-34 peptide) and nucleotides (esp. ADP) in UMR-106 cells were investigated in a recent study: despite the link to G_S and adenylate cyclase, this synergy did not involve cAMP or cGMP accumulation, nor PKA activation, so that events further upstream at the level of G protein subunit interaction of $P2Y_1$ and PTH receptors were suggested (Buckley *et al.*, 2001b). The significance of this potentiation can be seen in its effects on transcription factor activation and gene expression: ADP in combination with PTH, but not alone, activated the transcription factor CREB (cAMP response element-binding protein), and both synergistically activated *c-fos* expression, which, as mentioned before, is strongly implicated in controlling the proliferation and differentiation of bone cells. These results contrasted with an earlier study on the human osteosarcoma SaOS-2 cell line, also predominantly expressing $P2Y_1$, but requiring the serum response element, in addition to the Ca/CRE (Ca^{2+}/cAMP response element), of the *c-fos* promoter for synergy upon co-stimulation with ADP and PTH (Bowler *et al.*, 1999). $P2Y_2$ receptors can also mediate synergistic *c-fos* induction by ATP/UTP and PTH in primary human osteoblasts (Bowler *et al.*, 1999). These synergies suggest a potentially highly targeted mechanism through which systemic PTH could initiate bone remodelling at specific sites in the skeleton by cooperating with the localised release of nucleotides.

Whether PTH can act synergistically with ATP and UTP in inhibiting bone nodule formation has not been investigated in the present study, but presents an interesting area of future work. As mentioned above, PTH combines with UTP and ATP to induce *c-fos* expression in primary human osteoblasts; thus, this synergistic effect is not only restricted to osteoblastic immortal cell lines. The action of PTH itself on osteoblastic function is complex and controversial. Effects depend on the differentiation stage and confluency of cultured osteoblasts, *e.g.* PTH may preferentially stimulate osteoblast differentiation in immature, low cell density, subconfluent osteoblasts, but inhibit it in more mature, high cell density, postconfluent osteoblasts (Isogai *et al.*, 1996). Additionally, PTH can have both anabolic and catabolic effects on mineralisation *in vitro* (30 day culture of murine MC3T3-E1 cells), depending on the time and duration of the treatment: continuous PTH treatment before day 20 decreased mineralisation, continuous treatment after day 20 had no effect, whereas intermittent short-term treatment only between day 20-25 increased mineralisation (Schiller *et al.*, 1999). Similarly, PTH was reported to have diverse effects on differentiation of rat osteoblastic cells and mediation through different signal transduction systems (cAMP/PKA and/or Ca^{2+}/PKC) depending on the exposure time (Ishizuya *et al.*, 1997). Thus, future investigations into any synergistic effects of PTH and nucleotides on bone nodule formation would require very careful planning of duration and timing of PTH treatment.

Nucleotides can also potentiate the mitogenic effects of growth factors, such as IGF or PDGF (Shimegi, 1996; 1998). Thus, one could speculate that in damaged bone tissues, increased local levels of PDGF and nucleotides released from activated platelets, endothelial cells and other cells, attract osteogenic cells to lesional sites and stimulate their proliferation (Shimegi, 1996). Extracellular nucleotides present in the bone microenvironment may thus be capable of modulating bone cells and controlling the remodelling process by interacting with both systemic hormones, such as PTH, and local growth factors.

It is unclear why no clear-cut mitogenic effects of nucleotides were observed in my study, whereas in the earlier studies (see below) nucleotide treatment could at least

double the number of proliferating cells. Although a variety of cell culture conditions were tried by changing the exposure time to nucleotides, the total time in culture, assessment of proliferation and conditions to render the cells quiescent, no consistent effects were found. Using [^{3}H]thymidine incorporation methods to study proliferation, UTP at 100 μM seemed to have a weak mitogenic effect when cultured for 2 d, but after 5 d of culture, ADP at 100 μM slightly stimulated proliferation, whereas adenosine and ATP seemed to inhibit proliferation. Quantification of total DNA content also suggested that adenosine and ATP might act as potential inhibitors of proliferation.

In contrast to these results, ATP and adenosine (10 – 100 μM) have previously been shown to stimulate proliferation of cloned osteoblast-like mouse MC3T3-E1 cells, whereas UTP had no effect. The actions of ATP appeared to be mediated via internal Ca^{2+} release, but were independent of PKC and/or PGE production (Shimegi, 1996). Conversely, another study reported that the mitogenic effects of ATP were mediated in part by ATP-stimulated PGE_2 synthesis (Suzuki et al., 1993). Furthermore, this group suggested that ATP stimulates phospholipase D (PLD) in osteoblast-like cells (Suzuki et al., 1995), and that phosphatidylcholine hydrolysis by PLD is involved in increased PGE_2 production (Watanabe-Tomita et al., 1997). ATP also stimulated Ca^{2+} influx, consistent with P2X receptors, which resulted in the release of arachidonic acid, production of PGE_2 and enhanced DNA synthesis in osteoblast-like cells (Suzuki et al., 1993).

Mitogenic actions of ATP, probably mediated via P2X receptors, were confirmed in a recent study on human osteoblast-like MG-63 cells: extracellular ATP (10 – 100 μM), but not UTP, increased DNA synthesis and enhanced the proliferative effect of PDGF and IGF-1 through P2X receptors. Based on antagonist studies, the authors proposed a $P2X_5$-mediated proliferative effect. Further evidence suggested that ATP-induced cell proliferation might be mediated through several protein kinase pathways, including tyrosine kinases and MAPK, but not the Ca^{2+}-dependent protein kinases or the PI-3 kinase pathways (Nakamura et al., 2000). A preliminary study recently reported that P1-purinoreceptors for adenosine can mediate both stimulatory and inhibitory effects on proliferation of MG-63 cells, depending on the P1 receptor subtype, A_1 or A_2, and the

concentration of adenosine (Shafique and Caswell, 2001). This would agree with my findings, showing that adenosine can have a slight inhibitory effect on cell proliferation at 100 μM.

Osteoblasts might change their expression profile as they differentiate, since studies on single cells and cell populations of human osteoblasts revealed a heterogeneity of receptor expression (Dixon *et al.*, 1997). As described above, studies that reported proliferative effects of nucleotides used established clonal osteoblastic cell lines, derived from tumours or normal osteogenic tissue, and no primary cell cultures. Although primary cell cultures should most closely reflect osteoblastic cell populations present *in vivo*, they are, by nature, very heterogeneous, and cells isolated on different occasions may give inconsistent results, as it was the case in this study. Primary populations of osteoblasts clearly comprise more than one lineage and different ratios of more, or less, differentiated cells, as demonstrated recently in a study on gene expression in calvarial osteoblasts (Candeliere *et al.*, 2001).

In conclusion, this chapter provides evidence for a functional role of the $P2Y_2$ receptor in negatively modulating bone formation by rat osteoblastic cells, which contrasts with the proposed role of the $P2Y_1$ receptor in bone resorption. Since the effective concentrations of ATP and UTP were in the low micromolar range, this could reflect the *in vivo* situation where nucleotides are likely to occur transiently in the bone microenvironment. Osteoblasts are capable of releasing ATP, resulting in nanomolar concentrations in the medium (Bowler *et al.*, 1998b; own preliminary results); however, concentrations measured in the entire medium are unlikely to accurately reflect concentrations occurring at the cell surface. These are probably in the micromolar range before breakdown by ecto-nucleotidases takes place. So far, release of UTP has been reported for a range of cells such as astrocytoma cells, epithelial cells and platelets, but not osteoblasts (Lazarowski and Harden, 1999). However, UTP could easily be generated extracellularly from other nucleotides through the action of ecto-nucleotidases, *e.g.* UDP + ATP → UTP + ADP via ecto-nucleoside diphosphokinase (Lazarowski *et al.*, 2000; Zimmermann, 2000). Interestingly, UTP can also act through P2Y receptors to

up-regulate ATP release from human osteoblasts, providing a possible positive feedback mechanism (Bowler *et al.*, 2001).

CHAPTER 5

INTERACTION OF LOW PH AND OSTEOLYTIC AGENTS

INTRODUCTION

Acid-base balance

The machinery of cells is very sensitive to changes in H^+ concentration, therefore the mechanisms regulating the intracellular (pH_i) and extracellular (pH_e) H^+ concentration are particularly important. pH_i, which can be measured by microelectrodes or pH-sensitive fluorescent dyes, regulates a variety of intracellular processes and although different from pH_e, it is still sensitive changes in the extracellular fluid. Several systems in the body maintain the pH of the body fluids within a narrow range (pH 7.36 to 7.44): the pH of arterial plasma is normally pH 7.40 and that of venous plasma slightly lower. Technically, acidosis is present whenever the pH is below 7.4, and alkalosis whenever the pH is above 7.4. Most chemical reactions in the body produce or absorb protons. For example, proteins are metabolised into amino acids, which are utilised in the liver for gluconeogenesis, leaving as products ammonium ions (NH_4^+) and bicarbonate (HCO_3^-). NH_4^+ becomes incorporated into urea, whereas HCO_3^- buffers the protons formed intracellularly, so that little NH_4^+ and HCO_3^- escape into circulation. In contrast, metabolism of sulphur-containing amino acids produces sulphuric acid (H_2SO_4), and of others phosphoric acid (H_3PO_4), both strong acids that enter the circulation and present a major H^+ load of about 50 mEq/d. Much larger amounts of H^+ (12,500 mEq/d) are formed indirectly from carbon dioxide (CO_2), generated by metabolism in the tissues and largely hydrated to carbonic acid (H_2CO_3). However, most of the CO_2 is excreted in the

lungs. Common sources of extra acid loads are strenuous exercise (lactic acid production), diabetic ketosis, ingestion of acidifying salts, such as NH_4Cl, but also food products such as cola drinks, which contain phosphoric acid and have a pH of 3 (Barzel, 1995). The main sources of dietary alkali are fruits and vegetables, as Na^+ and K^+ salts of weak acids. A common cause of alkalosis is the loss of acid from the body as a result of vomiting of HCl-rich gastric juice.

The lungs and kidneys ultimately remove excess CO_2 and H^+ generated in the body, but in the short-term, several buffer systems help to maintain pH_i and pH_e at very constant levels. The three major buffers in the blood are plasma proteins, haemoglobin and the bicarbonate system, whereas pH_i is mainly controlled by protein- and phosphate buffer systems, and pH_e by bicarbonate buffering. For the bicarbonate system, *in vivo* and *in vitro* (in physiologically buffered media), the following equilibrium exists:

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

The system is one of the most effective buffer systems in the body, because the amount of dissolved CO_2 is controlled by respiration. Thus, when H^+ (in the form of strong acid = metabolic acidosis) is added to the blood, HCO_3^- declines as more H_2CO_3 is formed. If the extra H_2CO_3 were not converted to CO_2 and H_2O, and the CO_2 excreted in the lungs, the H_2CO_3 concentration would raise and the pH drop considerably.

In contrast, a rise in arterial P_{CO_2} due to decreased ventilation causes respiratory acidosis. Since CO_2 is in equilibrium H_2CO_3, which in turn is in equilibrium with HCO_3^-, the plasma HCO_3^- raises and a new equilibrium is reached at a lower pH.

In vitro, physiologically buffered tissue culture media formulated with Earle's salts contain 2.2g $NaHCO_3$/l (DMEM contains 3.7g $NaHCO_3$/l), and are normally used in combination with a 5% CO_2 atmosphere to produce an operating pH close to 7.25 at equilibrium. Addition of protons (in the form of concentrated HCl) to the medium reduces the HCO_3^- concentration and the equilibrium pH at constant P_{CO_2}, thus mimicking *in vivo* metabolic acidosis. Conversely, increasing P_{CO_2} reduces pH, while

the HCO_3^- concentration remains more or less constant, a model of *in vivo* respiratory acidosis (see also Figure 3.1).

Acidosis and the skeleton

The importance of the skeleton in the maintenance of acid-base balance has long been recognised. Besides serving as a Ca^{2+} reservoir, the skeleton contains 80% of the body's carbonate and 35% of the body's sodium, which, in response to a metabolic acid challenge, can be acutely released to buffer protons in an acellular physicochemical reaction (Barzel, 1995). Acidosis has been associated with mineral loss since the early part of this century, and may contribute to pathological conditions such as renal osteodystrophy and osteoporosis. It has long been thought that buffering of acid loads was only due to short-term physicochemical reactions; however, as will be discussed below, it is now widely accepted that acidosis predominantly activates more long-term cellular mechanisms, involving both osteoclasts and osteoblasts, leading to bone loss.

A number of *in vitro* studies, using both bone organ cultures and isolated osteoclast cultures, confirmed the role of acidosis in bone resorption. First studies using whole-organ calvarial or long bone cultures suggested that, especially in short-term (3 h), a pH_e decrease resulted in a net Ca^{2+} efflux due to physicochemical dissolution of either hydroxyapatite, brushite ($CaHPO_4$), or calcium carbonate ($CaCO_3$) from bone (Dominguez and Raisz, 1979; Bushinsky *et al.*, 1983; Bushinsky *et al.*, 1986). However, in longer-term cultures, Ca^{2+} release in response to acidosis appeared to be osteoclast-mediated. Goldhaber & Rabadjija (1987) demonstrated hat acidification of culture media with HCl produced large dose-dependent increases in Ca^{2+} release from mouse calvariae over 7 days. The effect was blocked by calcitonin and also by indomethacin, suggesting that osteoclast-mediated resorption was involved, and that the effect was prostaglandin mediated (Goldhaber and Rabadjija, 1987). Bushinsky *et al.* first reported that metabolic acidosis was more potent than respiratory acidosis in stimulating resorption in 4 d calvarial cultures, suggesting that decreased medium HCO_3^-, and not just a fall in pH, is necessary to enhance net Ca^{2+} efflux from calvariae (Bushinsky, 1989), which was

confirmed in a more recent study (Meghji *et al.*, 2001). A low HCO_3^- concentration (as in metabolic acidosis) might be expected to facilitate the exchange of intracellular HCO_3^- for Cl^- in osteoclasts, leading to enhanced HCl secretion and increased resorption. Increasing CO_2 (as in respiratory acidosis) would not be expected to stimulate the HCO_3^- /Cl^- exchanger. The issue whether metabolic or respiratory acidosis are more potent stimulators of bone resorption remains controversial: in short-term isolated osteoclast cultures, rat osteoclasts were more sensitive to stimulation by CO_2 acidosis than by HCO_3^- acidosis, resulting in deeper and bigger pits (Arnett *et al.*, 1994).

However, isolated osteoclast cultures demonstrated the direct effect of pH on resorption most strongly: in non-physiologically buffered culture media (using HEPES only), resorption pit formation increased progressively as medium pH was reduced from 7.4 to 6.8 (Arnett and Dempster, 1986). The remarkable sensitivity of rat osteoclasts to extracellular protons when cultured in physiologically HCO_3^-/CO_2-buffered media was demonstrated more recently: small shifts in pH_e (from 7.25 to 7.00) resulted in a steep sigmoidal response curve, suggesting that rat osteoclasts are essentially 'switched on' or 'switched off' by very slight alterations in H^+ concentration (Arnett and Spowage, 1996). The stimulatory effects of low pH (< pH 7.2, maximal at ~ pH 6.9-7.0) have been observed in osteoclasts derived from numerous species, including mouse (Morrison and Arnett, 1998; Meghji *et al.*, 2001), rabbit (Shibutani and Heersche, 1993) and chick, although for the latter, differences were reported in the optimal pH for osteoclast activity (Arnett and Dempster, 1987; Walsh *et al.*, 1990; Morrison and Arnett, 1997).

Additionally, a number of osteolytic agents have been shown to act synergistically with low pH, most notably PTH (Arnett and Dempster, 1986), $1,25(OH)_2D_3$ (Murrills *et al.*, 1998) and ATP (Morrison *et al.*, 1998). The interaction with ATP was particularly interesting because one receptor subtype of the P2 receptor family, the $P2X_2$ receptor, has been shown to require extracellular acidification to show its full sensitivity to ATP (King *et al.*, 1996), and was thus thought to be involved in the stimulation of osteoclasts by low pH and ATP. The mechanism by which acidosis 'switches on' osteoclasts is still unclear, although studies reported acid-induced upregulation of CA II, calcitonin receptor

and TRAP mRNA (Asotra *et al.*, 1994; Biskobing and Fan, 2000), and vacuolar H^+-ATPase (Nordström *et al.*, 1997), as well as increased podosome formation and attachment to bone (Teti *et al.*, 1989a; Murrills *et al.*, 1993). The finding that acid-induced bone resorption, in the absence of ATP, could be partly inhibited by the general P2 receptor antagonist suramin and by apyrase, an ecto-nucleotidase scavenging any ATP present, additionally suggested that the pH effect might be dependent on trace levels of extracellular ATP, and that the $P2X_2$ receptor might be the putative 'pH receptor' on osteoclasts (Morrison *et al.*, 1998).

This chapter aimed at answering a number of questions arising from the role of acidosis in osteoclast function. (1) Human osteoclastoma-derived osteoclasts are known to resorb mineralised substrates more agressively than normal mammalian osteoclasts. To investigate whether this could be due to pertubation in the response of the these osteoclasts to pH_e, or whether human osteoclasts are as pH-dependent as osteoclasts from other species, the effect of pH_e changes on osteoclasts disaggregated from human osteoclastoma tissue was investigated. (2) The interaction between RANKL and low pH was studied using the disaggregated rat osteoclast assay. RANKL, in addition to its effects on osteoclastogenesis (see Chapter 1), can also directly activate mature osteoclasts to resorb bone (Fuller *et al.*, 1998; Lacey *et al.*, 1998; Burgess *et al.*, 1999), and it was investigated whether the pro-resorptive action of RANKL is as pH-dependent as the action of other osteolytic agents. (3) In an attempt to identify putative 'pH receptors' on osteoclasts, the expression of acid-sensitive ion channels (ASIC) on osteoclasts was studied. The first ASIC was cloned four years ago (Waldmann *et al.*, 1997), and characterisation of further H^+-gated ion channels suggested that they represent members of amiloride-sensitive Na^+ channel/degenerin superfamily of ion channels. Expression induces an amiloride-sensitive cation channel which is transiently activated by rapid extracellular acidification. ASIC is now called ASIC1 and is activated by $pH < 7.0$, whereas ASIC2, or mammalian neuronal homolog of degenerins (MDEG1) requires a more acidic pH. A third channel, dorsal root ganglion ASIC (or DRASIC or ASIC3), is activated by $pH < 4.5$. Since the pH activation profile of ASIC1 strongly resembles the

pH response of osteoclasts, expression was studied on osteoclasts using a set of commercially available polyclonal antibodies.

Studies on the interaction of RANKL and low pH were performed in collaboration with Nichola Zanellato, who worked in our lab on her BSc project.

METHODS

Human osteoclastoma cultures

The effect of pH_e alterations was studied on osteoclasts disaggregated from human osteoclastoma tissue. Fresh osteoclastoma tissue was obtained at surgery (kindly provided by Dr Ann Sandison, Royal National Orthopaedic Hospital, Stanmore, UK). Tumours (~5 mls volume) were chopped into small pieces in serum-free MEM and digested with collagenase type I solution (3 mg/ml in MEM) for 30 min at 37°C, with mixing every 5 min. The mixture was passed through a 100 μm sieve by gentle use of a 5 ml syringe plunger, and rinsed thoroughly with MEM. Cells were resuspended in serum-free MEM and spun at 200g for 10 min. MEM was discarded, the cell pellet resuspended, washed again, and MEM discarded. For cell freezing, the cell pellet was resuspended in 10% DMSO and 40% FCS in MEM, frozen in 1 ml vials and stored in liquid nitrogen. For culture, cells were quickly thawed, washed with MEM, spun down and reconstituted in cold tissue culture medium (MEM/10% FCS). Cells were sedimented on to 5 mm dentine discs for 2 h, transferred to 6-well plates with pre-equilibrated test media, and cultured for 24 h in 5% CO_2 - 95% air. MEM was modified by the addition of small amounts of HCl or NaOH (12, 9, 6, 3, 0, -3, -6, -9, -12, -15, -18 or -21 mEq/l H^+ (minus sign represents addition of OH^-). Each group contained 8 replicates. At the end of the experiment, discs were fixed with 2% glutaraldehyde, stained for TRAP, and medium pH and P_{CO_2} were measured by blood gas analyser. TRAP-positive osteoclasts and resorption pits were assessed 'blind' by transmitted/reflected light microscopy (for detailed method refer to Chapter 3: Disaggregated rat osteoclast assay). Pit depth was estimated using reflected light microscopy and the fine focus control (in μm) on the microscope.

The osteoclastoma cells used in the culture for which results are presented here were kindly provided by Dr Steve Nesbitt (Bone and Mineral Centre, UCL).

Disaggregated rat osteoclast assay

RANKL was a generous gift of Dr Colin Dunstan (Amgen Inc, USA). The effects of RANKL (at 1, 10, 100 ng/ml) and pH (~ pH 7.0 and ~ pH 7.4) on resorption pit formation by mature rat osteoclasts were studied using the disaggregated rat osteoclast assay, as described in Chapter 3. pH was modified by direct addition of concentrated HCl or 6 M NaOH, resulting in an operating pH close to pH 7.0 and pH 7.4, respectively. The latter was assumed to reflect the 'physiological' pH present in blood. In addition to the number of TRAP-positive multinucleated osteoclasts and the number of discrete resorption pits, the total area covered by resorption, and the area of individual resorption pits were assessed 'blind' via a colour image video output, using 'dot count' morphometry.

Statistical comparisons were made by one-way analysis of variance (ANOVA); representative data are presented as means ± SEM for 5 replicates. Significance was assumed at $p < 0.05$. Results are presented for a representative experiment that was repeated three times.

Immunocytochemistry for ASIC

Antibodies against ASIC1, ASIC2, and DRASIC and control peptide for ASIC1 were obtained from Alpha Diagnostic International (USA) at stock concentrations of 1mg/ml, and used at 1:200 dilution. Pre-absorption of ASIC1 antibody was performed overnight with 10-fold excess peptide. Osteoclasts were isolated as described before (Chapter 2), and cultured in LabTek 8 chamber slides or on dentine discs for 24 h in pH 6.9 or pH 7.2. Immunocytochemical procedures were performed as described for immunostaining with anti-P2 receptor antibodies in Chapter 2, with the exception that alternative primary antibody diluents were tested, *i.e.* with or without the addition of Triton X-100 (0.1% in 10% NHS). Additionally, cultures on dentine discs were not treated with methanol at -20°C when followed by staining for F-actin in resorbing osteoclasts with rhodamine-conjugated phalloidin (Molecular Probes, Netherlands) for 30 min prior to confocal

microscopy. Osteoclasts on dentine discs were monitored with a Leica TCS NT confocal laser scanning microscope (Leica, Germany), using standard filter settings and sequential scanning. The confocal microscope was kindly provided by Prof Mike Horton, and demonstrated by Dr Gudrun Stenbeck (Bone and Mineral Centre, UCL).

RESULTS

Acid activation of osteoclasts derived from human osteoclastoma

The response of osteoclasts derived from human osteoclastoma to changes in pH_e was very similar to that of normal rat osteoclasts (Arnett and Spowage, 1996). Culture medium was modified by addition of 12, 9, 6, 3, 0, -3, -6, -9, -12, -15, -18 or -21 mEq/l H^+ (minus sign represents addition of OH^-), resulting in pH values from pH 6.83 to pH 7.37. Figure 5.1 shows that pit formation was almost 'switched off' above pH 7.32 and was maximally activated at about pH 7.0 (**Fig. 5.1**). Osteoclastoma-derived cells seemed to resorb dentine more aggressively than osteoclasts from normal tissues. Single pits formed by osteoclastoma cells were typically up to 30 μm deep, compared with about 5 μm for rat osteoclasts and 10 μm for chick osteoclasts (Tim Arnett, personal communication) (**Fig. 5.2**).

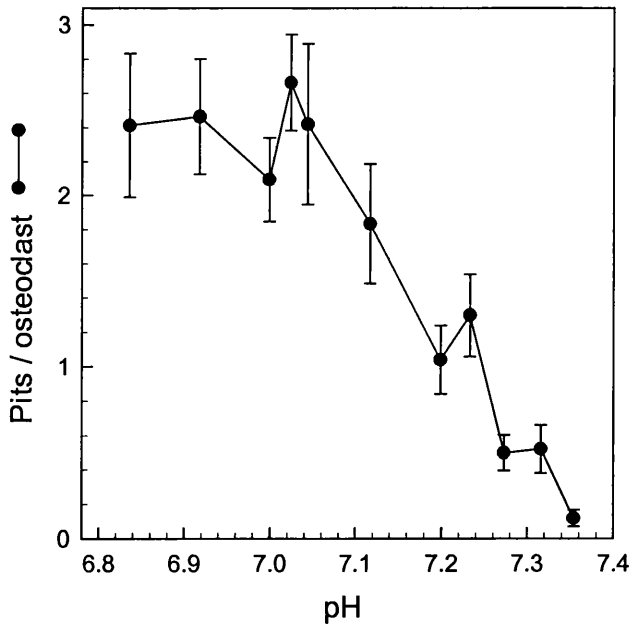


Figure 5.1

Stimulation of resorption pit formation by osteoclasts derived from human osteoclastoma as extracellular pH is reduced.

Culture medium was modified (from left to right) by addition of 12, 9, 6, 3, 0, -3, -6, -9, -12, -15, -18 or -21 mmol/l H^+ (minus sign represents addition of OH^-). Values are means ± SEM (n = 8).

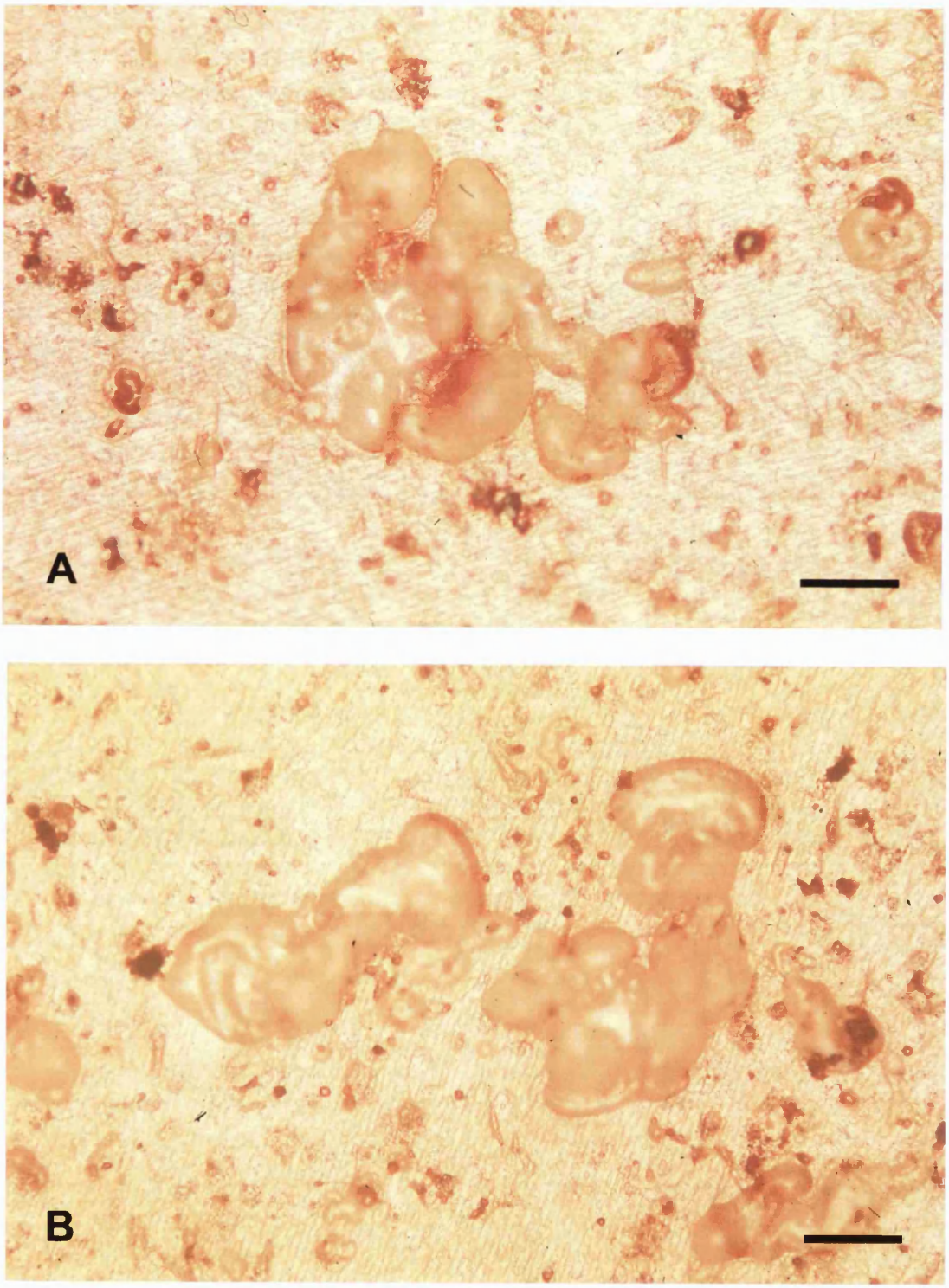


Figure 5.2

Resorption pits excavated by human osteoclastoma cells.

(A, B) Resorption pits up to 30 μm deep formed by osteoclasts derived from human osteoclastoma in 24 h cultures. Stained for TRAP; viewed by reflected light microscopy. Scale bars = 50 μm.

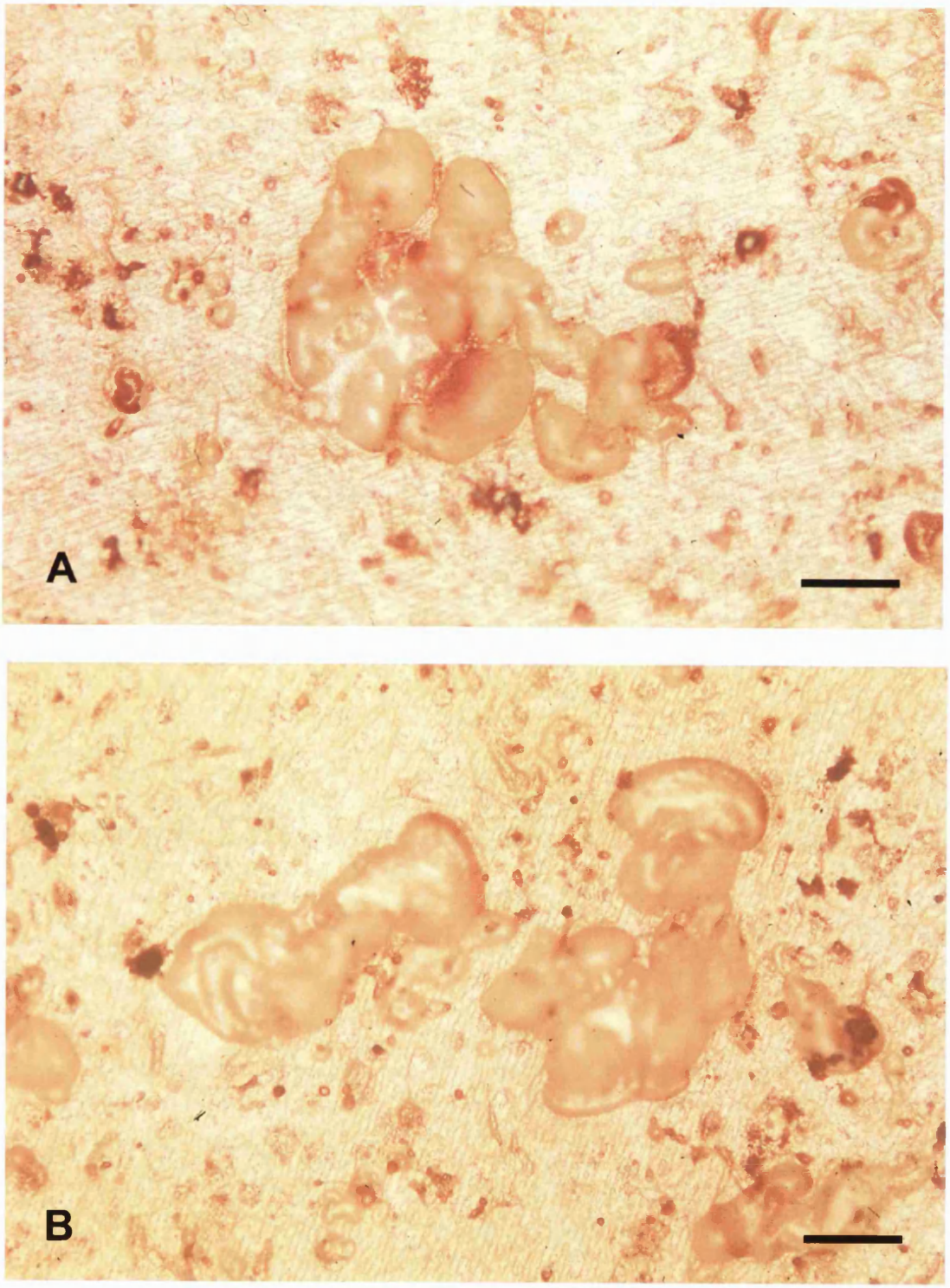


Figure 5.2

Resorption pits excavated by human osteoclastoma cells.

(**A, B**) Resorption pits up to 30 μm deep formed by osteoclasts derived from human osteoclastoma in 24 h cultures. Stained for TRAP; viewed by reflected light microscopy. Scale bars = 50 μm.

Interaction between RANKL and low pH

As demonstrated in Figures 5.3 and 5.4, results show clearly that RANKL can cause striking increases in osteoclastic resorption, but that this effect is dependent on extracellular acidification. Assays were analysed by assessing three separate parameters: number of resorption pits, number of osteoclasts, and area of resorption pits.

As shown in Fig. 5.3, at physiological pH (~ 7.4), basal resorption was very low and RANKL (10 or 100 ng/ml) caused small increases only, in absolute terms, in numbers of resorption pits. In the absence of RANKL, reduction of pH from pH ~7.4 to pH ~7.0 increased pit formation 16-fold. Combined treatment with RANKL (10 or 100 ng /ml) and pH reduction resulted in a synergistic increase in resorption: the total number of pits doubled compared to acidified control and increased 10-fold compared to equivalent RANKL treatments at pH 7.4. (**Fig. 5.3 A**). No significant effects of either pH or RANKL treatment were observed on the total numbers of osteoclasts per dentine disc (**Fig. 5.3 B**); the numbers of pits resorbed per osteoclast (**Fig. 5.3 C**) exhibited similar changes to those shown in Fig. 5.3 A.

Broadly similar responses were observed when resorption pit areas were taken into account, except that the magnitude of RANKL effects at pH ~7.0 was even greater (**Figs. 5.4 A,B**). Treatment with 10 and 100 ng RANKL/ml at pH ~7.0 stimulated osteoclasts to resorb 29- and 25-fold more than at pH ~7.4 (**Fig. 5.4 B**). The effect of RANKL at either pH was to increase the size of resorption pits: in the presence of 100 ng/ml RANKL, the area of individual resorption pits was approximately doubled (**Fig. 5.4 C**). Histological examination of dentine discs revealed that RANKL treatment frequently caused osteoclasts to excavate continuous, large 'snail-trail' pits (**Fig. 5.5**); this pattern of resorption was almost never observed in cultures stimulated by acidification alone.

Figure 5.3

Effects of RANKL and low pH on the numbers of pits and osteoclasts.

(**A, C**) pH-dependent effects of RANKL treatment on the numbers of discrete resorption pits excavated by rat osteoclasts cultured on dentine discs for 26 h; the stimulatory effects of low pH and RANKL were synergistic. (**B**) Total numbers of osteoclasts were unchanged by RANKL treatment and/or pH changes. Significantly different from control in the same pH group: $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$. Significantly different from the same RANKL concentration at physiological pH: ** $p < 0.01$, *** $p < 0.001$. Values plotted are means ± SEM ($n = 5$).

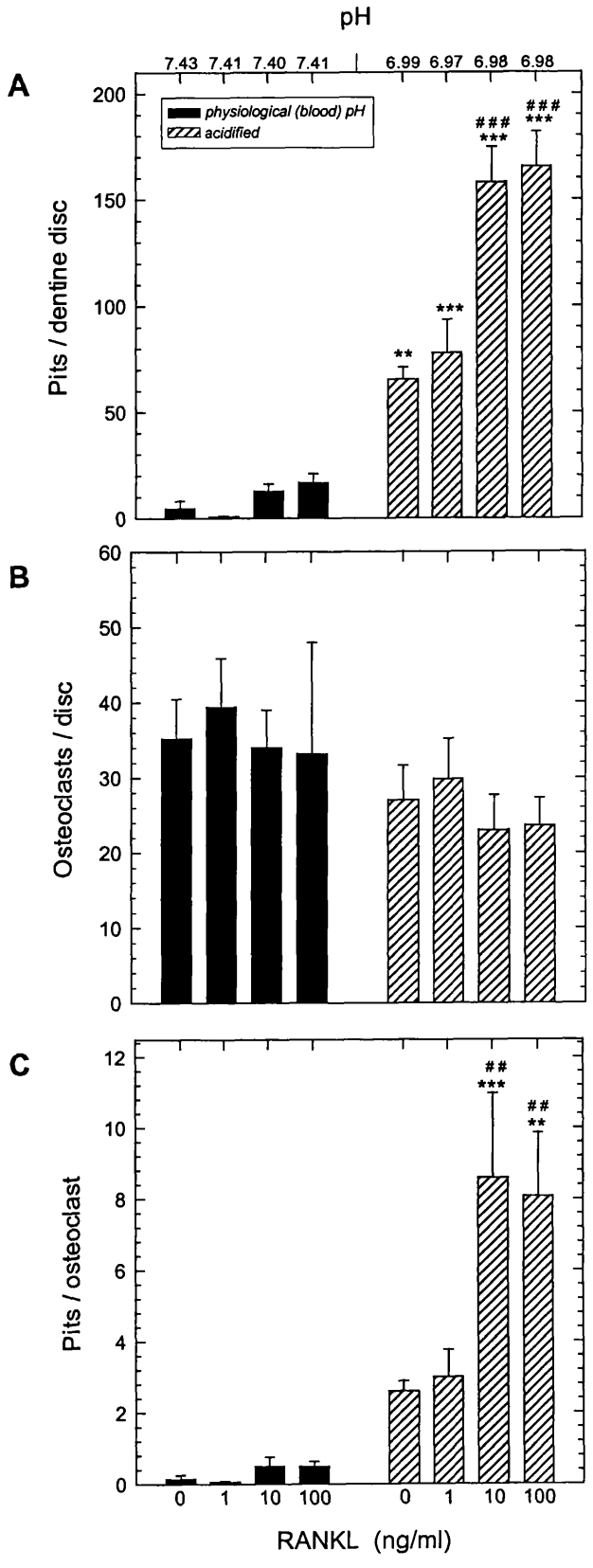
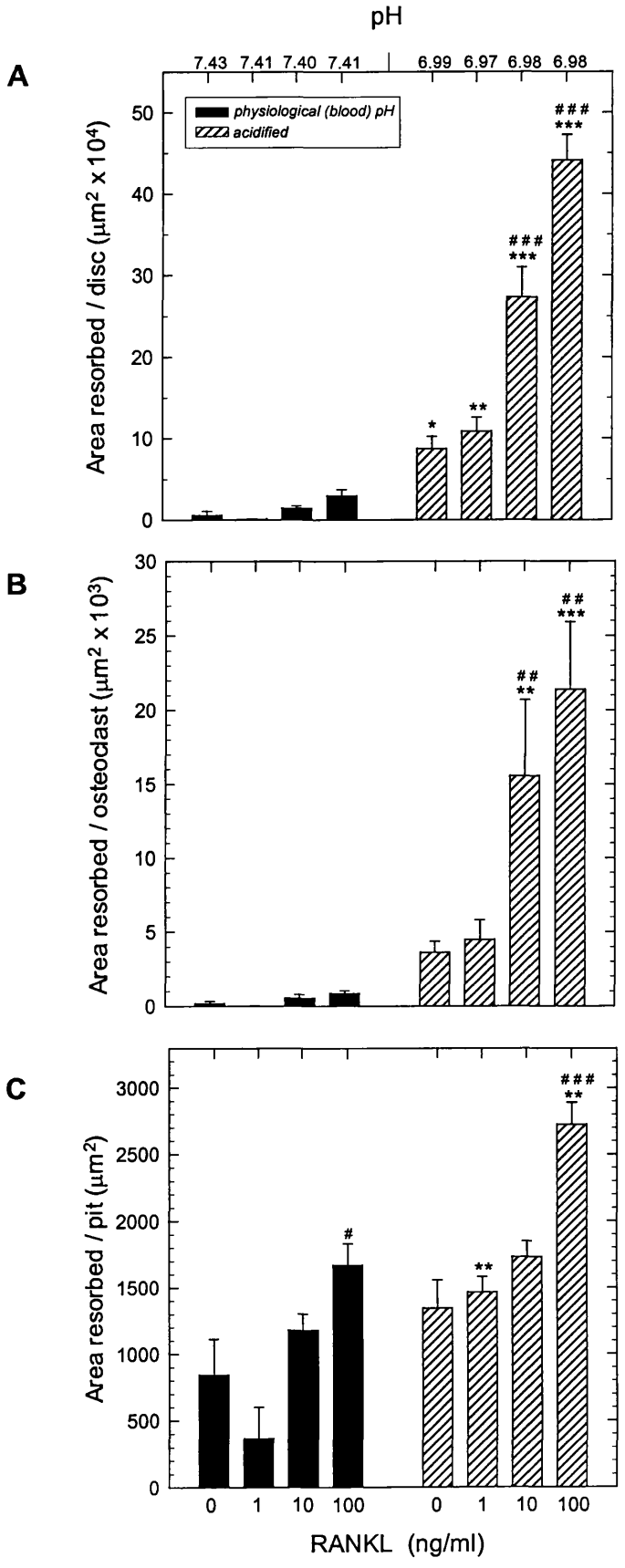

pH
7.43 7.41 7.40 7.41
6.99 6.97 6.98 6.98
A
physiological (blood) pH
acidified
Pits / dentine disc
200
150
100
50
0
**
***
###
***
###
***
B
Osteoclasts / disc
60
50
40
30
20
10
0
C
Pits / osteoclast
12
10
8
6
4
2
0
##
***
##
**
0 1 10 100 0 1 10 100
RANKL (ng/ml)

Figure 5.4

Effects of RANKL and low pH on resorption areas.

(**A, B**) Synergistic stimulatory effects of pH and RANKL treatment on the area resorbed per dentine disc (A) or per osteoclast (B). (**C**) Treatment with 100 ng/ml RANKL resulted in a doubling of resorption pit area at physiological or acidified pH. Significantly different from control in the same pH group: $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$. Significantly different from the same RANKL concentration at pH 7.4: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Values plotted are means ± SEM ($n = 5$).


pH
7.43 7.41 7.40 7.41
6.99 6.97 6.98 6.98
A
physiological (blood) pH
acidified
Area resorbed / disc (µm² x 10⁴)
B
Area resorbed / osteoclast (µm² x 10³)
C
Area resorbed / pit (µm²)
RANKL (ng/ml)
0 1 10 100 0 1 10 100

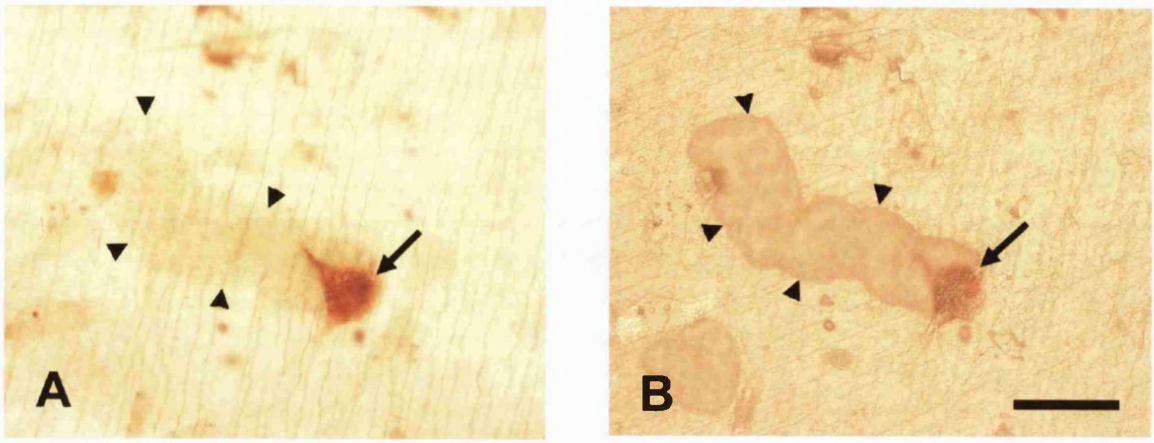


Figure 5.5

Large 'snail trail' pit excavated by osteoclasts treated with RANKL.

(A, B) Osteoclasts (arrow) excavated large 'snail-trail' pits (arrowheads) in the presence of RANKL, viewed by transmitted light microscopy (A) or reflective light microscopy (B). Scale bar = 50 μm.

Expression of ASIC in osteoclasts

Expression of three members of the H^+-gated ion channel family was studied on cultured osteoclasts using a set of polyclonal antibodies directed against ASIC1, ASIC2 and DRASIC. No membrane staining was observed for any of the proteins; however, immunostaining on osteoclasts both on plastic (**Fig. 5.6 A, B**) and on dentine (**Fig. 5.6 D-G**), the latter analysed by confocal microscopy, revealed strong perinuclear staining for ASIC1, with some additional staining observed in cytoplasmic vesicles in osteoclasts cultured on dentine discs (**Fig. 5.6 F,G**). 'Ring-like' perinuclear staining in osteoclasts could represent staining in the Golgi apparatus; thus, ASIC1 appears to be present in osteoclasts, but not functionally expressed in the membrane. Culture in acidified medium did not cause a translocation to the membrane. The staining for ASIC1 was strongly reduced by pre-absorption controls, implying specificity (**Fig. 5.6 C**), whereas ASIC2 and DRASIC showed no significant staining.

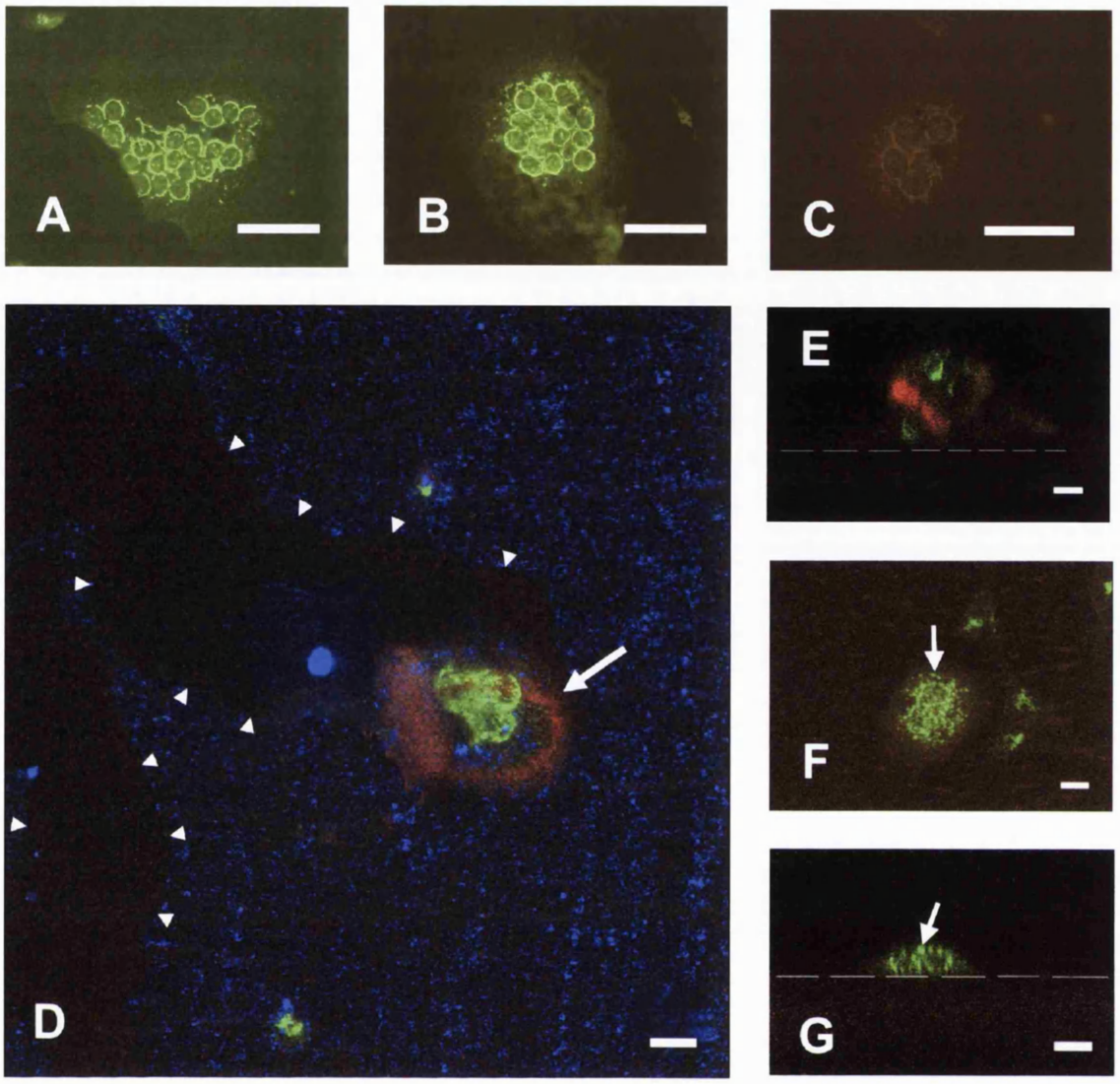


Figure 5.6

Immunostaining for H^+-gated ion channels in osteoclasts cultured on plastic or dentine.

(**A, B, C**) Localisation of ASIC1 on rat osteoclasts, cultured on plastic, visualised with fluorescein. Strong perinuclear immunostaining with anti-ASIC1 antibody (A, B), which was almost abolished in control cell (C) pre-absorbed with ASIC1 peptide. (**D-G**) Confocal microscopy of osteoclast immunostained with anti-ASIC1 (green) cultured on dentine. (**D, E**) Resorbing osteoclasts (arrow) were stained with rhodamine-phalloidin to demonstrate F-actin distribution (red). Pits are visualised by reflective light (blue) (arrowheads) (D). (E) Lateral view of the same cell shows the perinuclear staining pattern. (**F, G**) Sometimes, additional ASIC1 immunoreactivity was observed in vesicles (arrows). (G) Lateral view. Scale bars = 50 μm (A, B), 30 μm (C), 10 μm (D-G).

DISCUSSION

The results presented in this chapter indicate that acid-activation is a fundamental property of human, rodent and avian bone resorption systems.

Human osteoclastoma-derived osteoclasts are known to resorb mineralised substrates more aggressively than normal mammalian osteoclasts. Osteoclastoma, or 'giant-cell tumour of bone', is a benign bone neoplasm, which can occasionally metastasise. It was investigated whether osteoclastoma-derived osteoclasts show similar acid activation responses as those observed in rodents and chicken, or whether their high resorptive activity *in vivo* could be due to pertubation in the response of these cells to pH_e, such that osteoclastoma cells are not 'switched off' at a non-acidic pH > 7.2, but remain active. The data presented here indicate that there is no perturbation of the 'set point' for acid activation of human osteoclastoma-derived osteoclasts, since pit formation was maximally activated at about pH 7.0 and essentially 'switched off' above pH 7.3, resembling the pH activation profile observed in other species. Once activated, however, these cells resorbed dentine more aggressively than osteoclasts from normal tissues. Single pits formed by osteoclastoma osteoclasts were typically up to 30 μm deep, compared with about 5 μm for rat osteoclasts and 10 μm for chick osteoclasts. Osteoclastoma cells are relatively easy to obtain in high numbers compared to rat osteoclasts; thus, they would be an interesting system to examine downstream signalling events following pH activation.

In a second series of experiments, the interaction between low pH and RANKL was studied using the short-term rat osteoclast resorption assay. This study demonstrates that osteoclast activation by slight extracellular acidification (pH ~7.0) is a key requirement for the stimulatory action of RANKL on osteoclastic resorption to occur, and that RANKL acts synergistically with low pH to activate osteoclasts. It is noteworthy that at 'physiological' pH (*i.e.* blood pH, ~7.4), RANKL exerted only minimal stimulatory effects on resorption. The stimulatory effect of RANKL at 10 – 100 ng/ml on osteoclastic resorption is broadly in line with earlier reports of the activation of mature osteoclasts by

RANKL (Fuller *et al.*, 1998; Lacey *et al.*, 1998; Burgess *et al.*, 1999); however, the effects of extracellular pH were not investigated in these studies. Fuller *et al.* (1998) described increased cytoplasmic spreading and pseudopodial motility after RANKL treatment. Their studies also showed that RANKL alone is sufficient to fully account for the stimulation of bone resorption induced by PTH, replacing the need for osteoblastic cells mediating the PTH effect on osteoclasts. The study of Burgess *et al.* (1999) observed that RANKL induced re-arrangement of the osteoclast cytoskeleton, increased the area resorbed per osteoclast, and probably activated osteoclasts to undergo multiple resorption cycles. However, no significant effect of RANKL on the mean area of individual resorption events was seen, in contrast to the results presented here, where RANKL at 100 ng/ml increased not only the numbers of pits resorbed per osteoclast, but also doubled the size of individual resorption pits. Additionally, at pH ~7.0, RANKL treatment often caused osteoclasts to exhibit contiguous 'snail-trail' resorption pits, consistent with multiple resorption cycles as suggested above. Stimulation of bone resorption by RANKL is probably not due to enhanced osteoclast survival, since numbers of osteoclasts were unchanged by RANKL treatment.

The action of other osteolytic agents (*e.g.* 1,25-$(OH)_2D_3$, ATP and ADP) is also enhanced by acidification, as mentioned in the 'Introduction' to this chapter. Conversely, slight alkalinization markedly attenuates the osteolytic action of PTH, 1,25-$(OH)_2D_3$ and PGE_2 (Meghji *et al.*, 2001). Taken together, these results suggest a 'hierarchy' in the stimulation of osteoclastic activity: a low pH appears to be an essential initial requirement for the activation process; once this activation has occurred, further stimulation by a wide range of osteolytic agents can take place. However, the stimulatory effect of low pH can be blocked by a number of inhibitors, most notably calcitonin (Arnett and Dempster, 1987).

The mechanism underlying the effects of extracellular protons on osteoclastic resorption is poorly understood. Recent work from our lab has shown that, in the presence of bicarbonate, lowering pH_e always led to a parallel intracellular acidification in rat osteoclasts (Dunina-Barkovskaya *et al.*, 2001). Extracellular acidification also leads

to an increase in actin-rich podosomes and in the formation of actin-rich 'clear zones' within the osteoclast, indicating increased adhesion to the substrate (Teti *et al.*, 1989a; Murrills *et al.*, 1993). These findings agree with biochemical data suggesting possible regulatory effects of H^+ concenctration on actin polymerisation and filament formation (Selve and Wegner, 1987; Zimmerle and Frieden, 1988). Along with effects on CA II and TRAP mRNA expression (Asotra *et al.*, 1994; Biskobing and Fan, 2000), acidosis can also induce vacuolar H^+-ATPase activity (Nordström *et al.*, 1997), and cause Ca^{2+} efflux from the cell, resulting in reduced $[Ca^{2+}]_i$ (Teti *et al.*, 1989a). Therefore, acidosis, through upregulation of each one of these mechanisms, is likely to stimulate osteoclastic bone resorption and thus promote bone loss. Interestingly, calcitonin receptor mRNA expression in osteoclasts increases under acidic conditions, suggesting that, as osteoclasts become more active at low pH, an increase in calcitonin receptor expression may augment the capacity for rapid inhibition of bone resorption (Biskobing and Fan, 2000).

However, another possibility is that osteoclasts possess a specific 'pH-sensing apparatus', either located extracellularly (*i.e.* receptor-mediated), or intracellularly, *e.g.* similar to an 'pH-reponse element', the latter particularly interesting in context with studies showing that lowering pH_e in rat osteoclasts leads to a parallel decrease in pH_i (Dunina-Barkovskaya *et al.*, 2001). Work from our lab about three years ago showed that the full sensitivity of osteoclasts to activation by ATP is only evident at low pH (~6.9 - 7.0). This, and other data, suggested a role for the $P2X_2$ receptor in acid activation of osteoclasts. Using recombinant $P2X_2$ receptors expressed in *Xenopus* oocytes, small acidic and alkaline shifts (as little as 0.03 pH units) were able to enhance or diminish the response to ATP, respectively (Wildman *et al.*, 1997). No other P2 receptor family member is so sensitive to extracellular pH. 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 exceptional in having an imidazole side chain with a pK_b of 6.8. Thus, small pH shifts in the physiological range will change the charge and protonation of histidine side chains, with possible alterations in conformation of this domain. Acid-activation of rat osteoclasts was shown to be inhibited by apyrase and the general $P2X_{1,2,3,5}$ receptor antagonist suramin, 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 (Morrison *et al.*, 1998). *In vivo* studies have shown that suramin is indeed a potent inhibitor of bone resorption, through unknown mechanisms (Yoneda *et al.*, 1995); however, care must be taken when interpreting data using suramin, as it has a much broader and non-specific actions than just antagonising P2X receptors, *e.g.* it is also known to inhibit ecto-nucleotidases (Yegutkin and Burnstock, 2000).

Taken together, this data pointed to the possible involvement of the $P2X_2$ receptor subtype in acid-activation of bone resorption, but it remained unclear how H^+ and ATP could interact at the $P2X_2$ receptor to stimulate resorption. One possibility was that when the $P2X_2$ receptor is occupied by ATP, protons might modulate the receptor to allow the opening of a non-selective cation (including H^+) channel in the osteoclast basolateral membrane. This would decrease pH_i through H^+ influx, thus increasing intracellular H^+ supply for the action of H^+-ATPase. More protons could be pumped into the resorption lacunae and thus facilitate resorption (Arnett and King, 1997). However, an alternative possibility is that ATP and protons are operating at distinct receptor sites on the osteoclast cell membrane.

To address these open questions, I undertook preliminary studies on $P2X_2$-deficient mice, kindly provided by Roche Bioscience (Palo Alto, USA). However, only a limited number of animals were available, and problems with the genotype of wild-type animals occurred during the course of the study. Thus, experimental data are not included in this thesis. However, preliminary results were surprising: the $P2X_2$ receptor does not seem to represent the putative 'pH receptor' on osteoclasts, since cells derived from knockout mice were still strongly stimulated by acid alone. Interestingly, at neutral pH, ATP still stimulated resorption, but at acidic pH, ATP did not only have no stimulatory effect, but inhibited resorption to levels lower than the acid-control. Thus, $P2X_2$ knockout mice-derived osteoclasts appeared to show a blunted response to ATP at low pH, which raises even more questions as to what the exact roles of P2 receptors are in bone cell function. This will be further explored in Chapter 6 (General Discussion).

Acidosis accompanies many painful inflammatory and ischaemic conditions, thus the mechanism underlying pain caused by acid has received much attention (Steen *et al.*, 1992). Recent cloning of a H^+-gated channel (ASIC) demonstrated its expression on small neurons of dorsal root ganglia, consistent with a role as acid sensors in nociceptive sensory neurons (Waldmann *et al.*, 1997). Since the pH activation profile of ASIC1 strongly resembles the pH response of osteoclasts, expression was studied on osteoclasts. Immunostaining on osteoclasts revealed strong perinuclear staining for ASIC1, but no functional expression in the membrane. Culture in acidified medium did not cause a translocation to the membrane. Thus, ASIC1 appears to be specifically expressed in osteoclasts; however, whether it can also be functionally expressed in the membrane to serve as an extracellular pH-sensing receptor remains to be determined by other methods.

Protons may represent one of the only known direct stimulators of osteoclastic resorption, as most osteolytic agents act indirectly on osteoclasts via osteoblasts. They may also serve a pathophysiological role as a local osteoblast-osteoclast coupling factor (Arnett and Dempster, 1990). Lowering pH_e in mouse calvarial cultures stimulates PGE biosynthesis, which leads to formation and activation of osteoclasts (Rabadjija *et al.*, 1990). In calvarial cultures, resorption stimulated by HCO_3^- acidosis could be inhibited by both indomethacin, blocking PGE synthesis, and also by 5-lipoxygenase inhibitors, suggesting that leukotrienes are also involved in mediating the effect (Meghji *et al.*, 2001). In contrast, resorption pit formation by cultured osteoclasts is stimulated by cyclooxygenase inhibitors (Morrison and Arnett, 1996), and inhibited by prostaglandins (Arnett and Dempster, 1987).

Osteoblasts are also generally affected by acidosis, *e.g.* osteoblastic collagen synthesis and alkaline phosphatase activity are decreased in metabolic acidosis, more than in respiratory acidosis (Krieger *et al.*, 1992; Kaysinger and Ramp, 1998), consistent with studies reporting that acidosis inhibited bone nodule formation (Sprague *et al.*, 1994). Extracellular pH can also induces changes in osteoblastic gene expression: decreasing pH_e to 6.8 - 7.0 led to a reduction in the immediate early response gene *egr-1*, and in type 1 collagen, osteopontin and matrix gla protein mRNA, compared to control (Frick *et al.*, 1997; Frick and Bushinsky, 1998). Metabolic acidosis may therefore be

doubly destructive for bone: it inhibits osteoblastic bone formation, and stimulates bone resorption.

There are relatively few reliable measurements for interstitial pH in intact tissues. In normal subcutaneous tissue, pH values decrease as the distance from a blood vessel (pH ~7.4) is increased from 10 μm (pH ~7.2) to 30 μm (pH ~7.1), a distance equivalent to approximately one or three cell diameters, respectively (Martin and Jain, 1994). Analagous pH drops are likely to occur in the 'bone compartment', away from capillaries, although precise values will depend on the rate of metabolic acid efflux from cells and the local buffering power of the extracellular fluid. A recent study, using high-density cultures of osteoblastic cells, reported pH values of ~7.0 beneath the cell layer following IGF-1 application (Santhanagopal and Dixon, 1999). This model system may resemble, in some respects, an *in vivo* situation, in which acid production by osteogenic or stromal cells reduces local pH to a value optimal for osteoclastic bone resorption and for interaction with other osteolytic agents, such as RANKL or nucleotides. Factors such as IL-1, ATP, PTH and 1,25-$(OH)_2D_3$ are also known to enhance medium acidification by osteoblasts, which could conceivably stimulate osteoclasts (Aisa *et al.*, 1995; Kaplan and Dixon, 1996, Barrett *et al.*, 1997; Sankararajah *et al.*, 2000).

Long-term stimulatory effects of low pH on osteoclastic resorption may be relevant to *in vivo* situations. The powerful stimulatory effect of low pH on pit formation by mature rat osteoclasts does not appear to diminish with time in culture, provided that the cytotoxic effects of very low pH (*i.e.* <6.8) are avoided, and small pH shifts are not associated with any long-term changes in osteoclast number. Thus, acidosis occurring in tumours, inflamed tissues or rheumatoid arthritis would be likely to amplify the effects of osteolytic factors, resulting in local bone loss. It has been shown that the pH in inflammatory synovial fluid is lower than fluid from non-rheumatoid joints, suggesting that bone loss commonly found in rheumatoid arthritis could be caused by local acidification (Falchuk *et al.*, 1970).

It has been recognised since the early part of this century that metabolic acidosis due to acid ingestion or renal disease can result in profound bone loss (review: Arnett and

Dempster, 1990). However, metabolic acidosis is not only present in pathological conditions: humans eating a typical western diet develop a slight, but progressive increase in blood acidity and a decrease in plasma HCO_3^- concentrations (low-grade metabolic acidosis) (Barzel, 1995; Frassetto and Sebastian, 1996). Furthermore, local acidification could occur in periods of low bone blood flow and poor oxygenation, such as with ageing and the initial stages of fracture healing. A decreased blood flow may result in insufficient removal of metabolic waste products (*e.g.* lactate) and thereby decrease the pH of bone interstitial fluid. Although pH decreases would be small, accumulation of a slightly elevated rate of bone resorption and a slightly diminished rate of osteoblastic collagen synthesis over several years could contribute to osteoporosis. In this context, recent studies on postmenopausal women have shown that administration of $KHCO_3$, sufficient to neutralise endogenous acid, improved calcium and phosphorus balance, reduced bone resorption and increased the rate of bone formation (Sebastian *et al.*, 1994).

In summary, results from this chapter suggest that acidosis stimulates osteoclasts strongly in all resorption models and species studied to date, and that is appears to be an essential requirement for pit formation by RANKL and other osteolytic agents; however, it remains to be determined if there is a 'pH-sensing' apparatus on osteoclasts, or whether an acidic pH_e simply decreases pH_i, resulting in pro-resorptive changes such as enhanced actin-polymerisation amongst others. Extracellular pH is clearly an important influence on bone homeostasis and must additionally be considered a critical factor in the design, monitoring and interpretation of all bone resorption experiments.

CHAPTER 6

GENERAL DISCUSSION AND FUTURE WORK

The work presented in this thesis has focused mainly on purinergic signalling in osteoblasts and osteoclasts, in addition to investigating some new aspects of the effects of acid on bone. A detailed discussion of the results was provided at the end of each experimental chapter; thus, in this general discussion, I would like to summarise the major findings and discuss: (1) the results of each chapter in relation to each other; (2) the potential physiological/pathophysiological relevance; (3) future directions of research; and (4) some advantages and disadvantages of the techniques used in this thesis.

This project was planned based on a large number of pharmacological and electrophysiological studies suggesting the presence of P2 receptors on bone cells (see Chapter 1), and on a few functional studies reporting that ATP has both an effect on osteoclast formation and resorption (Bowler *et al.*, 1998a; Morrison *et al.*, 1998) and on bone formation (Jones *et al.*, 1997). However, it was neither known which P2 receptor subtypes were present on bone cells, nor which of these receptors might be mediating any effects on osteoclast and osteoblast function. Thus, in the first experimental chapter, using immunohistochemistry and *in situ* hybridisation, it was shown that osteoblasts, osteoclasts and chondrocytes express a wide range of P2 receptors, both of the P2X and P2Y receptor subtypes. Often, a cytoplasmic immunoreactivity was observed in osteoblasts and osteoclasts, which was surprising since receptors were expected to be functionally expressed in the plasma membrane. However, two recent studies, using green fluorescent protein (GFP)-tagged $P2X_1$- and $P2X_2$ receptor constructs, reported both cytosolic and plasma membrane localisation of these two receptor subtypes (Dutton

et al., 2000; Khakh *et al.*, 2001). GFP-tagged $P2X_1$ receptors, which were clustered in the cell membrane, became internalised following prolonged exposure to ATP, most likely into acidic endosomes (Dutton *et al.*, 2000). In contrast, ATP application produced no internalisation of GFP-tagged $P2X_2$ receptors, but areas of increased fluorescene, *i.e.* 'hot spots', in the plasma membrane (Khakh *et al.*, 2001). Thus, more detailed localisation studies, using techniques such as GFP-receptor constructs, but also electron and confocal microscopy, would be desirable in future immunohistochemical studies on bone cells.

The presence of at least two P2Y subtypes ($P2Y_1$- and $P2Y_2$-like receptors) on bone cells had been suggested in number of earlier studies, but the finding of P2X receptor expression is relatively new (especially the expression of the acid-sensitive $P2X_2$ receptor subtype). Chapters 3 and 4 explored which receptors might play a role in bone resorption and bone formation, employing a number of functional studies on rodent osteoclasts and osteoblasts.

Whilst studying osteoclasts, I found that ADP, at nanomolar to low micromolar concentrations, is a powerful stimulator of osteoclast formation and subsequent resorption, and presented evidence that this osteolytic effect is probably mediated via the $P2Y_1$ receptor subtype. The $P2Y_1$ receptor was found to be expressed on both osteoclasts and osteoblasts; thus, the osteolytic effects could be mediated directly, via receptors present on osteoclasts, or indirectly via receptors expressed on osteoblasts, which then in turn release pro-resorptive local factors. It is evident from a number of earlier studies that both osteoclasts and osteoblast respond to ADP application with elevated $[Ca^{2+}]_i$ levels; however, receptor activation might result in different downstream events. Levels of $[Ca^{2+}]_i$ are thought to play an important role in osteoblast regulation and might be involved in activating signals ultimately responsible for stimulating bone resorption. Therefore it is likely that the large $[Ca^{2+}]_i$ transients induced in osteoblasts by extracellular nucleotides have profound effects on bone remodelling.

Most osteolytic stimuli, including the hormones PTH and $1,25\text{-}(OH)_2D_3$, are indeed known to act indirectly via osteoblasts, and not directly on osteoclasts. One of the

osteolytic factors released by osteoblasts has recently been identified to be "Receptor activator of nuclear factor κB ligand" (RANKL) in a number of studies; RANKL is both sufficient and necessary for osteoclast formation, and is also a potent activator of mature osteoclasts. Additionally, diacylglycerol (DAG), an intracellular signalling molecule produced by cleavage of phosphoinositides in response to stimulation of cell surface receptors, is recognised as a source of arachidonic acid, the precursor of prostaglandins; DAG can also act as an activator of PKC, which in turn can stimulate PLA_2 and the production of arachidonic acid. Therefore, this pathway, which involves PGE production, might also be important in osteoblast-osteoclast interactions in response to P2 receptor activation, and is consistent with my result that ADP-induced resorption in the calvarial resorption assay can be blocked by addition of indomethacin.

My studies on osteoblast function revealed that both ATP and UTP, at physiological concentrations of 1-10 μM, are potent inhibitors of bone nodule formation, whereas neither adenosine nor ADP had an effect in this assay. ATP and UTP are potent agonists at two P2Y receptor subtypes: $P2Y_2$ and $P2Y_4$. In contrast to an earlier study (Maier *et al.*, 1997), I found no evidence for the expression of $P2Y_4$ mRNA or protein on osteoblasts (and osteoclasts), thus suggesting that the $P2Y_2$ receptor might be responsible for the inhibitory effects of UTP and ATP on bone nodule formation.

The finding that UTP and ATP, but not ADP, play a role in osteoblast function is especially interesting when seen in context with Chapter 3: the low-dose effects of extracellular nucleotides on bone resorption and formation appear to be mediated via different P2 receptor subtypes, since ADP, signalling through the $P2Y_1$ receptor, is a powerful stimulator of osteoclast formation and activity, whereas UTP (which does not affect osteoclast function), signalling via the $P2Y_2$ receptor, could play a role as a inhibitor of bone formation. A mechanism for the differential purinergic regulation of osteoblasts and osteoclasts has been suggested before, based on $[Ca^{2+}]_i$ responses of osteoclasts and osteoblastic cells to nucleotides: application of extracellular ATP (50 or 100 μM) inhibited the Ca^{2+} response to a subsequent application of ATP in rat osteoblastic UMR-106 cells, but not in rabbit osteoclasts, suggesting that osteoclasts can

adapt to the extracellular ATP, whereas osteoblastic cells cannot adapt and desensitise (Luo *et al.*, 1997b).

Effects of ADP on resorption and osteoclast formation were observed in the nanomolar to low micromolar range, whereas higher concentrations had no effect, resulting in an unusual bell shaped response curve. In contrast, the inhibitory effects of ATP and UTP on bone nodule formation were observed at slightly higher concentrations, starting at 1 μM (UTP) and 10 μM (ATP), with maximal effects at 100 μM. Higher concentrations into the submillimolar range were not tested, but should be considered in future experiments. ATP at even higher, millimolar concentrations has been shown to induce apoptosis in both osteoclasts (Morrison *et al.*, 1998) and human osteoblasts (Gartland *et al.*, 2001b) via the $P2X_7$ receptor. Taken together, it might be an intriguing possibility that nucleotides, once released from cells, target different receptors depending on their concentrations, and can thus produce highly selective spatial effects, similar to classic concept of 'morphogens' in developmental biology, inducing cells to take on different fates according to their position to the source of release.

After having finished my studies of the effects of ADP on osteoclast function, a new receptor of the P2Y family was cloned: the '$P2Y_{12}$' receptor (Hollopeter *et al.*, 2001). It was identified as the second platelet ADP receptor (the first being $P2Y_1$), which had formerly been called $P2Y_{ADP}$, P_{2T} or $P2Y_{AC}$, because in contrast to $P2Y_1$, it couples to inhibition of adenylate cyclase (AC) through G_i proteins and subsequent alterations in cAMP levels. ADP activation of the $P2Y_{12}$ receptor is required for platelet aggregation and acts in combination with $P2Y_1$, which, through G_q, leads to platelet shape change and aggregation. Although first studies show that the $P2Y_{12}$ receptor has a very restricted distribution in platelets and to a smaller extent in brain (bone tissue was not tested), the possibility cannot be completely ruled out that the $P2Y_{12}$ receptor is expressed on osteoclasts and might mediate some of the potent ADP effects. Clearly, further agonist and antagonists studies are required, in addition to the development and testing of anti-$P2Y_{12}$ antibodies, but the $P2Y_1$ receptor remains the strongest candidate for mediating the effects of ADP on osteoclasts. The application of ADP has always been linked to

increased $[Ca^{2+}]_i$ levels in osteoclasts, consistent with the involvement of a G_q protein, whereas $P2Y_{12}$ is coupled to a change in cAMP levels through adenylate cyclase, and not to a change in $[Ca^{2+}]_i$ levels. Potent $P2Y_1$ antagonists such as MRS 2179 have not been tested on the $P2Y_{12}$ receptor yet, thus further knowledge in this area will clarify the situation for effects of ADP on osteoclasts.

It is still largely unknown which signalling events, downstream from an initial rise in $[Ca^{2+}]_i$ levels, are activated in osteoclasts by ADP application. Using rat osteoblastic UMR-106 cells, co-stimulation with ADP and PTH led to increased levels of CREB phosphorylation, and synergistically increased expression of the immediate early gene *c-fos* (Buckley *et al.*, 2001b); thus, both stimuli converged on the Ca/CRE promoter element of *c-fos*. Similar effects, but different pathways, were observed in primary human osteoblasts and human osteoblast-like SaOS-2 cells, where nucleotides alone had little effects on cAMP levels, but acted synergistically with PTH to elevate *c-fos* levels. In contrast to UMR-106 cells, in SaOS-2 cells both the Ca/CRE and the serum response element of the *c-fos* promoter were required to drive this synergy (Bowler *et al.*, 1999). Thus, multiple pathways, both Ca^{2+}-dependent and -independent, exist to couple dual activation of separate G protein-coupled receptors (P2Y and PTH) in osteoblasts. Clearly, similar experiments are required to study signal transduction events in osteoclasts to investigate which genes are ultimately 'switched on' or 'off' by nucleotide activation. This would also help to generally clarify the changes that occur in osteoclastic gene expression and protein synthesis once the osteoclast has been activated. As discussed before, nucleotides probably occur only transiently in the bone microenvironment, thus ADP, ATP and UTP must all act relatively quickly on their target cells to induce signalling events.

In this context, an interesting area of future work in the P2 receptor field, but also other receptor families, is the oligomerisation of G protein-coupled receptors, a receptor family including both P2Y and PTH receptors. A range of approaches, mostly co-immunoprecipitation techniques, has recently provided evidence that G protein-coupled receptors can exist as oligomeric complexes, both as homo-oligomers, comprising

multiple copies of the same gene product, and hetero-oligomers, containing more than one receptor. Oligomerisation could occur for example in form of intramolecular disulphide bonds, since most G protein-coupled receptors contain cysteine residues in their extracellular loops. However, there is still little direct information on the fraction of receptors existing as oligomers in intact cells, on how ligands can effect G protein-coupled receptor oligomerisation, and what the functional consequence of receptor oligomerisation might be, *e.g.* if receptor oligomers produce subtly different pharmacological profiles (for a recent review, see Milligan, 2001). So far, only one paper on this subject has emerged from the purine field, showing that dopamine and adenosine A_1 receptors form functionally interacting heteromeric complexes (Gines *et al.*, 2000), but considering the number of P2Y receptor family members existing and the presence of mostly more than one P2Y receptor on cells, including bone cells, more seems likely to come.

ATP can also act synergistically with TNF-α in the activation and maturation of dendritic cells (Ogilvie *et al.*, 2000; Schnurr *et al.*, 2000). Dendritic cells have specialised antigen-presenting functions and initiate primary T-cell mediated immune responses; they are, similarly to osteoclasts, also monocyte-derived. However, relatively high ATP concentrations of 100 –500 μM were used, and based on agonist studies, both P2X and P2Y receptors were implied (Schnurr *et al.*, 2000). However, a similar synergism between ATP/ADP and TNF-α in osteoclast formation and activation would highlight a possible role for nucleotides in inflammatory bone loss. The dominant process leading to bone loss in conditions such as rheumatoid arthritis (RA) is osteoclastic bone resorption. This is widely accepted to be driven by pro-inflammatory cytokines, particularly IL-1, IL-6 and TNF-α, released from monocytes and other cells in the inflamed synovium (Gough *et al.*, 1998). These cytokines were found to be elevated in both the synovial fluid and the serum of patients with RA. Bone loss in early RA occurs at bone sites proximal and distal to the joint inflammation, but there is also a systemic effect of inflammation on bone loss, leading to secondary osteoporosis (Gough *et al.*, 1998). Nucleotides would only be expected to be involved in the first process (local bone loss) because of their fast degradation once released. Inflammation is also characterised

by increased release of nucleotides and low pH, in addition to cytokine release. These three stimuli are all known to stimulate osteoclast formation and resorption. The known synergism between nucleotides and low pH could thus be additionally enhanced by TNF-α/ATP synergism, resulting in further focal bone destruction. It is an intriguing possibility that some or all these stimuli could combine on a pathway to indirectly stimulate release of RANKL or PGE_2 from neighbouring and invading cells, both very powerful osteoclastogenic agents. Osteoclasts themselves might also represent a possible source for ATP release into the medium, especially in inflammatory conditions: stimulation of the macrophage cell line RAW 264.7, which has recently been employed to generate osteoclasts *in vitro*, with lipopolysaccharides induced them to release ATP. ATP then activated nitric oxide release through $P2X_7$ receptors, presenting another possible regulator of bone cell function (Sperlagh *et al.*, 1998).

Increased release of nucleotides from virtually all tissues has also been reported in hypoxia and ischemia (Bodin and Burnstock, 2001b). Recent experiments from our lab have shown that hypoxia is a major stimulator of osteoclast formation and bone resorption in mouse marrow cultures and calvarial organ cultures (Gibbons *et al.*, 2001). Hypoxia is well known to act as a general stimulator of the recruitment and/or activation of cells derived from marrow precursors, and is present in ageing poorly vascularised yellow fatty marrow, as well as in inflamed tissue, tumours and fracture sites. Thus, the stimulation of osteoclast function by hypoxia might also be partly mediated by increased nucleotide release acting on P2 receptors, as well as production of angiogenic and osteolytic cytokines such as RANKL, TNF-α, IL-1 and VEGF.

However, it remains unanswered how exactly, *i.e.* whether through receptors in the plasma membrane or through other intracellular mechanisms, stimuli like hypoxia and extracellular acidification affect cell function. Some attempts to answer these issues were made in Chapter 5. In contrast to earlier suggestions, it seems now unlikely that the $P2X_2$ receptor is the putative 'pH-receptor' on osteoclasts. The acid-sensing ion channel (ASIC) was shown to be present intracellularly in osteoclasts; however, future experiments will need to be undertaken to know whether ASIC is the extracellular acid

sensor on osteoclasts, including electrophysiology, application of ASIC antagonists in bone resorption assays, ASIC-knockout models amongst others. An important area of future research into both hypoxia and acid effects will be to identify the molecular and transcriptional changes in response to both stimuli in osteoclasts. This has recently been achieved in a study on 'alkaline response genes' in yeast cells: analysis by gene filter cDNA macroarrays and Northern blot identified a number of possible candidate genes encoding a variety of proteins (membrane proteins, ion pumps, transcription factors, phosphate metabolism proteins *etc.*) that increase substantially in response to a shift from acidic (pH 4) to alkaline pH (pH 8) (Lamb *et al.*, 2001). Thus, although rather extreme pH values were studied, this study and the molecular tools available to date provide an exciting approach for the study of osteoclasts that have been exposed to different pH and/or hypoxia conditions, in addition to studying what genes become highly expressed when ADP/ATP is applied to osteoclast cultures.

Interestingly, my preliminary results showed that although $P2X_2$-deficient mice-derived osteoclasts retained their acid sensitivity, they exhibited a 'blunted' response to a combined application of acid and ATP *in vitro*, instead of the acid-ATP synergism observed in wild-type cultures. This may complicate the whole picture of P2 receptor function in bone cells as to what the exact function of the $P2X_2$ receptor is in osteoclast biology, and why a lack of $P2X_2$ receptor expression inhibits acid-induced resorption when ATP is applied, rather than just keeping it at control levels. Data indicate that ATP, at pH 7.2, is still capable of stimulating resorption, thus this effect is probably mediated by a different receptor; likely candidates include the $P2Y_1$ receptor, at which both ATP and ADP are potent agonists, and which does seem to play a major role in bone resorption (Chapter 3), or, as proposed in earlier studies, the $P2X_4$ or $P2Y_2$ receptor subtypes, which are also present on osteoclasts. However, an involvement of the $P2Y_2$ receptor in bone resorption seems unlikely, because UTP has not been shown to stimulate osteoclast function (Bowler *et al.*, 1998a; data from this thesis).

It can only be speculated what might happen under acidic conditions in $P2X_2$ deficiency. In many gene knockout cases, the targeted gene arose through duplication

and subsequent divergence of an ancestral gene, suggesting that other, presumably related, gene(s) can compensate for the mutated gene. Due to this 'gene redundancy', it seems likely that related genes share partially overlapping functions. The $P2X_2$ receptor shares approximately 44% and 46% identity with the $P2X_3$ and $P2X_4$ receptor subtypes, respectively, and below 40% with the other P2X subtypes (Humphrey *et al.*, 1998). Thus, the $P2X_4$ receptor may compensate partially for the loss of $P2X_2$. In contrast to $P2X_2$, none of the other P2X subtypes shows enhanced ATP responsiveness when the pH is dropped to pH 6.5; instead, 2-fold reduced ATP potency at rat $P2X_4$ receptors has been reported at pH 6.5 compared to pH 7.5, but without altering maximal activity of ATP, which was only reduced at further acidification to pH 5.5 (Wildman *et al.*, 1999). However, even if the $P2X_4$ is the receptor compensating for $P2X_2$ in osteoclasts, a reduced ATP potency at pH 6.5 does not explain the inhibitory response after combined ATP and acid application. Clearly, further experiments are required in the future to clarify this phenomenon. Unfortunately, only a small number of animals were available when my preliminary studies were performed, but it might also be interesting to study if ADP can still synergise with low pH, or whether it produces a similar blunted response as ATP. Possible gene redundancy can be explored by double or even triple gene knockouts, for example a $P2X_2/P2X_4$-deficient mouse, although this is very time-, cost- and animal-consuming.

Additionally, P2 receptor-deficient animals will need to be assessed for skeletal changes by detailed histomorphometric analysis of the bones, and by whole body scans to check for changes in bone mass and density. $P2Y_1$-deficient mice, which have been generated by two groups in France (Fabre *et al.*, 1999; Léon *et al.*, 1999), were not reported to display an obvious skeletal phenotype. However, an initial lack of phenotype has been reported for other knockout mice in the bone field, before bone remodelling in these mice was challenged. For example, bone development and the skeleton in osteopontin-null mice were normal, but surprisingly, these mice were resistant to ovariectomy-induced bone resorption, showing that osteopontin is essential for postmenopausal osteoporosis in women (Yoshitake *et al.*, 1999). Similarly, osteopontin is required for the bone loss induced by mechanical stress and also PTH (Ihara *et al.*,

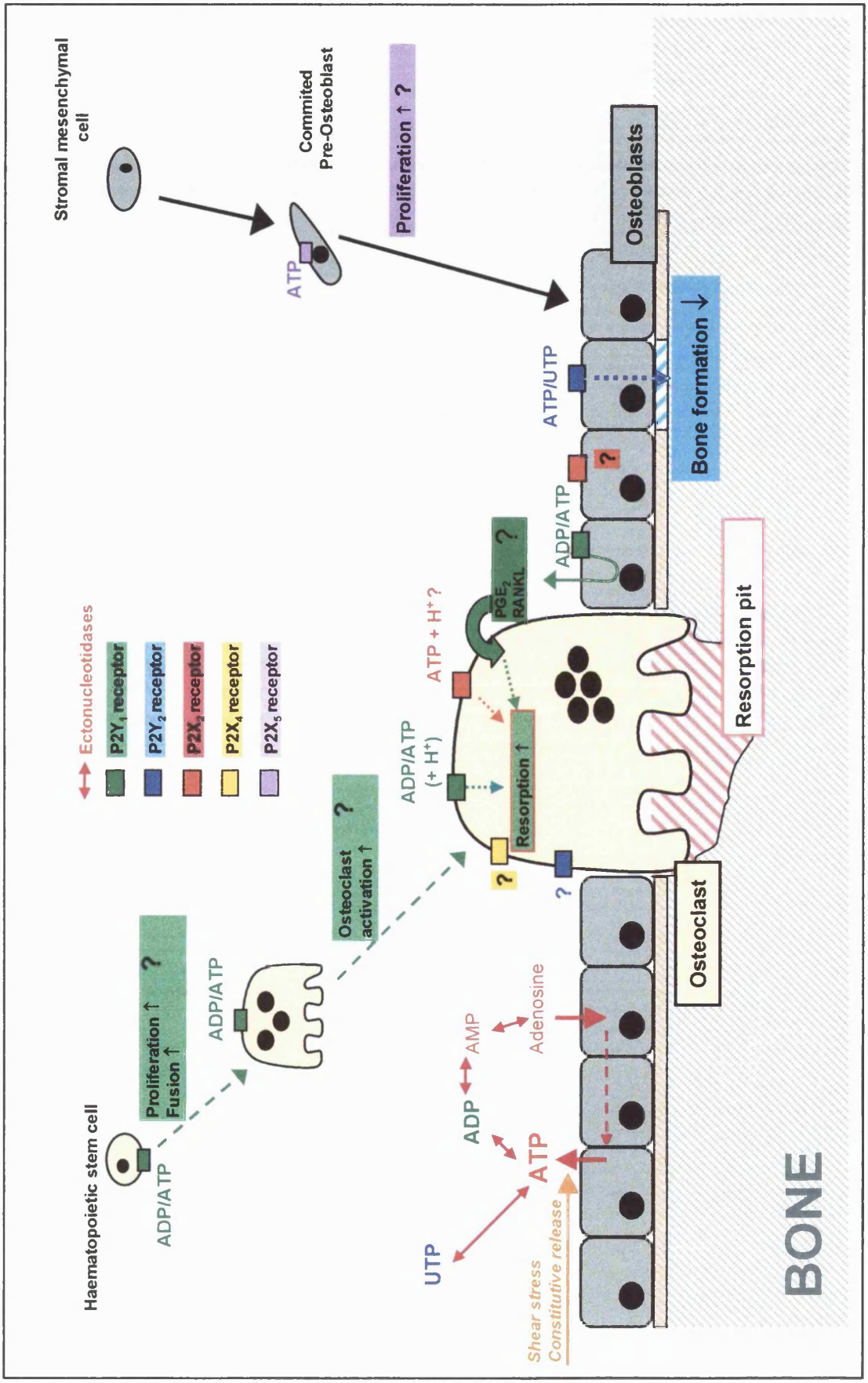
2001; Ishijima *et al.*, 2001). Thus, P2 receptor knockout mice could be challenged by similar treatments.

Potent and selective P2 receptor antagonists, *e.g.* the potent $P2Y_1$ antagonist MRS 2179, could also be tested generally as anti-resorption drugs *in vivo*. $P2Y_1$-mediated stimulation of bone resorption by ADP might play a role in several pathophysiological conditions accompanied by bone loss. This could be investigated *in vivo*, *e.g.* by performing ovariectomy, to assess a possible function in postmenopausal osteoporosis, or by inducing inflammation, to assess a role in inflammatory bone loss. However, designing and administering antagonists/drugs to selectively reach the skeleton represents a major problem, with one of the exceptions being bisphosphonates, which once administered, directly target bone cells.

In summary, data from this thesis have helped to elucidate functions of nucleotides in bone remodelling, acting via specific P2 receptor subtypes on both osteoclasts and osteoblasts. A speculative model for the role and interactions of P2 receptors on bone cells, based on results of this thesis, is shown in Figure 6.1 (**Fig. 6.1**). Please note that this figure does not include a number of earlier discoveries, e.g. $P2X_7$-induced apoptosis in osteoblasts and osteoclasts, due to the limited space. Since both ADP and ATP are potent stimulators of bone (and also cartilage) resorption, and ATP and UTP inhibitors of bone formation, at physiological concentrations, nucleotides seem to have an overall destructive effect on the skeleton, resulting in net bone loss, and could present interesting targets for drug developments in the future.

Figure 6.1

Speculative model for the role and interaction of P2 receptors on bone cells.



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APPENDIX I: ABBREVIATIONS

ABBREVIATION	FULL NAME
$1,25\text{-}(OH)_2D_3$	1,25-dihydroxyvitamin D_3
2-meSADP	2-methylthioadenosine 5'-diphosphate
2-meSATP	2-methylthioadenosine 5'-triphosphate
α,β-meATP	α,β-methylene adenosine 5'-triphosphate
β,γ-meATP	β,γ-methylene adenosine 5'-triphosphate
AA	Amino acid
ABC	ATP binding cassette
AC	Adenylate cyclase
AMP	Adenosine 5'-monophosphate
ADP	Adenosine 5'-diphosphate
AP-1	Activator protein-1
ATP	Adenosine 5'-triphosphate
BGJb	Biggers, Gwatkin and Heyner medium
BMP	Bone morphogenetic protein
BMU	Bone remodelling unit
C-	Carboxy-
Ca^{2+}	Calcium
Ca/CRE	Ca^{2+}/cAMP response element
CA II	Carbonic anhydrase II
cAMP	Cyclic adenosine 5'-monophosphate
Cbfa1	Core binding factor 1
CFTR	Cystic fibrosis transmembrane regulator
Cl^-	Chloride
CNS	Central nervous system
CO_2	Carbon dioxide

CPC	Cresolphthalein complexone
CREB	cAMP-response element binding protein
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylen-diamine tetraacetic acid
eNOS	Endothelial nitric oxide synthase
ER	Estrogen receptor
F-actin	Filamentous actin
FBS	Foetal bovine serum
FCS	Foetal calf serum
GFP	Green fluorescent protein
gla	γ-carboxylated glutamate
GTP	Guanosine 5'-triphosphate
HBSS	Hank's balanced salt solution
HCl	Hydrochloride acid
HCO_3^-	Bicarbonate
H_2CO_3	Carbonic acid
IFN-γ	Interferon-γ
IGF	Insulin like growth factor
Ihh	Indian hedgehog
IL-1	Interleukin-1
IP_3	Inositol 1,4,5-triphosphate
K^+	Potassium
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage-stimulating growth factor
MMP	Matrix metalloproteinase
MNC	Mononuclear cells
MRS 2179	*N*6-methyl-2'-deoxy-adenosine-3',5'-bisphosphate
N-	Amino-

Na^+	Sodium
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NF-κB	Nuclear factor kappa B
NHS	Normal horse serum
NO	Nitric oxide
NOS	Nitric oxide synthase
ODF	Osteoclast differentiation factor
OPG	Osteoprotegerin
OPGL	Osteoprotegerin ligand
P_i	Phosphate (inorganic)
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PGE_2	Prostaglandin E_2
PI-3	Phosphatidylinositol-3 kinase
PKC	Protein kinase C
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
PPADS	Pyridoxal-5'-phophate-6-azophenol-2',4'-disulfonate
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone related protein/peptide
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor κB
RANKL	Receptor activator of nuclear factor κB ligand
RGD	Arg-Gly-Asn
SDS	Sodium dodecylsulphate
TGF	Transforming growth factor
TNF	Tumour necrosis-related factor
TRAF	TNF receptor-associated factors

TRANCE	TNF-related activation-induced cytokine
TRAP	Tartrate resistant acid phosphatase
UDP	Uridine 5'-diphosphate
UTP	Uridine 5'-triphosphate
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide
V-type	Vacuolar-type
Zn^{2+}	Zinc

APPENDIX II: PUBLISHED PAPERS

ELSEVIER

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Expression of P2 Receptors in Bone and Cultured Bone Cells

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Extracellular nucleotides acting through P2 receptors elicit a wide range of responses in many cell types. There is increasing evidence that adenosine triphosphate (ATP) may function as an important local messenger in bone and cartilage. In this study, we used immunocytochemistry, employing novel polyclonal antibodies against $P2X_{1-7}$ receptors, and in situ hybridization, using oligonucleotide probes corresponding to $P2X_{2,4}$ and $P2Y_{2,4}$ messenger RNAs (mRNAs), to localize P2 receptors on undecalcified bone sections and on cultured osteoblasts and osteoclasts. We provide the first direct evidence that the $P2X_2$ receptor subtype is expressed on osteoclasts, osteoblasts, and chondrocytes. We also obtained evidence for the expression of $P2X_5$ and $P2Y_2$ receptors on osteoblasts and chondrocytes, and for $P2X_4$ and $P2X_7$ receptors on osteoclasts. Our results confirm earlier reports of $P2Y_2$ and $P2X_4$ expression in human osteoclastoma and rabbit osteoclasts, respectively, and are consistent with ATP responses observed on bone cells using electrophysiological techniques. Our novel finding that $P2X_2$ is expressed by osteoclasts is of particular interest. $P2X_2$ is the only P2 receptor subtype that requires extracellular acidification to show its full sensitivity to ATP, and our recent functional studies have shown that the stimulatory action of ATP on resorption pit formation by mature osteoclasts is amplified greatly at low pH. These findings point to fundamental new mechanisms for the local modulation of bone resorption. (Bone 27:503–510; 2000)

Key Words: P2 receptors; $P2X_2$; Adenosine triphosphate; (ATP); Osteoclasts; Osteoblasts; Chondrocytes.

Introduction

In recent years, the biological effects of extracellular purine nucleotides acting through P2 receptors have been studied in many cell and tissue types, and adenosine triphosphate (ATP) is now recognized as an important messenger molecule for cell-cell communication.[6] P2 receptors are classified into two main families: the P2X receptor family, the members of which are ligand-gated nonselective cation channels; in contrast, members of the P2Y receptor family are G-protein-coupled receptors, the principal signal transduction pathway involves phospholipase C, which leads to the formation of IP_3 and mobilization of intracellular Ca^{2+}. So far, seven P2X subtypes have been cloned and characterized, and about eight P2Y subtypes have been established in vertebrates.[26] Nucleotides can be released into extracellular space as a result of cell damage, by exocytosis from nerve and endothelial cells, and also by an ATP transport mechanism, perhaps via ABC proteins.

There is growing evidence that ATP may function as an important local messenger in bone and cartilage. The existence of P2 receptors on chondrocytes was demonstrated in the work of Russell and colleagues.[7,17] Cultured chondrocytes are also capable of releasing ATP constitutively at concentrations that may activate P2 receptors in the local microenvironment.[10,20] Cross-inhibition studies with different agonists have suggested that a mixed population of P2 receptors exists on rat osteoblast-like cells.[34] Heterogeneity of receptor expression was also demonstrated within a population of human osteoblasts, probably reflecting differences in the differentiation status of individual cells.[8] Recent studies have shown that ATP inhibits appositional bone formation by cultured primary rat osteoblasts,[11] and that cultured osteoblasts can release ATP when subjected to shear force.[5]

Initial studies using cultured rabbit osteoclasts have indicated that exogenous ATP was able to elicit an intracellular Ca^{2+} pulse via two different pathways: (1) Ca^{2+} influx across the cell membrane via Ca^{2+} channels; and (2) G-protein-coupled internal Ca^{2+} release.[33,35] In addition, ATP was reported to induce a transient intracellular pH decrease in osteoclasts that was Ca^{2+} independent.[36] Using in situ hybridization, the work of Bowler and colleagues showed that the $P2Y_2$ receptor was expressed on osteoclasts derived from human giant cell tumors.[3] The same group also reported that ATP exerted a small stimulatory effect on resorption pit formation by giant cell tumor osteoclasts, but that this effect was not mediated by the $P2Y_2$ receptor.[4] Electrophysiological experiments have provided evidence supporting the coexpression of both P2X and P2Y receptors on rat osteoclasts, with ATP activating both nonselective cation channels and Ca^{2+}-dependent K^+ channels.[30] Recent functional studies by our group showed the potent stimulatory effect of ATP on the formation and resorptive activity of normal mammalian osteoclasts.[23] The stimulatory effect on resorption was amplified greatly when osteoclasts were activated by culture in acidified medium, which may be consistent with the involvement of the $P2X_2$ receptor subtype, the only receptor of this family that requires extracellular acidification to show its full sensitivity to ATP.[32]

In the present study, we used a novel set of polyclonal antibodies against $P2X_{1-7}$ receptors and also oligonucleotide probes corresponding to $P2X_{2,4}$ and $P2Y_{2,4}$ mRNAs to investi-

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gate the expression of P2 receptors in sections of neonatal rat bone and in cultured rat bone cells.

Materials and Methods

Tissue Preparation

Two-day-old neonatal Sprague-Dawley rats were killed by cervical dislocation. Long bones and calvariae were removed immediately and the tissues were rapidly frozen by immersion in isopentane at −70°C and stored in liquid nitrogen. Cryostat sections of undecalcified, unfixed bone (10 μm) were prepared and collected on gelatin-coated or polysine-coated slides (BDH/Merck, UK) for immunocytochemistry or in situ hybridization, respectively. Tissues were kept frozen until used and air dried at room temperature prior to use. Unless otherwise specified, all reagents were purchased from Sigma (Poole, Dorset, UK).

Cell Culture

Primary rat osteoblastic cells were obtained by sequential enzyme digestion of excised calvarial bones from two-day-old neonatal Sprague-Dawley rats using a three-step process (1% trypsin for 10 min, 0.2% collagenase type II for 30 min, and 0.2% collagenase type II for 60 min at 37°C), rejecting the first two digests. The cells were resuspended in Dulbecco's modified Eagle medium (DMEM) + 10% fetal calf serum (Gibco, Paisley, UK), seeded at a concentration of 2×10^4 cells/chamber on LabTek 8 chamber slides (Nunc Life Technologies, UK) and incubated for 2–4 days until confluence. At confluence, these cells were mainly strongly positive for alkaline phosphatase, as assessed by cytochemical staining (Sigma Kit 86C).

Mixed cell populations containing osteoclasts were obtained by rapidly mincing the long bones of two-day-old neonatal Sprague-Dawley rats, killed by cervical dislocation, in MEM + 10% fetal bovine serum (Gibco, Paisley, UK), followed by vortexing. The resulting cell suspension was allowed to sediment for 60 min onto LabTek chamber slides. Chambers were rinsed twice with phosphate-buffered saline (PBS) before incubation with MEM for 4 h in a humidified atmosphere of 5% CO_2 and 95% air. Cultures were fixed in 4% paraformaldehyde in 0.1 mol/L PBS and processed for in situ hybridization or immunocytochemistry.

Western Blot

Specificity of the $P2X_2$ antibody was tested by immunoblotting with rat calvarial osteoblastic cell extracts. Cells were grown to confluence as described earlier, rinsed with PBS, and lifted from the culture plates with PBS containing 0.2% ethylene-diamine tetraacetic acid (EDTA) and spun down; the cell pellet was resuspended in 1 mL of ice-cold RIPA buffer (PBS containing 1% Nonidet, 0.5% deoxycholate, 0.1% sodium dodecylsulfate [SDS], 0.1 mg/mL PMSF, 30 μl/mL Aprotinin, and 1 mmol/L sodium orthovanadate). These lysates were passed through a 21G needle to sheer DNA and then spun for 10 min at 10,000g and 4°C. The supernatants were used for western blotting in a Mini-Protean 2 Electrophoresis and Trans-Blotting Cell (Bio-Rad, Richmond, CA) according to the manufacturer's instructions.

Proteins were loaded on Tris-HCl ready gels (10% gel) (Bio-Rad) and run under reducing conditions (10% SDS and 26 mmol/L dithiothreitol). Biotinylated molecular weight markers were obtained from Sigma (B2787). Proteins were transferred onto a Hybond ECL nitrocellulose membrane (Amersham, UK). The nitrocellulose was blocked at room temperature in PBS containing 3% milk powder and 0.05% Tween-20, and then incubated in the same solution containing 2.5 μg/mL $P2X_2$ antibody overnight at 4°C. For detection, the ECL chemiluminescence method was performed, using peroxidase-linked donkey antirabbit immunoglobulin G (IgG), peroxidase-linked streptavidin, and ECL western blotting reagents (all purchased from Amersham, UK). The signal was visualized on a Hyperfilm ECL (Amersham) and scanned with a Umax Powerlook 2 flatbed scanner.

Immunocytochemistry

The immunogens used for production of polyclonal antibodies were synthetic peptides corresponding to the carboxy termini of the cloned rat P2X receptors, covalently linked to keyhole limpet hemocyanin. The peptide sequences are as follows:

$P2X_1$: amino acids 385–399, ATSSTLGLQENMRTS
$P2X_2$: amino acids 458–472, QQDSTSTDPKGLAQL
$P2X_3$: amino acids 383–397, VEKQSTDSGAYSIGH
$P2X_4$: amino acids 374–388, YVEDYEQGLSGEMNQ
$P2X_5$: amino acids 437–451, RENAIVNVKQSQILH
$P2X_6$: amino acids 357–371, EAGFYWRTKYEEARA
$P2X_7$: amino acids 555–569, TWRFVSQDMADFAIL

The polyclonal antibodies were raised by multiple monthly injections of New Zealand rabbits with the peptides (performed by Research Genetics, Huntsville, AL). The specificity of the antisera was verified by immunoblotting with membrane preparations from CHO K1 cells expressing the cloned $P2X_{1-7}$ receptors. The antibodies recognized only one protein of the expected size in the heterologous expression systems and were shown to be specific for receptor subtype.[25] For immunostaining of cryostat sections, the avidin-biotin (ABC) technique was employed according to a recently developed protocol.[18,19] Air-dried serial sections of bones were fixed in 4% formaldehyde and 0.02% picric acid in 0.1 mol/L phosphate buffer (pH 7.4) for 2 min. After washing in PBS for 20 min, endogenous peroxidase activity was blocked by treating the sections with 0.5% H_2O_2 and 50% methanol for 10 min. Nonspecific binding sites were blocked by 20 min preincubation in 10% normal horse serum (NHS) in PBS containing 0.05% merthiolate, followed by incubation with the primary antibodies diluted to 5 μg/mL or 2.5 μg/mL in antibody diluent (10% NHS in PBS + 2.5% NaCl) at 4°C overnight. Subsequently the sections were incubated with biotinylated donkey antirabbit IgG (Jackson ImmunoResearch Lab, West Grove, PA) diluted 1:500 in 1% NHS in PBS for 1 h, and then with ExtrAvidin peroxidase diluted 1:1000 in PBS for 1 h at room temperature. For color reaction, a solution containing 0.05% 3,3′-diaminobenzidine (DAB), 0.04% nickel ammonium sulfate, 0.2% β-D-glucose, 0.004% ammonium nitrate, and 1.2 U/mL glucose oxidase in 0.01 mol/L PBS was applied for 6 min. Contrast Green (Kirkegaard & Perry Laboratories) was used as a counterstain. Sections were washed three times with PBS after each of the aforementioned steps except after the preincubation. Control experiments were carried out with primary antibody or secondary or tertiary stages omitted from the staining procedure and the primary antibodies preabsorbed with the peptides for immunizing the rabbits.

For immunofluorescent staining of the cells, fixed cells were treated with methanol at −20°C for 7 min. The cells were preincubated in 10% NHS in PBS for 30 min at room temperature, followed by incubation with the primary antibodies diluted 1:1000 to 1:2000 in 10% NHS in PBS at 4°C overnight. Biotinylated donkey antirabbit IgG (Jackson ImmunoResearch), diluted 1:500 in 1% NHS in PBS, was applied for 1 h followed by fluoresceinated streptavidin (Amersham, UK) or Texas Red-

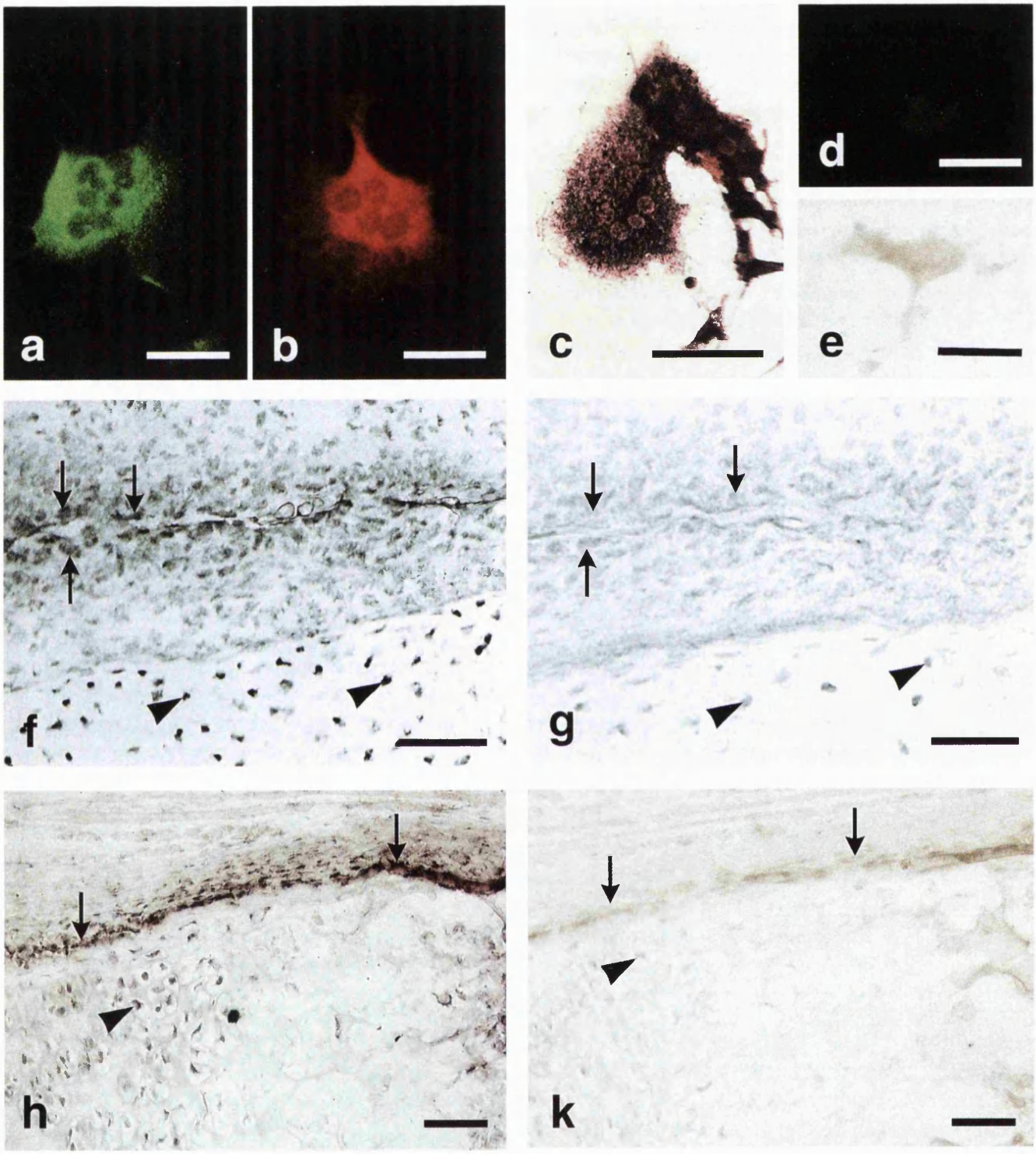

Figure 1. Localization of $P2X_2$ receptor subtype on bone sections and bone cells. (a, b) Specific immunostaining of cultured rat osteoclasts with anti-$P2X_2$ antibody, visualized with fluorescein (a) or Texas Red (b), which was abolished after preabsorption of anti-$P2X_2$ antibody with $P2X_2$ peptide (d). (c, e) Specific in situ localization of $P2X_2$ receptor probe on cultured rat osteoclast (c) was almost abolished in control preparations hybridized in the presence of excess unlabeled probe (e). (f, g) Serial sections of rat calvaria showing immunostaining of osteoblasts (arrows) and chondrocytes (arrowheads) with anti-$P2X_2$ antibody (f); immunostaining of osteoblasts (arrows) and chondrocytes (arrowheads) in control rat calvaria section was almost abolished after preabsorption of anti-$P2X_2$ antibody with $P2X_2$ peptide (g). (h, k) Specific in situ localization of $P2X_2$ receptor probe on osteoblasts (arrows) and growth plate chondrocytes (arrowheads) in rat long bone section (h); localization was greatly reduced in control preparations hybridized in the presence of excess unlabeled probe (k). Scale bars = 25 μm (a, b, d, e) and 50 μm (c, f, g, h, k).

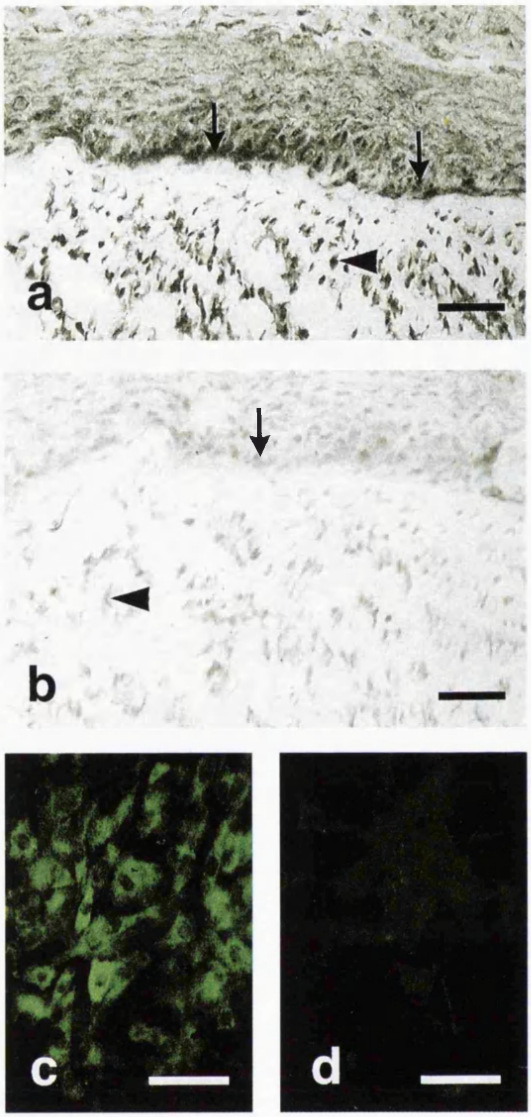

Figure 2. Localization of $P2X_5$ receptor subtype on rat long bone sections and rat osteoblasts. (a, b) Immunostaining with anti-$P2X_5$ antibody on serial sections of rat long bone: (a) Localization of immunostaining on osteoblasts (arrows) lining the bone surface and chondrocytes (arrowhead), which was almost abolished in the control section (b) preabsorbed with $P2X_5$ peptide. (c) Strong immunostaining in cultured rat calvarial osteoblastic cells with anti-$P2X_5$ antibody, visualized with fluorescein. (d) Immunostaining on cultured osteoblastic cells was greatly reduced in the control preparation preabsorbed with $P2X_5$ peptide. Scale bars = 50 μm (a–d).

labeled streptavidin (Amersham) diluted 1:200 in PBS for 1 h at room temperature.

In Situ Hybridization

Antisense oligonucleotides (45-mer) directed against receptor subtype-specific sequences were designed for use in in situ hybridization experiments. These sequences correspond to the C-terminal 15 amino acids of the rat $P2X_2$ and $P2X_4$ subtypes, the peptidic sequences used for the generation of the antipeptide antibodies, and against the third extracellular domain of the rat $P2Y_2$ and $P2Y_4$ subtypes. The oligonucleotide sequences were as follows:

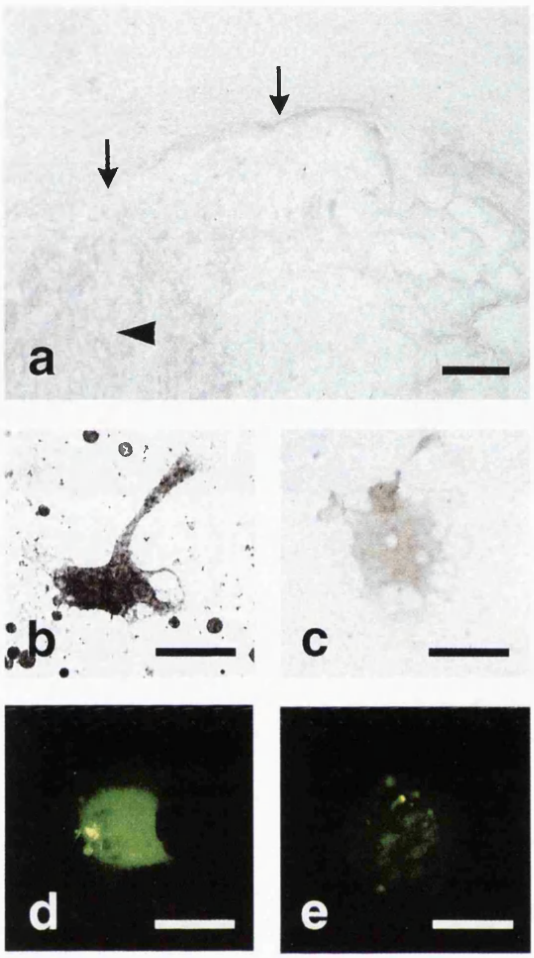

Figure 3. Localization of $P2X_4$ receptor subtype on a rat long bone section and on cultured rat osteoclasts. (a) In situ hybridization in rat long bone sections revealed no localization of $P2X_4$ receptor mRNA on osteoblasts (arrows) or chondrocytes (arrowheads). (b) Specific in situ localization of $P2X_4$ receptor probe on cultured rat osteoclast. (c) Control rat osteoclast hybridized in the presence of excess unlabeled probe showed greatly reduced localization of $P2X_4$ receptor probe. (d) Strong cytoplasmic immunostaining on cultured rat osteoclasts with anti-$P2X_4$ antibody, visualized with fluorescein. (e) Reduced anti-$P2X_4$ immunostaining in control rat osteoclast preparations preabsorbed with $P2X_4$ peptide. Scale bars = 50 μm (a) and 25 μm (b–e).

Rat $P2X_2$: 5′-AAGTTGGGCCAAACCTTTGGGGTCCGTG-GATGTGGAGTCCTGTTG-3′
Rat $P2X_4$: 5′-CTGGTTCATCTCCCCCGAAAGACCCT-GCTCGTAGTCTTCCACATA-3′
Rat $P2Y_2$: 5′-GATGGCGTTGAGGGTGTGGCAACTGAG-GTCAAGTGATCGGAAGGA-3′
Rat $P2Y_4$: 5′-GACAATGTTCAGCACATGACAGTCAGCT-TGCAACAGTCTTGCCTG-3′

The aforementioned primers were labeled at the 3′ end with digoxigenin dUTP using an oligonucleotide tailing kit (Roche Diagnostics, UK). Digoxigenin, a naturally occurring plant steroid, is not found in animal tissues, so cytoplasmic localization of the immunoproduct is considered to be specific.

Following fixation in 4% formaldehyde in PBS for 10 min, slides were dehydrated in graded ethanol and air-dried. The hybridization buffer contained 2× SSC buffer (Gibco), 0.1 mg/mL sheared and denatured salmon sperm DNA, 0.1 mg/ml total RNA, 50% de-ionized formamide, 1× Denhardt's solution,

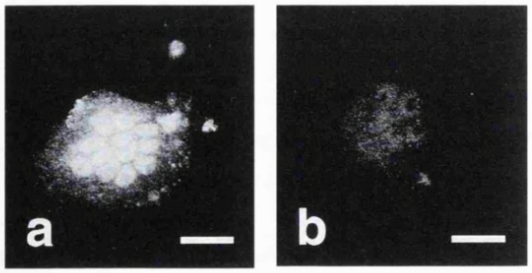

Figure 4. Localization of $P2X_7$ receptor subtype on cultured rat osteoclast, visualized with fluorescein. (a) Strong nuclear immunostaining of rat osteoclasts with anti-$P2X_7$ antibody that was almost abolished in the control cell (b), which was preabsorbed with $P2X_7$ peptide. Scale bars = 25 μm (a, b).

and 1 ng/μL digoxigenin-labeled probe. Before hybridization, prehybridization was done at 37°C for 2 h in a humidified chamber without digoxigenin-labeled probe. The slides were then incubated at the same temperature for 16 h with digoxigenin-labeled probe.

After washing with decreasing salt solutions (twice with 2× SSC for 5 min at room temperature, twice with 2× SSC for 15 min at 37°C, twice with 1× SSC for 15 min at 37°C, twice with 0.5× SSC for 30 min at 37°C), slides were blocked in 2% normal sheep serum in wash buffer (0.1 mol/L Tris-HCl and 0.15 mol/L NaCl, pH 7.4) for 2 h at room temperature. They were then incubated with anti-digoxigenin antibody (diluted 1:1000 in 2% normal sheep serum in wash buffer) conjugated with alkaline phosphatase for 2 h. The color reaction was made with 45 μl of 4-nitroblue tetrazolium salt, 35 μL of 5-bromo-4-chloro-3-indolylphosphate solution in 10 mL detection buffer (0.1 mol/L Tris-HCl, 0.1 mol/L NaCl, and 0.05 mol/L $MgCl_2$, pH 9.5) in the dark for up to 16 h. Negative controls were performed by hybridizing in the presence of 100-fold excess of unlabeled probe and by hybridizing without adding the labeled probe.

Results

Immunocytochemical Localization of P2X Receptors

Sections of neonatal rat long bones and calvariae showed different staining patterns with each P2X receptor antibody. In long bones and calvariae, osteoblasts on bone surfaces and chondrocytes showed specific immunostaining for $P2X_2$ and $P2X_5$ receptors, whereas in control preparations pretreated with the relevant P2X peptide immunostaining was abolished or greatly reduced (Figures 1f,g and 2a,b).

Specific immunostaining for the $P2X_1$ receptor was only observed in smooth muscle cells of blood vessels. No specific immunostaining for either $P2X_3$, $P2X_4$, or $P2X_6$ receptors was seen in sections of long bones and of calvariae; however, adjacent skeletal muscle tissue was strongly positive for $P2X_6$. Antibodies against $P2X_7$ produced no staining in the bone sections, except in the keratinizing and exfoliating layers of skin.

Fluorescence staining revealed immunostaining for P2 receptors in osteoclasts isolated from neonatal rat long bones and in primary rat calvarial osteoblastic cells. In essentially all cultured osteoclasts, the most prominent, specific immunostaining was observed with the $P2X_2$ antibody (**Figure 1**a,b); in controls pretreated with the $P2X_2$ peptide, staining was completely abolished (Figure 1d). Cytoplasmic staining was observed using the $P2X_4$ antibody in osteoclasts (**Figure 3**d). A small proportion of cultured osteoclasts also showed nuclear staining. However, in control preparations, the cytoplasmic staining was abolished,

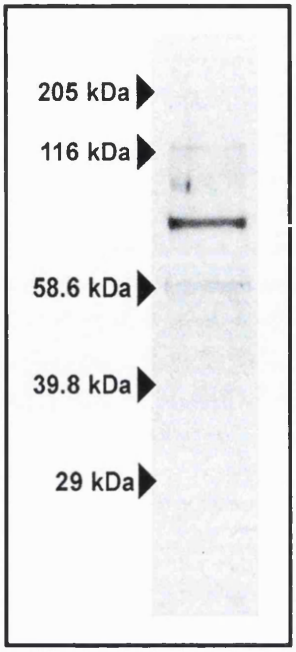

Figure 5. Western blotting of rat calvarial osteoblastic cell lysates. The blot was probed with polyclonal antibody to a rat $P2X_2$ receptor peptide. A single $P2X_2$ reactive band of approximately 70–80 kDa was detected. Molecular weight markers are indicated on the left side.

whereas nuclear staining remained, indicating that this was nonspecific (Figure 3e). Strong nuclear staining was seen with the $P2X_7$ antibody (**Figure 4**a) in all osteoclasts; in control preparations, the reaction was greatly reduced (Figure 4b). No specific immunostaining was observed for $P2X_1$, $P2X_3$, $P2X_5$, or $P2X_6$ receptors in cultured osteoclasts.

Rat osteoblastic cells cultured from neonatal rat calvariae showed strong staining with anti-$P2X_2$ and anti-$P2X_5$ antibodies (**Figure 2**c); in controls pretreated with the $P2X_2$ and $P2X_5$ peptides, staining was completely abolished or reduced, respectively (Figure 2d). No specific immunostaining was observed for $P2X_1$, $P2X_3$, $P2X_4$, $P2X_6$, or $P2X_7$ receptors in cultured osteoblasts.

To verify the finding of abundant $P2X_2$ expression in bone, immunoblotting was performed with rat calvarial osteoblastic cells. A single $P2X_2$ reactive band at approximately 70–80 kDa was detected (**Figure 5**). This band was in the expected weight range, as determined by immunoblotting with membrane fractions of cell lines expressing recombinant $P2X_2$ receptors.[25]

In Situ Hybridization

We investigated the expression of $P2X_2$, $P2X_4$, $P2Y_2$, and $P2Y_4$ mRNA in sections of rat long bone and calvariae and in rat osteoclasts and osteoblastic cells. In situ hybridization on long bone and calvarial sections using $P2X_2$ and $P2Y_2$ receptor probes revealed intense, specific localization over osteoblasts on bone surfaces and over chondrocytes, both in the growth plate in long bones and in cartilage in the skull (Figure 1h and **Figure 6**a). Use of the $P2X_4$ and $P2Y_4$ receptor mRNA probes resulted only in weak signals that were not consistently detectable in bone sections (Figure 3a). Negative controls of serial sections performed by hybridizing in the presence of 100-fold excess of unlabeled probe significantly reduced the signals for all probes (Figures 1k and 6b).

On essentially all rat osteoclasts, in situ hybridization re-

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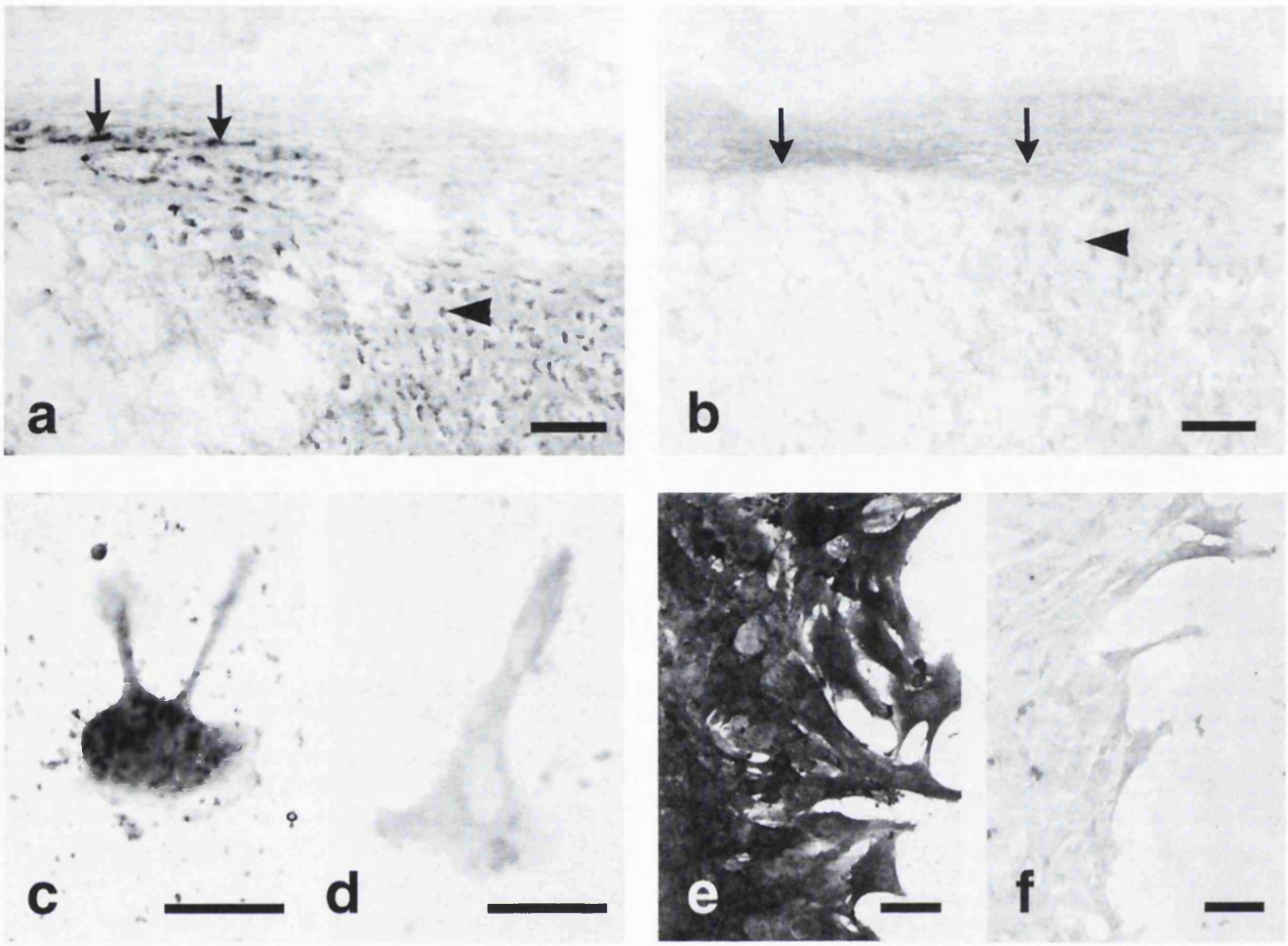

Figure 6. In situ localization of $P2Y_2$ receptor mRNA in a serial section of rat long bone and cultured cells. (a) Specific localization of $P2Y_2$ receptor probe on osteoblasts lining the bone surface (arrows) and on chondrocytes in the growth plate (arrowhead). (b) Signal was greatly reduced in osteoblasts (arrows) and chondrocytes (arrowhead) in control preparation hybridized in the presence of excess unlabeled $P2Y_2$ probe. (c) Specific localization of $P2Y_2$ receptor probe on cultured rat osteoclast, which was almost abolished in control (d) hybridized in the presence of excess unlabeled $P2Y_2$ probe. (e) Specific localization of $P2Y_2$ receptor probe on cultured primary osteoblastic cells from rat calvaria, which was almost abolished in control (f) hybridized in the presence of excess unlabeled $P2Y_2$ probe. Scale bars = 50 μm (a, b) and 25 μm (c, d, e, f).

vealed intense specific localization of $P2X_2$, $P2X_4$, and $P2Y_2$ receptor mRNAs (Figures 1c, 3b, and 6c), whereas the $P2Y_4$ receptor probe and negative controls showed significantly less staining (Figure 1e, 3c, and 6d). $P2X_2$ and $P2Y_2$ receptor mRNA was also highly expressed on primary rat calvarial osteoblastic cells (Figure 6e), in contrast to $P2X_4$ and $P2Y_4$ receptor mRNAs, which were undetectable. Negative controls showed reduced staining of cultured osteoblasts (Figure 6f).

Discussion

In this study we obtained the first direct evidence for the expression of the $P2X_2$ receptor in bone cells, using immunocytochemistry and in situ hybridization techniques. Earlier studies on the general distribution of P2X subunits showed that the $P2X_2$ subunit is located primarily in neural tissues in the central and peripheral nervous systems, but has now been found to be present in other tissues, including vascular smooth muscle.[13] We demonstrated immunostaining and in situ hybridization signals for the $P2X_2$ receptor on bone sections and on cultured bone cells including osteoclasts and primary cultures of osteoblastic rat calvarial cells.

Our finding of the $P2X_2$ receptor subtype on osteoclasts, shown by two independent methods, is of particular interest. We recently reported that extracellular ATP at relatively low concentrations (0.2–2 μmol/L) stimulated resorption pit formation by rat osteoclasts and that this stimulatory effect was amplified greatly by culture in acidified media (pH 6.9–7.0). Furthermore, pit formation by acid-activated osteoclasts in the absence of ATP was inhibited by the ecto-ATPase apyrase and by suramin, an antagonist of P2 receptors.[23] These results suggest that low levels of extracellular ATP might play a fundamental role in modulating the resorptive function of mammalian osteoclasts. They also point to the possible involvement of the $P2X_2$ receptor in this stimulatory effect, because this is the only P2 receptor subtype that requires extracellular acidification to show its full sensitivity to extracellular ATP.[14,15,31,32] The pH-activation profile of the recombinant $P2X_2$ receptor expressed in *Xenopus* is similar to the pH-activation profile for resorption pit formation by rat osteoclasts.[2] In addition, the apparent lack of desensitization to ATP and the suramin antagonism are consistent with the involvement of the $P2X_2$ receptor.[23] However, our recent experiments show that adenosine diphosphate (ADP), a potent $P2Y_1$ receptor agonist, stimulates bone resorption in a similar or even more

potent manner than ATP; $P2Y_1$ expression on bone is currently under investigation (Hoebertz et al., manuscript in preparation). We also detected expression of the $P2X_4$ receptor subtype in osteoclasts by immunocytochemistry and in situ hybridization, confirming a recent report that detected $P2X_4$ receptor mRNA in rabbit osteoclasts by polymerase chain reaction (PCR).[24]

Our immunocytochemical studies also revealed the apparent nuclear expression of the $P2X_7$ receptor subtype on cultured osteoclasts. Nuclear localization of this receptor subtype has recently been reported for epithelial cells using the same polyclonal antiserum[9]; the functional significance of this observation is under investigation. In addition, osteoblasts, both in culture and in bone sections, showed immunostaining for the $P2X_5$ receptor subtype. Studies of stratified epithelia showed that $P2X_5$ immunoreactivity was restricted to the metabolically active, differentiating cell layers in epithelia and hair follicles, whereas $P2X_7$ receptors were associated with keratinizing cells undergoing cell death.[9] Our results suggest that $P2X_5$ receptors may also participate in the regulation of osteoblastic differentiation and proliferation. $P2X_7$ (or P2Z) is a bifunctional receptor, which, in the absence of divalent cations, mediates the formation of large cytolytic membrane pores. The function of the receptor has been associated with lytic and apoptotic events.[6] Osteoclasts have been reported to die in a manner reminiscent of apoptosis when exposed to high concentrations of ATP (2 mmol/L).[23] The same ATP concentration has also been shown to cause formation of pores in murine osteoclasts and macrophages, but not in osteogenic or chondrogenic cells.[22]

It has been suggested that P2X receptors are involved in gating some of the intracellular supply of H^+ needed for resorption.[1] There is indeed evidence for an ATP- and ADP-induced pulsed decrease in rabbit osteoclast intracellular pH, together with an intracellular Ca^{2+} pulse,[36] indicating the existence of two different intracellular signaling pathways after ATP application. Electrophysiological experiments have provided evidence for the coexpression of both P2X and P2Y receptors on rat osteoclasts, with ATP activating both nonselective cation channels and Ca^{2+}-dependent K^+ channels.[30]

We demonstrated the first localization of $P2Y_2$ (formerly P2U) receptor mRNA in bone sections and its expression not only by osteoblasts, but also by chondrocytes. These findings are consistent with earlier work that detected $P2Y_2$ mRNA in osteoclastoma giant cells by in situ hybridization, and in human bone derived cells by PCR.[3] Our findings are also consistent with studies on the effects of extracellular nucleotides on intracellular Ca^{2+} levels in osteoblast-like cells, both of human and rat origin, which suggested the existence of two different P2 receptor subtypes (including $P2Y_2$) linked to Ca^{2+} mobilization.[27,28]

Our finding that the $P2X_2$, $P2X_5$, and $P2Y_2$ receptors were strongly expressed by osteoblasts both in bone sections and in vitro implies that exogenous ATP could therefore stimulate resorption pit formation via direct effects on mature osteoclasts or indirect effects on other bone cells such as osteoblasts, or both. Osteoblasts have been shown to release ATP under shear stress conditions via a nonlytic mechanism[5] and osteoclasts have been reported to express a novel member of the ATP binding cassette superfamily.[29] Our failure to detect $P2Y_4$ expression in rat osteoblastic cells contrasts with the recent identification of $P2Y_4$ transcripts in human osteoblastic cells by PCR[21]; this may reflect a species difference or changes in cellular differentiation states.

Our localization studies also showed the presence of P2 receptors in chondrocytes, in agreement with suggestions made some time ago.[7,17] Cultured chondrocytes are also capable of constitutively releasing ATP at concentrations that may activate P2 receptors in the local microenvironment.[10,20] Extracellular nucleotides have also been shown to enhance growth-factor-induced proliferation of chondrocytes,[12] and stimulation of the $P2Y_2$ receptor on human articular chondrocytes increases interleukin (IL)-1-mediated prostaglandin E_2 release.[16]

In summary, the immunohistochemical and in situ hybridization results presented in this study are consistent with a wide range of earlier experimental findings and demonstrate, for the first time, the presence of $P2X_2$ receptors on osteoblasts and osteoclasts. The identification of $P2X_2$ receptors on osteoclasts provides a possible mechanism for the critical activation of osteoclasts at low pH.

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Extracellular ADP is a powerful osteolytic agent: evidence for signaling through the $P2Y_1$ receptor on bone cells

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ABSTRACT **There is increasing evidence that extracellular nucleotides act on bone cells via P2 receptors. This study investigated the action of ADP and 2-methylthioADP, a potent ADP analog with selectivity for the $P2Y_1$ receptor, on osteoclasts, the bone-resorbing multinuclear cells. Using three different assays, we show that ADP and 2-methylthioADP at nanomolar to submicromolar levels caused up to fourfold to sixfold increases in osteoclastic bone resorption. On mature rat osteoclasts, cultured for 1 day on polished dentine disks, peak effects on resorption pit formation were observed between 20 nM and 2 μM of ADP. The same concentrations of ADP also stimulated osteoclast and resorption pit formation in 10-day mouse marrow cultures on dentine disks. In 3-day explant cultures of mouse calvarial bones, the stimulatory effect of ADP on osteoclast-mediated Ca^{2+} release was greatest at 5–50 μM and equivalent to the maximal effects of prostaglandin E_2. The ADP effects were blocked in a nontoxic manner by MRS 2179, a $P2Y_1$ receptor antagonist. Using in situ hybridization and immunocytochemistry, we found evidence for $P2Y_1$ receptor expression on both osteoclasts and osteoblasts; thus, ADP could exert its actions both directly on osteoclasts and indirectly via $P2Y_1$ receptors on osteoblasts. As a major ATP degradation product, ADP is a novel stimulator of bone resorption that could help mediate inflammatory bone loss in vivo.—Hoebertz, A., Meghji, S., Burnstock, G., Arnett, T. Extracellular ADP is a powerful osteolytic agent: evidence for signaling through the $P2Y_1$ receptor on bone cells. *FASEB J.* 15, 1139–1148 (2001)**

Key Words: P2 receptors · ADP · ATP · osteoclast · bone resorption · $P2Y_1$

BONE IS A dynamic tissue, being continuously remodeled by the coordinated actions of osteoclasts and of cells in the osteoblast lineage. Osteoclasts, cells responsible for bone resorption, are polarized multinuclear cells, derived from hematopoietic precursors of the monocyte-macrophage series. Osteoblasts, the bone-forming cells, originate from mesenchymal stem cells; osteocytes, cells thought to mediate mechanical responsiveness of bone, differentiate from osteoblasts to form a network of cells within bone matrix. In bone loss disorders, the normal remodeling process becomes unbalanced, which can result in excessive osteoclastic bone resorption and fragile bones. The complex mechanisms by which systemic and local factors influence osteoclastic formation and activation, key steps in the bone remodeling sequence, are still not well understood.

ATP and other extracellular nucleotides are now recognized as important messenger molecules for cell-cell communication (1). It has recently become evident that extracellular nucleotides, signaling through P2 receptors, could play an important role in bone remodeling (2). Receptors for nucleotides and nucleosides were originally divided into two groups: P1 receptors for which adenosine and AMP are major agonists, and P2 receptors for adenosine 5′-diphosphate (ADP), ATP, and uridine 5′-triphosphate (UTP). The P2 receptors are further classified into two main families: the ionotropic P2X receptors are a family of ligand-gated nonselective cation channels; in contrast, the metabotropic P2Y receptors are coupled to G-proteins, which activates signal transduction pathways involving inositol 1,4,5-trisphosphate-dependent mobilization of intracellular Ca^{2+}. Nucleotides can be released into the extracellular fluid in a number of ways. ATP, the nucleotide with the widest spectrum of biological activity and present intracellularly at ~2–5 mM, could be released as a result of cell damage, via synaptic vesicles from nerve cells, by active secretion via "ATP binding cassette" (ABC) transport proteins and sulfonylurea receptors, or by release from activated platelets and leukocytes at a site of tissue injury and inflammation.

We have previously shown that ATP is capable of stimulating osteoclasts to perform their pathophysiological function, namely the formation of resorption pits (3). In addition, we recently reported, using in situ hybridization and immunocytochemistry, that bone cells express several P2 receptors, of both the P2X and the P2Y families (4). This result was consistent with

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earlier findings that both osteoclasts and osteoblasts respond to extracellular nucleotides with an increase in intracellular Ca^{2+} (5–9) and, in the case of osteoclasts, also with a decrease in intracellular pH (10).

Few studies to date have related the presence of P2 receptors to specific functions of bone cells. The aim of this study was to investigate the effects of a wider range of P2 receptor agonists and antagonists on bone resorption and to determine which receptors were involved in mediating any effects. We show for the first time that extracellular ADP, the first degradation product of ATP, is a powerful stimulator of bone resorption and acts at nanomolar to submicromolar concentrations, as assessed by three independent methods in two different species. We also provide evidence that this stimulation of osteoclastic function is probably mediated via the $P2Y_1$ receptor. This is the first study that links a specific P2 receptor to a key functional action of an extracellular nucleotide on bone and could point to a fundamental new mechanism in the local modulation of bone resorption.

MATERIALS AND METHODS

Materials

Culture media and buffers were purchased from Life Technologies (Paisley, U.K.). Reagents for in situ hybridization experiments were purchased from Boehringer (Mannheim, Germany). All other reagents were purchased from Sigma (Poole, U.K.) unless stated otherwise. Stock solutions of nucleotides were prepared in PBS and stored at −80°C. Untreated elephant ivory was kindly provided by HM Customs and Excise (London Heathrow Airport).

Resorption pit formation assay

The effects of extracellular nucleotides on resorption pit formation by mature rat osteoclasts were studied by using modifications of an assay described previously (11). All experiments were performed with minimum essential medium supplemented with Earle's salts, 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (complete mixture abbreviated MEM). In most experiments, MEM was acidified by the direct addition of small amounts of concentrated hydrochloric acid (10 mEq/l H^+, equivalent to 85 μl of 11.5 M HCl per 100 ml of 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 (12). Elephant ivory (dentine) was prepared by cutting 250-μm-thick transverse wafers using a low-speed diamond saw (Buehler, Coventry, U.K.); 5-mm-diameter disks were cut from wet dentine wafers by using a standard paper punch, washed extensively by sonication in distilled water, and stored dry at room temperature. Before use, dentine disks were sterilized by brief immersion in ethanol, after which they were allowed to dry and were then rinsed in sterile PBS.

Mixed cell populations containing osteoclasts were obtained by mincing rapidly the pooled long bones of 2-day-old Sprague-Dawley rat pups, killed by cervical dislocation (n = 5), in 5 ml MEM, followed by vortexing for 30 s. The resulting cell suspension was allowed to sediment for 45 min onto 5-mm dentine disks, prewetted with 50 μl of MEM, in 96-well plates (100 μl of cell suspension/disk). Disks 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 5 ml of acidified MEM and five replicate dentine disks; 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 Pco_2 were measured using a blood gas analyzer (Radiometer, Copenhagen, Denmark), with careful precautions to prevent CO_2 loss. Dentine disks were removed and fixed in 2% glutaraldehyde and then were stained for tartrate-resistant acid phosphatase (TRAP), a cytochemical marker for mature osteoclasts (Sigma Kit 387-A). The numbers of TRAP-positive multinucleated osteoclasts (three or more nuclei) and the number of stromal cells were assessed "blind," by using transmitted light microscopy. Discrete resorption pits were counted blind by scanning the entire surface of each disk with a reflected light microscope after restaining in 1% toluidine blue in 1% sodium borate for 2 min.

Mouse calvarial bone resorption assay

The method, which measures bone resorption as Ca^{2+} release from neonatal mouse calvariae, was similar to that described in detail by Meghji et al. (13). Briefly, 5-day-old MF1 mice were killed by cervical dislocation. The frontoparietal bones were removed and trimmed of any adhering connective tissue and interparietal bone, with care taken not to damage the periosteum. Dissected calvariae were pooled, washed free of blood and adherent brain tissue in HBSS, and then divided along the sagittal suture. Half-calvariae were cultured individually on 1-cm^2 stainless steel grids (Minimesh, FDP quality, Expanded Metal, West Hartlepool, U.K.) in 6-well plates with 1.5 ml of BGJb medium, 5% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, at the air-liquid interface in a humidified CO_2 incubator. After an initial 24-h preincubation period, the medium was removed and replaced with control or test media. Prostaglandin E_2 (PGE_2) and indomethacin were dissolved in ethanol vehicle for use; the final concentration of ethanol in cultures did not exceed 1 part in 500. Each experimental group consisted of five individual cultures. The cultures were then incubated for 72 h without further medium changes, and without opening the incubator door, so as to ensure constant CO_2 levels and minimize pH fluctuations. Culture medium acidification was achieved by adding small amounts of concentrated HCl to culture medium, as described before (11, 12), resulting in decreased HCO_3^- concentration (metabolic acidosis).

After 72 h, experiments were terminated by withdrawing culture medium and washing the bones once with PBS, followed by fixation in 95% ethanol/5% glacial acetic acid for 10 min. Incubator Pco_2 was determined by immediately measuring a culture medium sample with a blood gas analyzer. The mean final pH of each treatment group was determined by removing and pooling a 100-μl sample from each replicate; the pooled samples were then re-equilibrated with CO_2 in the incubator before measurement using the blood gas analyzer; slight differences in CO_2 tension between groups were normalized to the initially measured value using pH-Pco2 calibration curves constructed for BGJb medium, as described previously (12).

Ca^{2+} concentrations in culture medium at the end of experiments were measured colorimetrically via an autoanalyzer (Chem Lab Instruments, Essex, U.K.) using the procedure described earlier (13). The basal Ca^{2+} concentration of the BGJb medium after addition of 5% heat inactivated FCS was 2.00 mM. All measurements were performed blind on coded samples.

After fixation and decalcification with 95% ethanol/5% glacial acetic acid, calvariae were stained for TRAP and mounted whole in melted glycerol jelly for transmitted light microscopy.

Osteoclast formation assays

Long bones of 8-wk-old MF1 mice ($n = 2$), killed by cervical dislocation, were fragmented in 5 ml of unmodified MEM, followed by vortexing for 1 min. The resulting cell suspension was allowed to sediment for 2 h onto sterile 5-mm-diameter dentine disks, prewetted with 50 μl of MEM, in 96-well plates (100 μl of cell suspension/disk). Dentine disks were then removed and placed in test or control medium in a 6-well plate. Each test or control well contained 5 ml of nonacidified MEM with 10 nM 1,25-dihydroxyvitamin D_3, 10 nM dexamethasone, 20 ng/ml human recombinant macrophage colony-stimulating factor (M-CSF), 1 ng/ml RANKL (receptor activator of NF-κB ligand, also called OPGL, a kind gift of Dr. Colin Dunstan, Amgen, Thousand Oaks, CA), 100 nM PGE_2, and six replicate dentine disks. Cultures were incubated for 10 days in a humidified atmosphere of 5% CO_2/95% air, with medium changes every 2–3 days. For the first 7 days, NaOH was added to a running pH of 7.4, which has been shown to be required for optimal osteoclast formation (14); for the last 3 days, MEM was acidified by addition of HCl to ensure resorptive activity (14). Medium pH and P_{CO_2} were monitored during and at the end of experiments via a blood gas analyzer. After 10 days of incubation, the disks were fixed in 2% glutaraldehyde and were stained for TRAP (Sigma Kit 387-A). A control group of dentine disks was also removed, fixed, and stained after 3 days of incubation to check for the presence of any mature osteoclasts that might have been released during the initial cell preparation. The total number of TRAP-positive multinucleated osteoclasts and of discrete resorption pits was assessed blind by transmitted and reflected light microscopy.

As an alternative procedure, bone marrow cells were isolated from 8-wk-old MF1 mice, using a modification of a method described previously (15). The marrow cavity of the long bones was flushed into a dish by slowly injecting MEM at one end of the bone using a sterile 25-gauge needle. The resulting suspension was washed twice and resuspended and incubated overnight in a 75-cm^2 flask at a density of 3×10^6 cells/ml MEM containing M-CSF (5 ng/ml). After 24 h, nonadherent cells were harvested, washed, and resuspended (10^6/ml) in MEM containing M-CSF (30 ng/ml) and RANKL (10 ng/ml). This suspension was added to the wells of either 96-well plates containing dentine disks (100 μl) or 48-well plates (800 μl). After a 24-h preincubation period, dentine disks were transferred to 6-well plates (six replicates/well) and test media were added. Cultures were fed every 3 days by replacing half the medium with fresh medium and reagents. The functional absence of contaminating stromal cells was confirmed in cultures in which M-CSF was omitted; such cultures showed no cell growth. After 10 days of treatment, 48-well plates and dentine disks were fixed and assessed for TRAP or bone resorption as described above.

In situ hybridization and immunocytochemistry

Neonatal (2-day-old) Sprague-Dawley rats were killed by cervical dislocation. Long bones were removed immediately, frozen rapidly by immersion in isopentane at −70°C, and stored in liquid nitrogen. Cryostat sections of undecalcified, unfixed bone (10 μm) were prepared and collected on polysine-coated slides (BDH/Merck, Poole, Dorset, U.K.) for in situ hybridization. Tissues were kept frozen until used and were air-dried at room temperature prior to use.

Mixed cell suspensions containing osteoclasts, obtained from the long bones of 2-day-old neonatal Sprague-Dawley rats as described above, were allowed to sediment for 60 min onto LabTek 8-chamber slides. Chambers were rinsed twice with PBS before incubation with MEM for 4 h in a humidified atmosphere of 5% CO_2/95% air. Cultures were fixed in 4% paraformaldehyde in 0.1 M PBS and processed for in situ hybridization or immunocytochemistry. Primary rat osteoblastic cells were obtained by sequential enzyme digestion of excised calvarial bones from 2-day-old rats using a three-step process as described previously (4). Cells were cultured in LabTek 8-chamber slides until confluence (up to 4 days) and fixed and processed as described above.

For immunofluorescent staining of the osteoclasts, fixed cells were treated with methanol at −20°C for 7 min. The cells were preincubated in 10% normal horse serum (NHS) and 0.1% Triton X-100 in PBS for 30 min at room temperature, followed by overnight incubation at 4°C with the affinity-purified anti-$P2Y_1$ antibody (a kind donation of Carlos Matute, Leioa, Spain) (16) at 2 μg/ml in the same solution. Biotinylated donkey anti-rabbit immunoglobulin G (Jackson ImmunoResearch Lab, West Grove, PA), diluted 1:500 in 1% NHS in PBS and 0.1% Triton X-100, was applied for 1 h, followed by fluoresceinated streptavidin (Amersham, Bucks, U.K.) diluted 1:200 in PBS for 1 h at room temperature. Control experiments were carried out with antiserum that had been preadsorbed overnight at 4°C with the immunogenic peptide (15 μM = 31 μg/ml) (16).

For in situ hybridization experiments, an antisense oligonucleotide (45 mer) directed against $P2Y_1$ receptor subtype-specific sequence was designed. This sequence corresponds to the third extracellular domain of the rat $P2Y_1$ subtype. The oligonucleotide sequence is as follows:

5′-AGGTGGCATAAACCCTGTCGTTGAAATCACACATT-TCTGGGGTCT-3′

The above primer was labeled at the 3′-end with digoxigenin dUTP by using an oligonucleotide tailing kit. Digoxigenin, a naturally occurring plant steroid, is not found in animal tissues, so cytoplasmic localization of the immunoproduct is considered to be specific.

After fixation in 4% formaldehyde in PBS for 10 min, slides with cryostat sections or cultured and fixed cells were dehydrated in graded ethanol and air-dried. The hybridization buffer contained 2× SSC buffer, 0.1 mg/ml sheared and denatured salmon sperm DNA, 0.1 mg/ml tRNA, 50% deionized formamide, 1× Denhardt's solution, and 1 ng/μl digoxigenin-labeled probe. Before hybridization, prehybridization was done at 37°C for 2 h in a humidified chamber without the digoxigenin-labeled probe. The slides were then incubated at the same temperature for 16 h with the digoxigenin-labeled probe.

After washing with decreasing salt solutions (twice with 2× SSC for 5 min at room temperature, twice with 2× SSC for 15 min at 37°C, twice with 1× SSC for 15 min at 37°C, and twice with 0.5× SSC for 30 min at 37°C), slides were blocked in 2% normal sheep serum in wash buffer (0.1 M Tris-HCl, 0.15 M NaCl; pH 7.4) for 2 h at room temperature. They were then incubated with anti-digoxigenin antibody (diluted 1:1000 in 2% normal sheep serum in wash buffer) conjugated with alkaline phosphatase for 2 h. The color reaction was made with 45 μl of 4-nitroblue tetrazolium salt, 35 μl of 5-bromo-4-chloro-3-indolyl-phosphate solution in 10 ml of detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M $MgCl_2$; pH 9.5) in the dark for up to 16 h. Negative controls were performed by hybridizing in the presence of 100-fold excess of unlabeled probe and by hybridizing without adding the labeled probe.

Statistics

Statistical comparisons were made by one-way analysis of variance or the Mann-Whitney test; representative data are presented as means ± SE for five or six replicates. Results are presented for representative experiments that were each repeated at least three times.

RESULTS

Effect of ADP on mature rat osteoclasts

Extracellular ADP exerted a reproducible, biphasic effect on resorption pit formation by rat osteoclasts in acid-activated 26-h cultures. **Figure 1** shows typical biphasic concentration results for the effects of ADP: in this experiment, high stimulatory effects were evident in the low nanomolar range (20–200 nM), with up to twofold increases in pit formation (Fig. 1). In other experiments, ADP increased resorption pit formation up to threefold (**Fig. 2**). At higher ADP concentrations of 20–200 μM, there was no stimulatory effect on resorption. Numbers of osteoclasts and of mononuclear cells (i.e., cells of osteoblastic/fibroblastic morphology) were unaltered by ADP treatment.

The stimulatory effect of ADP on rat osteoclast resorption pit formation was observable clearly only when culture medium (MEM) was acidified to a running pH of ≤7.0 by addition of H^+ as HCl (Fig. 2). In the absence of ADP, acidification (pH reduction from 7.08 to 6.82) elicited a fourfold increase in resorption; in nonacidified MEM (pH 7.11), ADP caused a modest twofold stimulation. However, culturing osteoclasts in acidified MEM with addition of 1 μM ADP resulted in a 3-fold increase in the number of pits formed per osteoclast compared with an acidified control and a 13-fold increase compared with a nonacidified control, suggesting a synergy between the stimulatory effects of acidification and ADP. Similar acidification dependency was observed with all active P2 receptor agonists tested, and thus all further experiments were conducted at low pH.

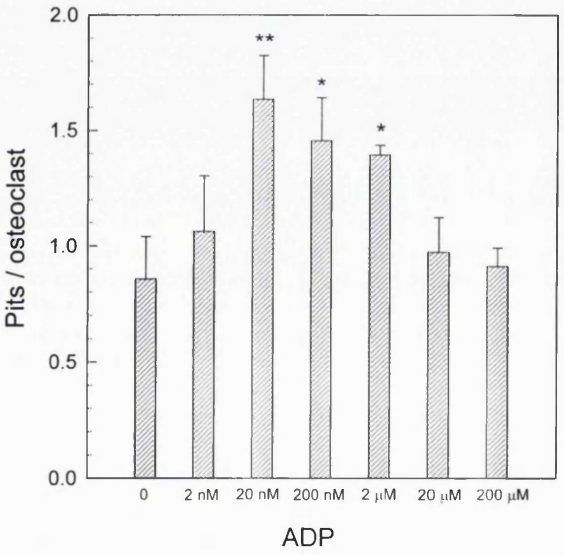

Figure 1. Effect of ADP on resorption pit formation by rat osteoclasts. Osteoclasts were cultured on 5-mm dentine disks in acidified medium (pH ~6.9) for 26 h. Large stimulatory effects were evident in the low nanomolar range (20–200 nM), at which ADP caused twofold increases in pit formation. Numbers of mononuclear cells and osteoclasts were unaltered by ADP treatment: treatment with 0, 0.002, 0.02, 0.2, 2, 20, or 200 μM ADP resulted in 2785 ± 198, 2726 ± 159, 2564 ± 241, 2491 ± 181, 2257 ± 281, 2642 ± 289, and 2782 ± 439 mononuclear cells and 60.4 ± 4.1, 35.4 ± 6.1, 39.6 ± 3.6, 37.8 ± 3.8, 43.6 ± 2.2, 46.4 ± 4.8, and 41.6 ± 6.7 osteoclasts/disk, respectively (all nonsignificant). Values are means ± SE ($n = 5$). Significantly different from control: $^*P < 0.05$; $^{**}P < 0.01$.

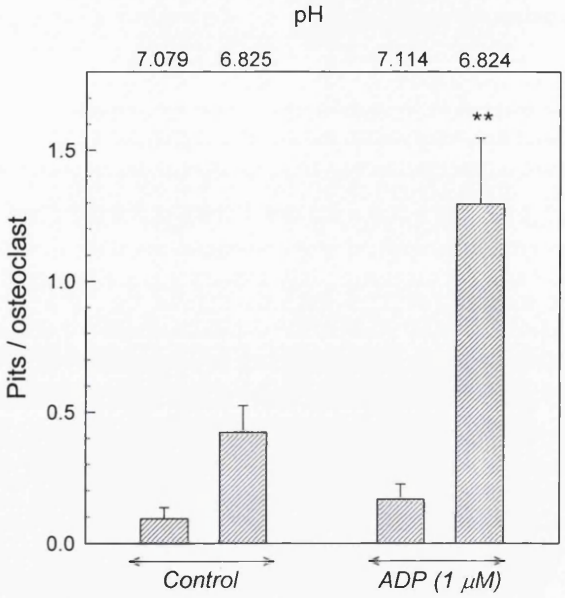

Figure 2. Comparison of the effects of ADP on resorption pit formation by rat osteoclasts cultured in unmodified medium (pH ~7.1) or in acidified medium (pH ~6.8) for 26 h. The figure shows potentiation of ADP-stimulated resorption at low pH. The number of osteoclasts/disk was 42 ± 7.6, 57 ± 7.3, 77 ± 10, and 69.3 ± 8.4 and the number of mononuclear cells/disk was 3291 ± 210, 3473 ± 200, 3491 ± 393, and 3346 ± 303 for each treatment group, respectively (all nonsignificant). Values are means ± SE ($n = 5$). Significantly different from acidified control: $^{**}P < 0.01$.

2-MethylthioADP (2-MeSADP), a highly selective $P2Y_1$ receptor agonist, was able to mimic the ADP effect (**Fig. 3**). At 200 nM, the effect of 2-MeSADP was somewhat greater than that of ADP, but the difference was not statistically significant. However, no stimulatory effect was observed with 1 μM 2-MeSADP, suggesting that this analog stimulates resorption effectively within a very narrow concentration range. Further degradation products of ADP, namely AMP and adenosine, had no significant effect on bone resorption, thus indicating that ADP itself is the signaling agent (**Fig. 4**).

ADP is the major agonist at the $P2Y_1$ receptor, but it is also a less potent agonist at the $P2X_1$ receptor. We therefore investigated the action of $P2X_1$ agonists, α,β-methylene-ATP (α,β-MeATP, EC_{50} 1.5 μM) and β,γ-methylene-ATP (β,γ-MeATP, EC_{50} 2 μM) (17) on bone resorption (**Fig. 5**). Neither α,β-MeATP nor β,γ-MeATP at concentrations of 0.5 and 5 μM had a significant stimulatory effect on bone resorption, thus suggesting a lack of involvement of the $P2X_1$ receptor.

To further study the involvement of the $P2Y_1$ receptor, we tested the compound MRS 2179 (N^6-methyl-2′-deoxyadenosine-3′,5′-bisphosphate; a kind gift from Dr. K. A. Jacobson, National Institutes of Health, Bethesda, MD), the most potent $P2Y_1$ receptor antagonist reported to date (18, 19). The twofold stimulatory

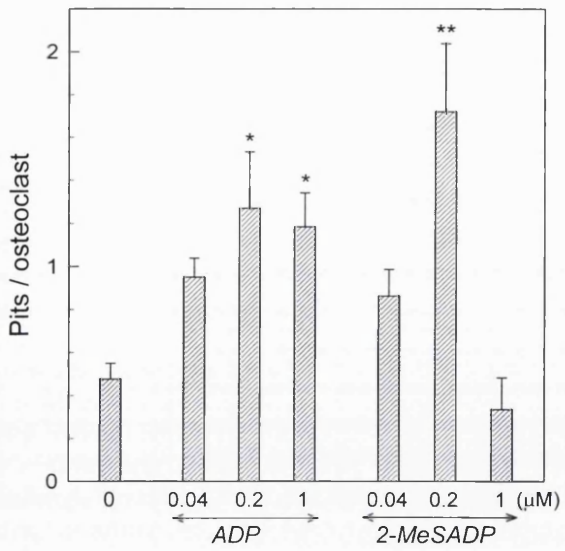

Figure 3. Effect of the selective $P2Y_1$ agonist 2-MeSADP on resorption pit formation by rat osteoclasts. Osteoclasts were cultured on 5-mm dentine disks in acidified medium (pH ~6.9) for 26 h. 2-MeSADP mimicked the ADP effect with a peak effect at 0.2 μM, increasing resorption pit formation up to fourfold. The number of osteoclasts/disk was 12.2 ± 1.2, 13.8 ± 1.6, 15.2 ± 3.4, 13.8 ± 1.7, 17.2 ± 2.1, 13.2 ± 1.8, and 17.5 ± 4.5 and the number of mononuclear cells/disk was 3264 ± 89, 3724 ± 227, 3531 ± 221, 3759 ± 44, 2836 ± 271, 3339 ± 58, and 2494 ± 504 for each treatment group, respectively (all nonsignificant). Values are means ± SE (n = 5). Significantly different from control: $^*P < 0.05$; $^{**}P < 0.01$.

effects of ADP at 0.2 μM could be blocked in a nontoxic manner by MRS 2179 at 0.02–20 μM (**Fig. 6**), whereas the control values were unchanged by this antagonist. Numbers of osteoclasts and mononuclear cells were unaltered by any treatment. Note that although baseline levels of resorption pit formation and cell numbers vary somewhat among individual assays, as would be expected for primary cell cultures of this nature, relative treatment/control effects were highly reproducible.

Effect of ADP on osteoclast-mediated Ca^{2+} release from mouse calvariae

In 72-h cultures of mouse calvariae, extracellular ADP also caused a dramatic increase in bone resorption. Peak effects were observed in the range 5–50 μM, but large stimulatory effects were observed at concentrations as low as 50 nM (0.05 μM). The peak effects of ADP in the mouse calvarial culture were observed at approximately 10-fold higher concentrations than in the disaggregated rat osteoclast system, reflecting the generally lower sensitivities exhibited by intact organ cultures. In the presence of 5 μM extracellular ADP, osteoclast-mediated Ca^{2+} release was increased up to sixfold compared with controls (**Figs. 7** and **8**). The peak stimulatory effects of ADP were equivalent to the maximal effects of PGE_2 at 1 μM (Fig. 8). Extracellular ADP appeared to be somewhat more effective than extracellular ATP, which stimulated Ca^{2+} release up to fourfold at 5 μM compared with the control (Fig. 7). Similarly to the stimulatory effects on mature rat osteoclasts, the $P2Y_1$ selective agonist 2-MeSADP mimicked the ADP effect and increased Ca^{2+} release fourfold compared with the control, with a peak effect at 5 μM (Fig. 7). At 50 μM, 2-MeSADP increased Ca^{2+} release only twofold, again suggesting that 2-MeSADP effectively stimulates resorption in a relatively narrow concentration range. The selective $P2Y_1$ antagonist MRS 2179 inhibited ADP-induced bone resorption (Fig. 8). The stimulatory effect of ADP at 5 μM could be reduced 3.6-fold by MRS 2179 at 5 μM.

In addition, ADP-stimulated Ca^{2+} release was completely blocked by the cyclooxygenase inhibitor indomethacin at 0.1 and 1 μM (Fig. 8), suggesting that the effect may be mediated by prostaglandins, as for other established resorption stimulators in this system.

Effect of ADP in mouse marrow cultures

In 10-day mouse marrow cultures on dentine disks, extracellular ADP at low concentrations reproducibly stimulated the formation of TRAP-positive osteoclasts and resorption pits (**Fig. 9**). The concentration of RANKL (1 ng/ml) in these experiments was chosen to permit relatively low level osteoclast formation without masking potential stimulatory effects of other agents. Effects of ADP were observed in the range 0.2–2 μM

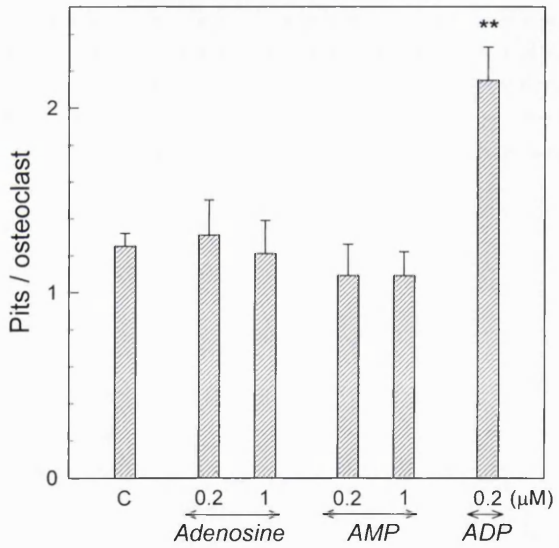

Figure 4. Effects of further degradation products of ADP, namely, AMP and adenosine, on resorption pit formation by rat osteoclasts. Osteoclasts were cultured on 5-mm dentine disks in acidified medium (pH ~6.9) for 26 h. Adenosine and AMP had no effect on bone resorption compared with ADP at 0.2 μM, indicating that ADP itself is the signaling agent. The number of osteoclasts/disk was 43.6 ± 8.9, 53.6 ± 4.7, 48.8 ± 6.7, 37.2 ± 5.3, 38.6 ± 3.7, and 28.4 ± 3.2 and the number of mononuclear cells/disk was 3599 ± 290, 3994 ± 323, 2869 ± 311, 3455 ± 59, 3759 ± 168, 3372 ± 333, and 4192 ± 400 for each treatment group, respectively (all nonsignificant). Values are means ± SE (n = 5). Significantly different from control: $^{**}P < 0.01$.

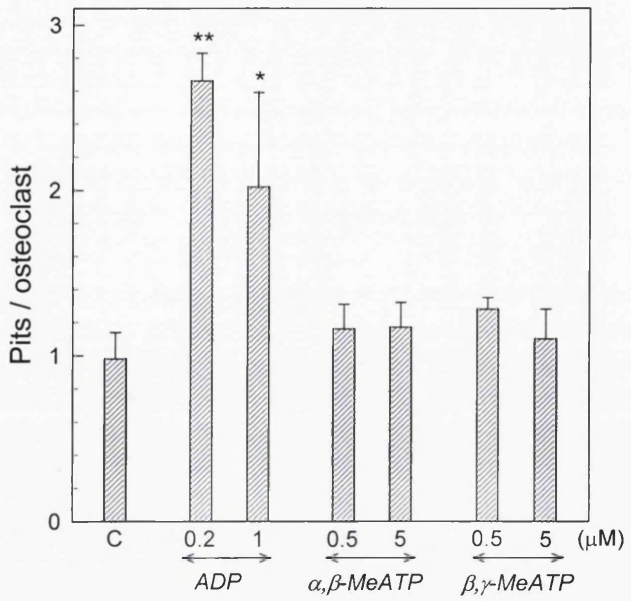

Figure 5. Agonists for the $P2X_1$ receptor subtype (α,β-MeATP and β,γ-MeATP) were unable to mimic the stimulatory effect of ADP on resorption pit formation, thus excluding the involvement of the $P2X_1$ receptor. Osteoclasts were cultured on 5-mm dentine disks in acidified medium (pH ~6.9) for 26 h. The number of osteoclasts/disk was 24.4 ± 4.2, 19.5 ± 1.3, 19.8 ± 3.6, 26.8 ± 1.2, 23.8 ± 2.2, 27.2 ± 2.7, and 19.6 ± 2.5 and the number of mononuclear cells/disk was 3042 ± 212, 2846 ± 304, 3135 ± 145, 2568 ± 313, 2361 ± 397, 2212 ± 298, and 2514 ± 158 for each treatment group, respectively (all nonsignificant). Values are means ± SE (n = 5). Significantly different from control: $^*P < 0.05$; $^{**}P < 0.01$.

ADP. Lower concentrations were without effect. In the presence of 2 μM ADP, osteoclast formation was increased twofold, but resorption was increased up to fivefold compared with the control, presumably reflecting stimulation of newly formed mature osteoclasts and consistent with our findings for rat osteoclasts in short-term cultures. ATP at 2 μM was slightly more effective than ADP in stimulating osteoclast formation (2.4-fold increase) and showed a similar 5-fold stimulation of resorption. In the control groups that were fixed and stained for TRAP after 3 days of incubation, osteoclasts and resorption pits were never observed, indicating that the osteoclasts and resorption pits observed after 10 days of culture resulted entirely from formation of new osteoclasts.

In experiments with stromal cell-free marrow cultures derived from marrow cells that were initially nonadherent, ADP at concentrations of 0.2 μM (on dentine disks) and 2 μM (on plastic) stimulated the formation of TRAP-positive multinuclear cells up 2.7-fold. ADP at 20 μM was without effect. In cultures on dentine disks, resorption was also stimulated 4.2-fold by ADP at 0.2 μM, in line with results from the mixed cell cultures described above. Numbers of mononuclear hemopoietic cells were unchanged by ADP or ATP treatment (control: 1879 ± 266, ADP 0.2 μM: 2075 ± 216, ADP 2 μM: 2269 ± 335, ADP 20 μM: 2269 ± 300, ATP 0.2 μM: 2653 ± 309, ATP 2 μM: 2218 ± 312).

$P2Y_1$ expression in bone cells

We investigated the expression of $P2Y_1$ receptor mRNA in sections of rat long bone and in cultured rat osteoclasts and osteoblasts, and the expression of the $P2Y_1$ receptor protein in cultured rat osteoclasts. In situ hybridization on long bone sections using $P2Y_1$ receptor probe revealed intense, specific localization over osteoblasts on bone surfaces and over chondrocytes in the growth plate in long bones (**Fig. 10*E***). Negative controls of serial sections performed by hybridizing in the presence of 100-fold excess of unlabeled probe significantly reduced the signal (Fig. 10*F*). On cultured rat osteoclasts, in situ hybridization and immunocytochemistry revealed intense specific localization of $P2Y_1$ receptor mRNA and protein (Fig. 10*A,C*), whereas negative controls showed significantly less staining (Fig. 10*B*). In addition, cultured primary calvarial rat osteoblasts showed strong specific staining for the $P2Y_1$ receptor mRNA by in situ hybridization (Fig. 10*D*), consistent with the osteoblast staining observed in long bone sections.

DISCUSSION

The role of extracellular nucleotides signaling through P2 receptors in the bone remodeling process is still not

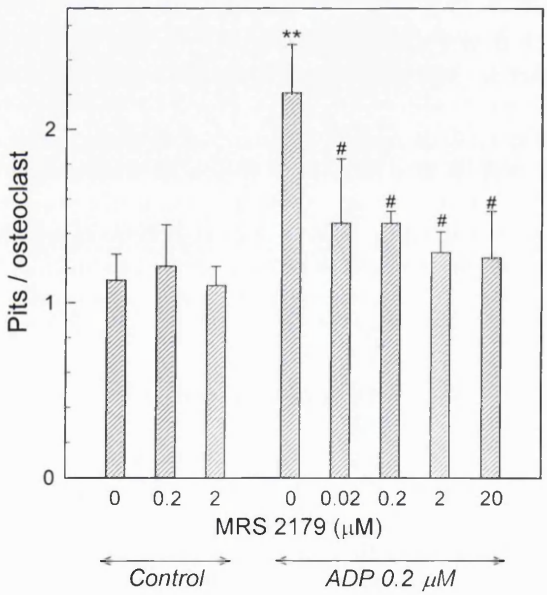

Figure 6. Inhibition of ADP-stimulated resorption pit formation by the $P2Y_1$ antagonist MRS 2179. ADP-induced stimulation of resorption pit formation at 0.2 μM was inhibited by MRS 2179 in a nontoxic manner. Control values were unchanged on addition of the antagonist. The number of mononuclear cells/disk was 4552 ± 572, 5134 ± 352, 4001 ± 439, 4311 ± 453, 3329 ± 57, 4572 ± 264, 4429 ± 345, and 4001 ± 413 and the number of osteoclasts/disk was 43.4 ± 6.8, 38 ± 3.5, 47.8 ± 6.7, 53.2 ± 2.8, 44 ± 4.7, 48.4 ± 4, 49 ± 4, and 47.2 ± 7 for each treatment group, respectively (all nonsignificant). Values are means ± SE (n = 5). Significantly different from control: $^{**}P < 0.01$. Significantly different from ADP at 0.2 μM: $^{\#}P < 0.05$.

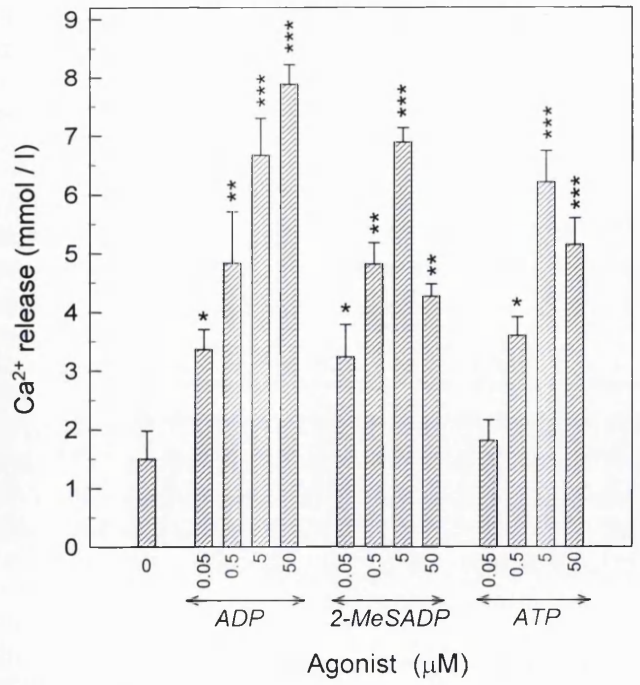

Figure 7. Stimulatory effect of ADP, 2-MeSADP, and ATP on Ca^{2+} release from mouse half-calvariae cultured for 3 days in acidified medium. ADP increased osteoclast-mediated Ca^{2+} release up to sixfold, with peak effects close to 5 μM. The $P2Y_1$ selective agonist 2-MeSADP was able to mimic the ADP effect. ADP appeared to be more potent than ATP. Values are means ± SE ($n = 5$). Significantly different from control: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

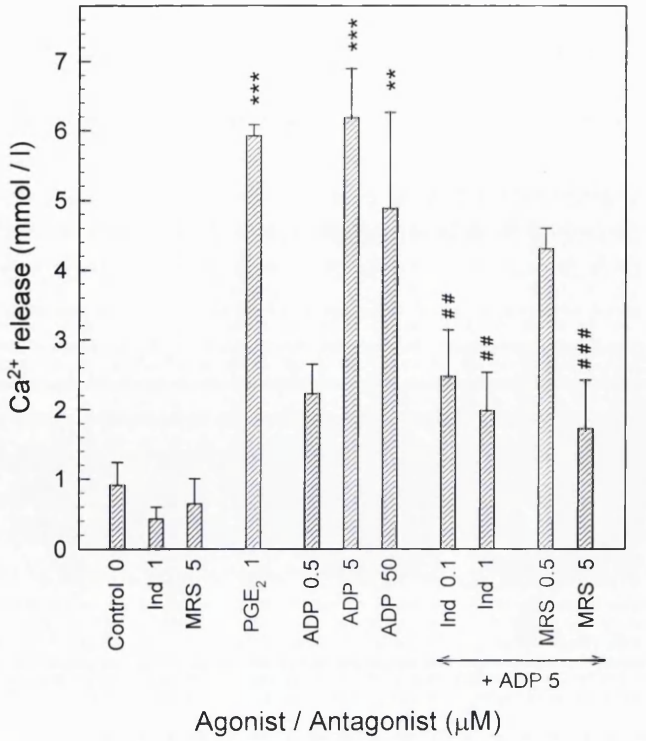

Figure 8. Inhibition of the stimulatory action of ADP on Ca^{2+} release from mouse half-calvariae by MRS 2179 (MRS) and indomethacin (Ind) and equivalence of the stimulatory action of ADP to the maximal effects of PGE_2 at 1 μM. Values are means ± SE ($n = 5$). Significantly different from control: $^{**}P < 0.01$; $^{***}P < 0.001$. Significantly different from ADP at 5 μM: $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$.

well understood. This is the first study that links a specific P2 receptor to a key functional action of an extracellular nucleotide on bone.

We report that extracellular ADP, a potent agonist at the G-protein-coupled $P2Y_1$ receptor, and 2-MeSADP, a selective $P2Y_1$ receptor agonist, are potent stimulators of bone resorption at nanomolar concentrations, as assessed by three independent methods in two different species. Further degradation products of ADP, namely AMP and adenosine, had no significant effect on bone resorption. This result indicates that ADP itself is the signaling agent and excludes the involvement of P1 receptors for adenosine and AMP. In addition, the stimulatory ADP effect could be blocked in a nontoxic manner by the compound MRS 2179, the most potent $P2Y_1$ receptor antagonist reported to date (18, 19). However, there is evidence that ADP can also act as an agonist at the $P2X_1$ receptor, which is a nonselective cation channel (20, 21). To discriminate between the two receptor subtypes, we used subtype-selective agonists and histochemistry and provided evidence that the stimulatory ADP effect is mediated via the $P2Y_1$ receptor rather than via the $P2X_1$ receptor. $P2Y_1$ receptor mRNA and protein were found to be expressed on both osteoclasts and osteoblasts. Using immunocytochemistry, we have recently studied the expression of $P2X_{1-7}$ receptors on bone cells and found no evidence for expression of the $P2X_1$ receptor on osteoclasts or osteoblasts (4). Extracellular ADP could therefore stimulate resorption directly, while signaling through the receptor expressed on mature osteoclasts, or indirectly via receptors expressed on osteoblasts, which in turn release activators of osteoclastic bone resorption, or both.

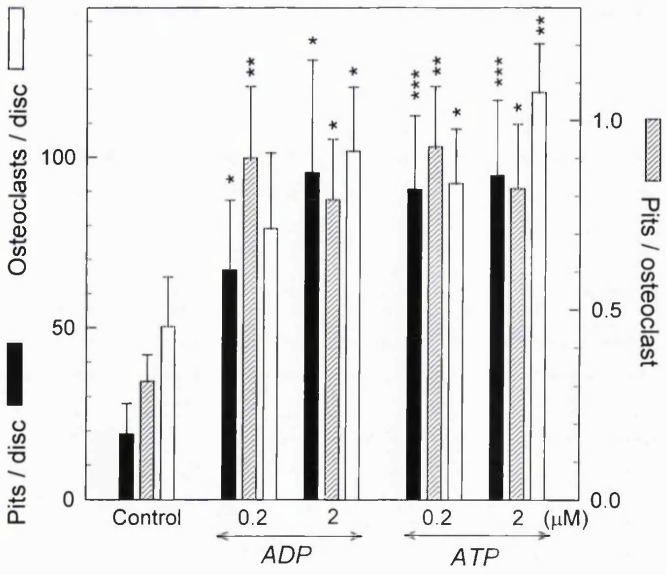

Figure 9. Effect of ADP and ATP on osteoclast formation and excavation of resorption pits in mouse marrow cultures maintained for 10 days on 5-mm dentine disks. Values are means ± SE ($n = 6$). Significantly different from control: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

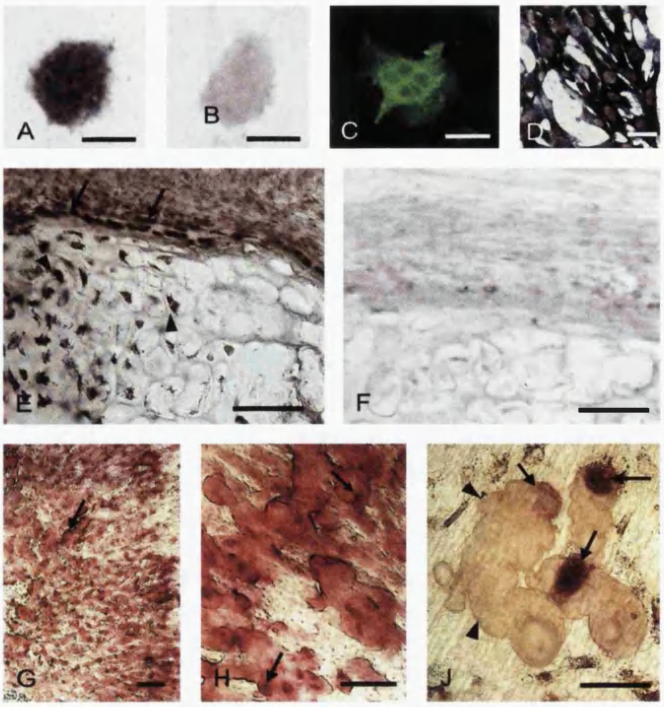

Figure 10. Specific in situ localization of $P2Y_1$ receptor probe on cultured rat osteoclasts (*A*) and cultured rat osteoblasts (*D*) and on osteoblasts (arrows) and chondrocytes (arrowheads) in frozen rat long bone section (*E*). Localization was reduced greatly in control preparations hybridized in the presence of excess unlabeled probe (*B, F*). Immunocytochemistry with $P2Y_1$ antibody revealed strong specific staining in cultured rat osteoclasts (*C*). Also shown are representative micrographs of whole-mount mouse half-calvariae stained to demonstrate TRAP after 72 h of culture stimulated with ADP at 5 μM (*G*) and 50 μM (*H*); arrows point to examples of TRAP-positive osteoclasts. *J*) A representative photomicrograph of a 26-h culture of rat long bone cells on dentine disks shows ADP-stimulated, TRAP-stained multinuclear osteoclasts (arrows) with corresponding resorption pits (arrowheads), visualized by reflective light microscopy. Scale bars = 20 μm (*A, B, C*), 40 μm (*J*), 50 μm (*D, E, F*), and 200 μm (*G, H*).

We observed the osteolytic effects of ADP and 2-MeSADP at concentrations as low as 20 nM; effects of nucleotides on osteoclasts at such low concentrations have not been reported before. Two earlier studies investigated the actions of ADP at much higher concentrations on osteoclasts: ADP at 50 μM has been shown to increase intracellular Ca^{2+} levels (8) and ADP at 100 μM induced an intracellular pH decrease in rabbit osteoclasts, probably by enhancing the Cl^-/HCO_3^- exchange across the osteoclast cell membrane (10). However, the present results indicate that ADP exerts its major functional action on osteoclasts at concentrations ~1000-fold lower than in these previous studies and that ADP at concentrations between 20 and 200 μM is without effect.

Our evidence for expression of the $P2Y_1$ receptor on osteoclasts appears to be consistent with recent electrophysiological data showing that 10–50 μM ATP, which is a partial agonist at the $P2Y_1$ receptor, can activate a K^+-selective outward current in rat osteoclasts that is dependent on P2Y receptor-mediated Ca^{2+} release from intracellular stores (9). A more recent study from the same group reported that the ADP analog ADPβS at 100 μM also elicits a Ca^{2+}-dependent K^+ current in rabbit osteoclasts, consistent with the presence of the $P2Y_1$ receptor (22). Several studies have described effects of extracellular ADP on osteoblasts, in accordance with the histochemical evidence of $P2Y_1$ receptor expression on osteoblasts reported in this study. ADP and 2-MeSADP in the submicromolar range caused a transient increase in intracellular Ca^{2+} in rat osteoblast-like UMR-106 cells (23, 24). This result is consistent with our findings that ADP and 2-MeSADP act at low concentrations. However, as with osteoclasts, the majority of studies on osteoblasts investigated the actions of ADP at concentrations of 10 μM and higher (5, 25, 26).

Bone resorption requires both the formation of mature osteoclasts from hematopoietic progenitors and their subsequent activation to form resorption pits. The present data suggest that ADP stimulates both formation and activation of osteoclasts, resulting in striking increases in resorption in 10-day mouse marrow cultures. However, the observed positive effect of ADP on osteoclast formation in mouse marrow cultures could also be due to a prolongation of osteoclast life span, in addition to enhanced recruitment of osteoclasts from progenitors. Our results obtained using cultures of nonadherent, stromal cell-free marrow cells suggest that ADP can act directly on osteoclasts and their precursors, in addition to any effects that may be mediated via stromal cells or osteoblasts.

Perhaps the most striking effects of ADP, 2-MeSADP, and also ATP were observed in mouse calvarial bone organ cultures. We found that ADP was roughly as potent as PGE_2, a reference osteolytic agent for this system, in activating osteoclastic resorption. However, resorption stimulated by ADP was blocked by the cyclooxygenase inhibitor indomethacin, suggesting a requirement for endogenous prostaglandin synthesis in this system, as is the case for other osteolytic agents such as protons (27, 28). It has long been known that adenine nucleotides can induce prostaglandin biosynthesis (29).

A previous study showed that ATP at low concentrations (0.2–2 μM) is a potent stimulator of the activation and formation of rodent osteoclasts (3), but this effect was not related to a specific P2 receptor subtype. The stimulatory effect on mature osteoclasts was evident only at low pH (~6.9), suggesting the possible involvement of the $P2X_2$ receptor, the only P2 receptor subtype that needs extracellular acidification to show its full sensitivity to ATP (30, 31). However, we show here that a similarly low pH is also required for ADP to show its full stimulatory effects on osteoclastic resorption. This result is also consistent with studies showing that proresorptive effects of 1,25-dihydroxyvitamin D_3, parathyroid hormone, PGE_2, and RANKL are acid-dependent (12, 32, 33). These findings point to a general dependency of osteolytic agents on slight local acidification, confirming that osteoclasts need to be "switched on" by low pH to resorb bone (11, 34).

To date the majority of studies on the effects of extracellular nucleotides on bone cells have focused on the actions of ATP, which is the nucleotide with the widest spectrum of biological activity and an agonist at all P2 receptor subtypes. On the basis of our findings that ADP appears to be somewhat more potent than ATP in stimulating mature osteoclasts, we suggest that, for several reasons, the $P2Y_1$ receptor may also be responsible for at least part of the stimulatory effect observed with ATP. First, ATP has been considered a potent agonist of the $P2Y_1$ receptor, based on studies using the cloned chick receptor (35). However, recent studies suggest that pure ATP is in contrast a weak competitive antagonist at the mammalian $P2Y_1$ receptor and that ATP actions were apparent only because of ADP contamination present or newly formed by ecto-ATPases (36, 37). This issue remains highly controversial; for example, potent ATP agonism at the $P2Y_1$ receptor has now been demonstrated on mammalian neurons (38) and, in the case of 2-MeSATP, an ATP analog, also on rat hepatocytes (39). Second, ATP could rapidly be hydrolyzed to ADP via ectonucleotidases present in the bone environment. Several studies have shown that ATP is rapidly hydrolyzed once present in the extracellular environment. For example, Sistare et al. showed that within 2 min approximately 25% of a 10 μM ATP solution is metabolized into ADP by rat osteoblast-like cells (24). Similarly, white blood cells can quickly metabolize 10 μM ATP into ADP and into further degradation products, including AMP (40). The presence of ectonucleotidases has also recently been demonstrated in bone marrow (41). Third, commercial ATP samples are often contaminated with traces of about 1–5% ADP; thus, an ATP concentration of 2 μM would result in up to 0.02–0.1 μM ADP. As we report here, these concentrations are sufficient to exert a peak stimulatory effect on bone resorption.

There are a number of potential sources for extracellular nucleotides in the bone environment: ATP is a ubiquitous intracellular constituent (2–5 mM inside the cell), and thus any cell could potentially serve as a source of extracellular ATP. ATP can be released into the extracellular space from intact cells by vesicular exocytosis (e.g., from nerve endings), or a channel-like pathway (e.g., ABC-proteins), but also from damaged cells and during tissue injury. Platelets can also release ADP itself. The granules in platelets contain up to 40 nM ATP and ADP/mg protein, and plasma concentrations of ATP/ADP of 20 μM have been measured after platelet activation (42). In addition, osteoblasts have been shown to release ATP under shear stress conditions (43).

Our finding that ADP, signaling through the $P2Y_1$ receptor, is a powerful activator of osteoclasts and may also induce recruitment of osteoclasts could be of relevance to several pathophysiological conditions that lead to increased bone resorption. First, inflammatory conditions such as rheumatoid arthritis lead to sustained systemic and localized bone loss, probably because of increased osteoclast activity. We show that ADP is as powerfully proresorptive as is PGE_2, an osteolytic agent that is also implicated in inflammatory bone loss (44). Release of nucleotides is increased under inflammatory conditions, suggesting an early role of extracellular nucleotides in the inflammatory process (45); thus, ADP could mediate a component of inflammatory bone loss. In addition, platelets play a key role in inflammation by being induced to release their granule contents, including adenine nucleotides (46).

A second pathological condition in which ADP-mediated bone resorption could play a major role is the bone loss associated with cancer metastases. Tumor cells are important sources of extracellular ATP (47); thus, localized ATP/ADP release could recruit and stimulate osteoclasts. It is important to note that inflamed and cancerous tissues are also characterized by low extracellular pH, which would facilitate the osteolytic action of ADP and ATP.

Nucleotides may also be implicated in a wider spectrum of connective tissue destruction. For example, net loss of the cartilage extracellular matrix occurs in all forms of arthritis, and extracellular ATP has been shown to stimulate cartilage resorption by acting on P2 receptors (48, 49).

In conclusion, our study points to a fundamental new mechanism for the local modulation of bone resorption by extracellular nucleotides at nanomolar concentrations. FJ

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Sequential Expression of Three Receptor Subtypes for Extracellular ATP in Developing Rat Skeletal Muscle

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ABSTRACT **In this study, we investigated the expression of the P2X receptor subtypes ($P2X_{1-7}$) during the development of skeletal muscle and in relation to acetylcholine receptors in the rat embryo and pup. By using immunohistochemistry, we showed that three receptor subtypes, $P2X_2$, $P2X_5$, and $P2X_6$, were expressed in developing skeletal muscle. The timing and pattern of receptor expression seemed to be precisely regulated. $P2X_2$, $P2X_5$, and $P2X_6$ were expressed in a sequential manner, which was consistent for all regional muscles tested (intercostal, paravertebral, and lower limb): $P2X_5$ expression appeared first (E15–E18) followed by $P2X_6$ (E16–E18), and finally $P2X_2$ (E18–adult). At no developmental stage did we observe colocalization of $P2X_2$ and acetylcholine receptors. In the case of $P2X_2$ and $P2X_6$, immunoreactivity was found to be widespread, immunopositive cells being apparent throughout the muscle. However, staining for $P2X_5$, both at the beginning and end of expression, was restricted to regions of muscle close to the myotendinous junctions. Because the timing of receptor expression is closely related to key events in skeletal muscle development, notably the generation of secondary myotubes and the redistribution of acetylcholine receptors, it is possible that ATP-signaling by means of P2X receptors could be involved in these processes.**

Key words: P2X receptors; ATP; skeletal muscle development; rat embryo; myotube formation; acetylcholine receptors

INTRODUCTION

In recent years, the biological effects of extracellular purine nucleotides acting through P2 receptors have been studied in many cell and tissue types and ATP is now recognized as an important messenger molecule in cell–cell communication (Burnstock, 1997). Thus far, attention has been largely focused on the role of purinergic signaling in mediating changes in short-term cellular activity. However, there is growing evidence that purinergic signaling may also play a role in long-term cellular communication, including cell proliferation, differentiation, and apoptosis (Neary et al., 1996; Abbracchio and Burnstock, 1998). These processes are central to embryonic development, and the specific functions of purine receptor subtypes during skeletal muscle development are the focus of interest in this study.

P2 receptors are classified into two main families, P2X and P2Y, based on molecular structure, transduction mechanisms, and pharmacological properties (Ralevic and Burnstock, 1998). The P2Y receptors are G protein–coupled receptors, which act principally by activating phospholipase C, leading to the formation of inositol 1,4,5-trisphophate and mobilization of intracellular Ca^{2+}. In contrast, P2X receptors are a ligand-gated ion channel family, and activation of these receptors by extracellular ATP elicits a flow of cations (Na^+, K^+, and Ca^{2+}) across the plasma membrane. To date, seven P2X receptor subunits ($P2X_{1-7}$) capable of assembling homo- or heteromultimeric receptors (Torres et al., 1999) have been cloned from mammalian species.

The functional importance of these receptor subtypes is yet to be fully understood, particularly with regard to their potential trophic actions. The $P2X_5$ receptor subunit has been shown to be expressed in the proliferating and differentiating cell layers of stratified squamous epithelial tissues (Gröschel-Stewart et al., 1999) suggesting that ATP signaling by means of the $P2X_5$ receptor may play a role in these processes. Conversely, the $P2X_7$ receptor subunit has been strongly linked to apoptosis (Surprenant et al., 1996; Collo et al., 1997; Gröschel-Stewart et al., 1999). There is increasing evidence to suggest that $P2X_7$ activation can induce apoptosis in several cell types and that this process is dependent on the caspase signaling cascade (Coutinho Silva et al., 1999; Ferrari et al., 1999).

Responses to ATP have been demonstrated in embryonic tissues, notably skeletal muscle. By using the patch clamp recording technique, a transmitter-like action of ATP on the cell membranes of myoblasts and myotubes cultured from 12-day chick embryos was first demonstrated by Kolb and Wakelam (1983). These results were confirmed by Hume and Thomas (Hume and

M. Ryten and A. Hoebertz contributed equally to the work presented in this study.

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Thomas, 1988; Thomas and Hume, 1990a,b, 1993; Thomas et al., 1991), who showed that ATP elicits a potent, P2-dependent depolarizing action on cultured chick myotubes. Similar responses, characteristic of P2 receptors, have been demonstrated in the mouse myoblast cell line, C2C12 (Henning et al., 1993; Henning, 1997). In chick muscle, ATP-responses were also shown to be developmentally regulated (Wells et al., 1995). ATP-elicited contractions were most apparent in early development (embryonic day 6 [E6]) and were absent by embryonic day 17. More recently, immunohistochemistry has been used to describe the developmentally regulated expression pattern of two members of the P2X family, $P2X_5$ and $P2X_6$, in the skeletal muscle of the chick embryo (Meyer et al., 1999).

However, the role of ATP and the identity of the P2X receptor subtypes involved in the development of mammalian skeletal muscle are still largely undefined. In this study, we use immunohistochemistry to investigate the expression of P2X receptors and their relationship to acetylcholine receptors (AChRs) in developing rat skeletal muscle. By using polyclonal antibodies raised against the seven different rat P2X receptor peptides (Oglesby et al., 1999), we demonstrate the timing and localization of receptor expression. In summary, we show the sequential and developmental expression of three receptor subtypes, $P2X_2$, $P2X_5$, and $P2X_6$, perhaps indicating that ATP acting by means of P2X receptors may play a key role in skeletal muscle formation.

RESULTS

$P2X_2$, $P2X_5$, and $P2X_6$ Immunoreactivity in Developing Rat Skeletal Muscle

Staining procedures were carried out for all seven of the P2X receptors at all embryonic stages; however, we found immunoreactivity in developing skeletal muscle only for $P2X_2$, $P2X_5$, and $P2X_6$. Control experiments, performed by preabsorbing the antibodies with the corresponding peptides, showed no immunostaining, confirming the specificity of our findings. The expression of these receptors was followed from E12 to postnatal day (P) 21 in various muscles, including the intercostal, paravertebral, and limb muscles. Results from the muscle groups examined showed that the timing of receptor expression was subtype-specific.

Before E15, there was no immunoreactivity for any of the P2X receptors in the developing muscle. Immunoreactivity for $P2X_5$, but not $P2X_2$ or $P2X_6$, began at E15. Staining with $P2X_5$ was restricted to a small number of cells at the ends of the muscles, close to the myotendinous junctions (Fig. 1A). Immunohistochemistry with an antibody against skeletal myosin on sequential sections from the same embryo confirmed that the staining was confined to the areas of developing muscle (Fig. 1B) described above. This skeletal marker was used throughout this study to identify areas of future muscle. Immunoreactivity for $P2X_5$ strengthened during development; by E16 staining was more widespread within muscles already shown to have positive immunoreactivity for $P2X_5$ and began to appear in muscles that had previously been negative (Fig. 1C,D). This stage also marked the appearance of $P2X_6$ immunoreactivity within skeletal muscle. $P2X_6$ staining was strong and extensive, immunopositive cells could be seen throughout muscle blocks and in all muscle groups tested (Fig. 2A). Immunoreactivity for $P2X_5$ and $P2X_6$ was still apparent at E18 (Figs. 2B,C,D, 3), although in the case of $P2X_5$, it had become restricted to the ends of developing muscle fibers (Fig. 6A). By E20, immunoreactivity for both $P2X_5$ and $P2X_6$ within the skeletal muscle had disappeared. However, it was at these final stages of prenatal development, E18 onward, that $P2X_2$ expression began to appear. Initially $P2X_2$ was expressed most strongly in the intercostal (Fig. 4C,D) and paravertebral muscles (Fig. 4B) and was almost entirely absent in the limb musculature (Fig. 4A). $P2X_2$ expression was most marked in the muscles of the lower limb at E20 (Fig. 5A). Immunoreactivity for $P2X_2$ continued for at least 1 week postnatally (Fig. 5B) but had reduced in strength to near adult expression by P14 (Fig. 5C,D).

In summary, we found three P2X receptors to be expressed in developing skeletal muscle, $P2X_2$, $P2X_5$, and $P2X_6$. These receptors were expressed sequentially, $P2X_5$ being the first receptor to be expressed (E15) followed by $P2X_6$ (E16) and finally $P2X_2$ (E18). This sequence of receptor expression was maintained for all muscles tested. Expression of $P2X_5$ and $P2X_6$ was restricted to in utero development, whereas staining for $P2X_2$ continued after birth. Immunoreactivity for the $P2X_5$ and $P2X_6$ receptor subtypes was most transient, E15-E18 and E16-E18, respectively, whereas $P2X_2$ expression was more sustained, being reduced to near adult expression in the second postnatal week (E18-P14).

Double-Labeling for $P2X_2$ and Acetylcholine Receptors

Double-labeling experiments for $P2X_2$ and AChRs, localized with α-bungarotoxin-Texas Red, were carried out at E18, E20, P7, P14, P21, and adult. As described above, $P2X_2$ was expressed from E18 to P14 (Fig. 5). AChR staining was restricted to large membrane clusters at the center of the muscle fibers, i.e., prospective endplate sites (Fig. 6B–D). Consequently, only a subset of clusters is seen in any one section. At no stage did we observe colocalization of $P2X_2$ and AchR clusters (Fig. 6B–D). This finding included adult muscle where endplates (positive for α-bungarotoxin) as well as extrasynaptic regions were $P2X_2$ negative.

$P2X_5$ Immunoreactivity in Cells Located Between Muscle Fibers

Although $P2X_5$ expression in developing skeletal muscle was restricted to the prenatal period, $P2X_5$-immunopositive cells were apparent within the muscle mass at P7. These cells appeared to be in between

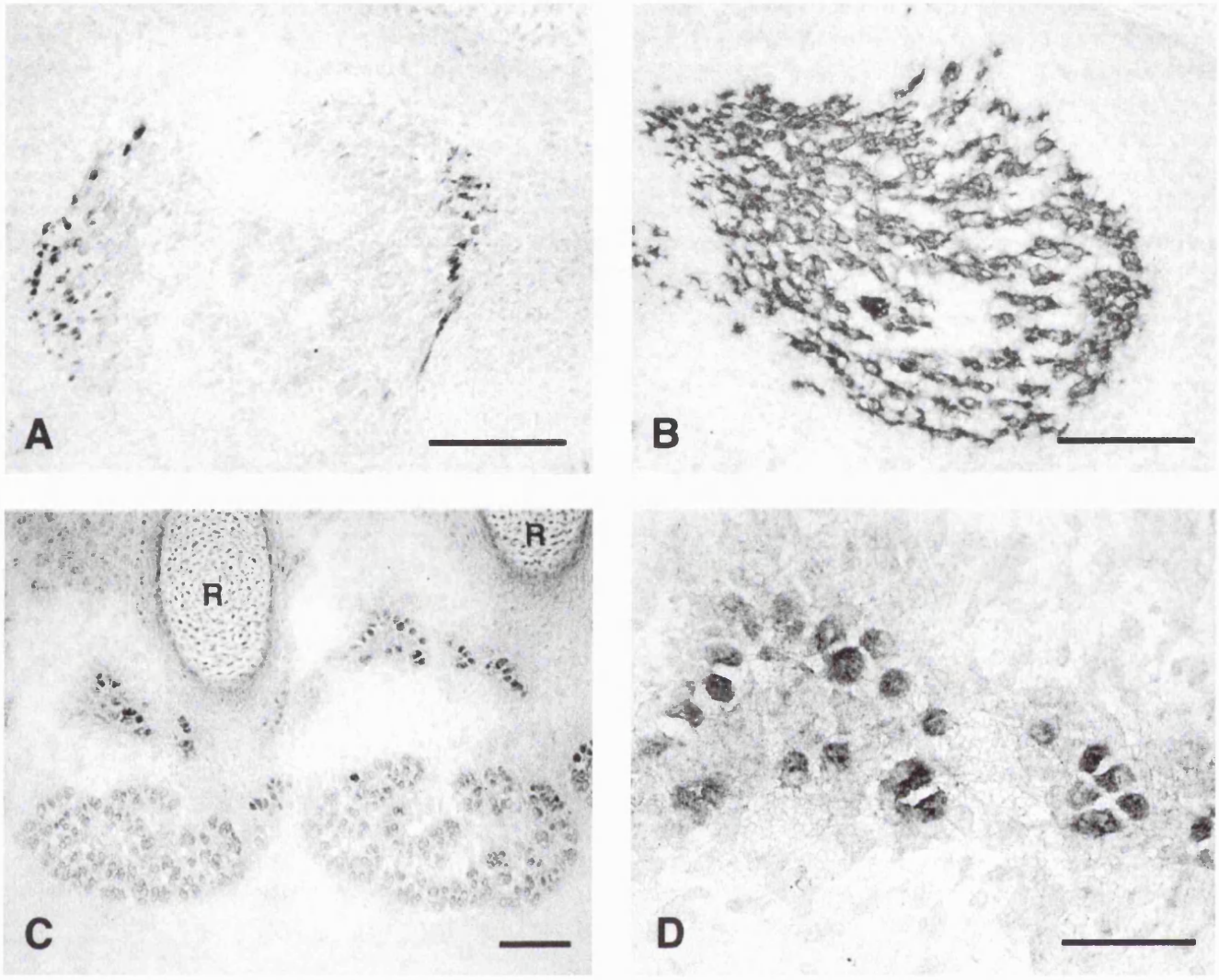


Fig. 1. Expression of $P2X_5$ in rat skeletal muscle in longitudinal sections of embryonic day (E) 15 and E16 embryos. **A:** Immunoreactivity for $P2X_5$ in a muscle block of the developing lower limb at E15 was restricted to a small number of cells at the ends of the muscles close to the myotendinous junctions. **B:** Sequential section showing skeletal muscle myosin staining. **C,D:** $P2X_5$ immunoreactivity, previously absent, began to appear in paravertebral muscles at E16, shown at higher magnification in (D). R, rib cartilage. Scale bars = 100 μm in A–D.

developing muscle fibers. This was confirmed by using double labeling with anti-skeletal muscle myosin (anti-PM) and anti-$P2X_5$, which showed a complete absence of overlapping expression (Fig. 7A). By P21, $P2X_5$ staining both within and outside muscle fibers had largely disappeared. To identify the $P2X_5$-positive cells present at P7, further double-labeling experiments were performed by using anti-neurofilament 200 or anti-smooth muscle actin. In both cases, there was limited coexpression with many cells remaining positive only for $P2X_5$ (Fig. 7B-D). This finding would suggest that $P2X_5$ stained a mixed population of cells at P7 including nerves, smooth muscle cells, and probably endothelial cells.

DISCUSSION

ATP, coreleased with acetylcholine, has been shown to modulate the developing neuromuscular synapses of *Xenopus* embryos (Fu and Poo, 1991; Fu, 1995) and to have direct transmitter-like actions on developing chick skeletal muscle (Kolb and Wakelam, 1983). In chick, ATP has potent P2-dependent, depolarizing actions on myoblasts in vitro (Hume and Honig, 1986) and causes muscular contraction in vivo (Wells et al., 1995). Furthermore, Wells et al. (1995) demonstrate that sensitivity to ATP is developmentally regulated and returns in denervated skeletal muscle. Experiments on the mouse myoblast cell line, C2C12, showing P2-dependent responses, suggest that ATP may also be

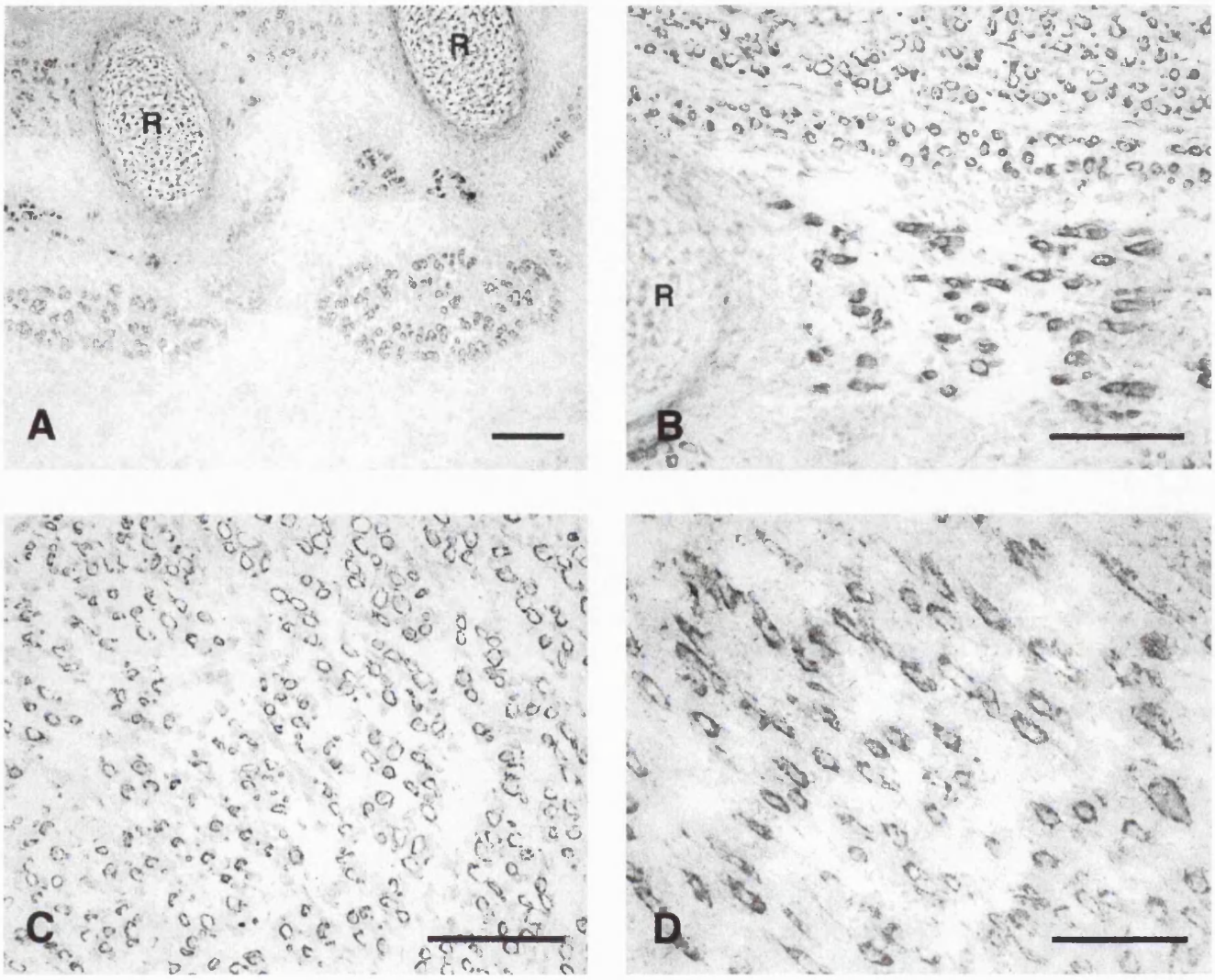


Fig. 2. Expression of $P2X_6$ in rat skeletal muscle at embryonic day (E)16 and E18. **A:** At E16, $P2X_6$ immunoreactivity appeared in all muscle groups tested, shown here in a longitudinal section of the embryo, in the intercostal and paravertebral muscles. **B–D:** Strong positive $P2X_6$ membrane staining of all muscle cells remained until E18, shown in an oblique section of the external intercostal muscles (B), a transverse section of the paravertebral muscles (C) and an oblique section of the lower limb muscles (D) of E18 embryos. R, rib cartilage. Scale bars = 100 μm in A–D.

an important signaling molecule in mammalian skeletal muscle development (Henning et al., 1993).

However, this work was performed before knowledge of the existence, let alone the properties of the P2X receptor subtypes (Ralevic and Burnstock, 1998). Our findings suggest that expression of the P2X receptors could account for the ATP sensitivity of developing skeletal muscle. By using immunohistochemistry, we show developmentally regulated expression of three P2X receptor subtypes, $P2X_2$, $P2X_5$, and $P2X_6$, in rat skeletal muscle. These receptors are expressed in a sequential manner, which was consistent for all muscles tested: $P2X_5$ was expressed first (E15-E18), followed by $P2X_6$ (E16-E18), and finally $P2X_2$ (E18-P14).

The temporal and spatial overlap in the expression of $P2X_2$, $P2X_5$, and $P2X_6$ receptor subunits, which we observed, raises the possibility that both homomeric and heteromeric receptors may be present in developing skeletal muscle. In sensory neurones of the nodose ganglion, $P2X_2$ and $P2X_3$ subunits are coexpressed (Vulchanova et al., 1997). The characteristics of the P2X receptors present on these cells can only be accounted for by the presence of a $P2X_{2/3}$ heteromeric receptor (Lewis et al., 1995). Biochemical studies have shown that 11 heteromeric receptors can be formed by pairwise combination of P2X subunits (Torres et al., 1999). However, of these only $P2X_{2/3}$, $P2X_{1/5}$, $P2X_{4/6}$, and $P2X_{2/6}$ have been demonstrated in functional studies (Lewis et al., 1995; Torres et al., 1998; Lê et al., 1998; King et al., 2000). Further double-labeling studies will be required to determine more closely the extent of P2X receptor coexpression in developing skele-

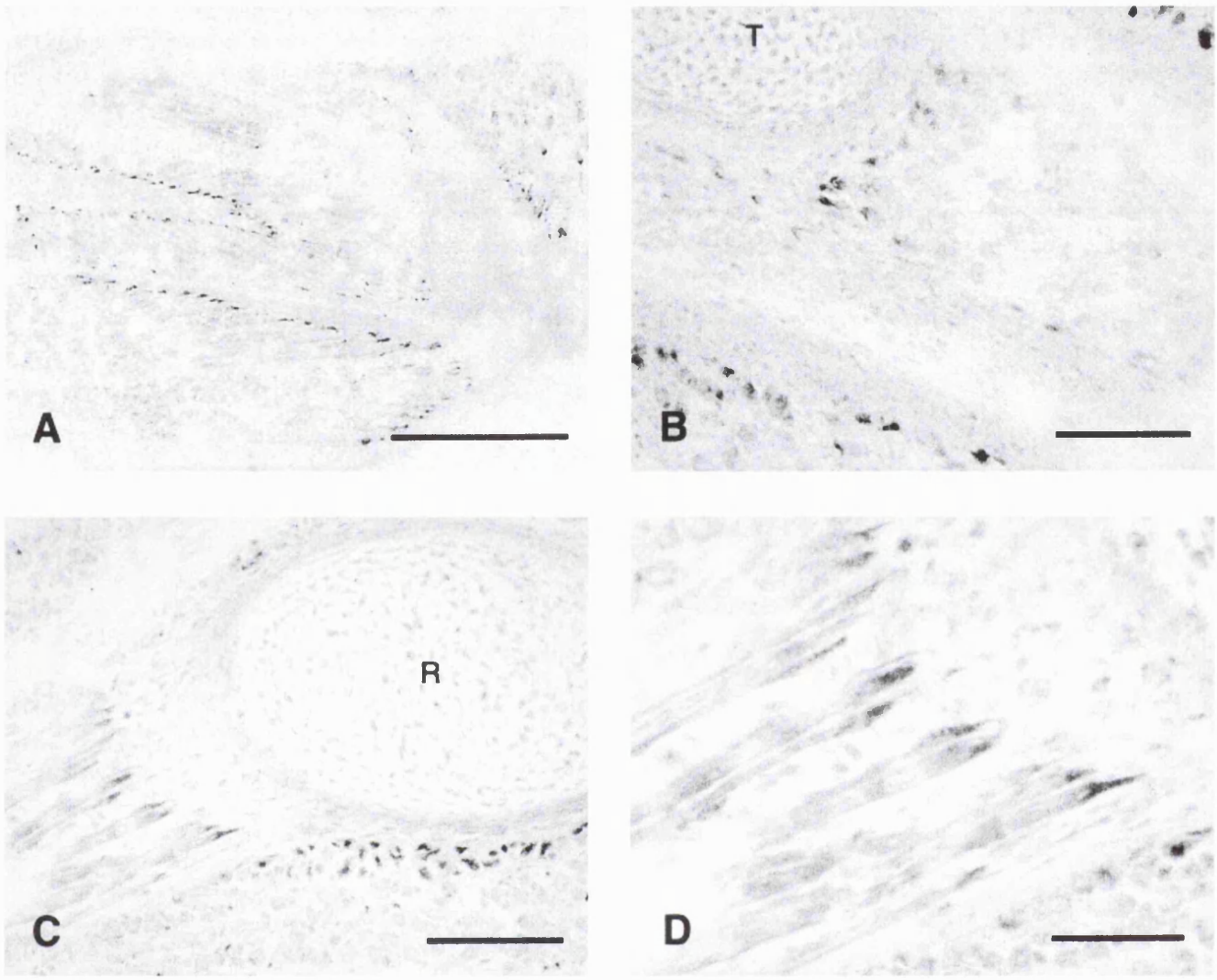


Fig. 3. Expression of $P2X_5$ in rat skeletal muscle at embryonic day (E) 18. At E18, $P2X_5$ immunoreactivity became restricted to cells at the end of the muscles, close to the developing cartilage/bones, shown in longitudinal sections of the lower limb (**A,B**) and external intercostal muscles (**C,D**). T, tibial cartilage; R, rib cartilage. Scale bars = 500 μm in A, 100 μm in B,C, 50 μm in D.

tal muscle. This issue is highlighted by our findings at P7, demonstrating the expression of both $P2X_2$ and $P2X_5$, but whereas $P2X_2$ stains skeletal muscle, $P2X_5$ is expressed on a range of other cell types, including smooth muscle and nerve fibers. However, it is worth noting that the study of Torres et al. (1999) suggests that $P2X_6$ does not form a homomeric receptor, suggesting that any functional role will be in coassembly with either $P2X_2$ or $P2X_5$ subunits.

The developmentally regulated expression of the P2X receptors suggests that the channels formed are of functional significance. The timing of receptor expression seems to be closely related to key events in skeletal muscle development (Fig. 8), notably secondary myotube formation and the establishment of mature neuromuscular junctions. We suggest that ATP signaling by means of $P2X_5$ and $P2X_6$ could be involved in the former event, whereas $P2X_2$ could be related to the latter.

Skeletal muscle cells are formed in two stages: primary myotubes develop first, and are followed after a delay by secondary myotubes, which will form the majority of muscle fibers in the adult tissue (Kelly and Zacks, 1969; Harris, 1981; Ontell and Kozeka, 1984; Ross et al., 1987). Secondary myotubes develop from the middle of the muscle in close association with the neuromuscular junctions of guiding primary myotubes (Duxson et al., 1989). They extend by the asynchronous fusion of myoblasts at their ends (Zhang and McLennan, 1995) to finally attach to the muscle tendons (Duxson and Usson, 1989). Immunohistochemical data from our study suggest the involvement of P2X receptors in

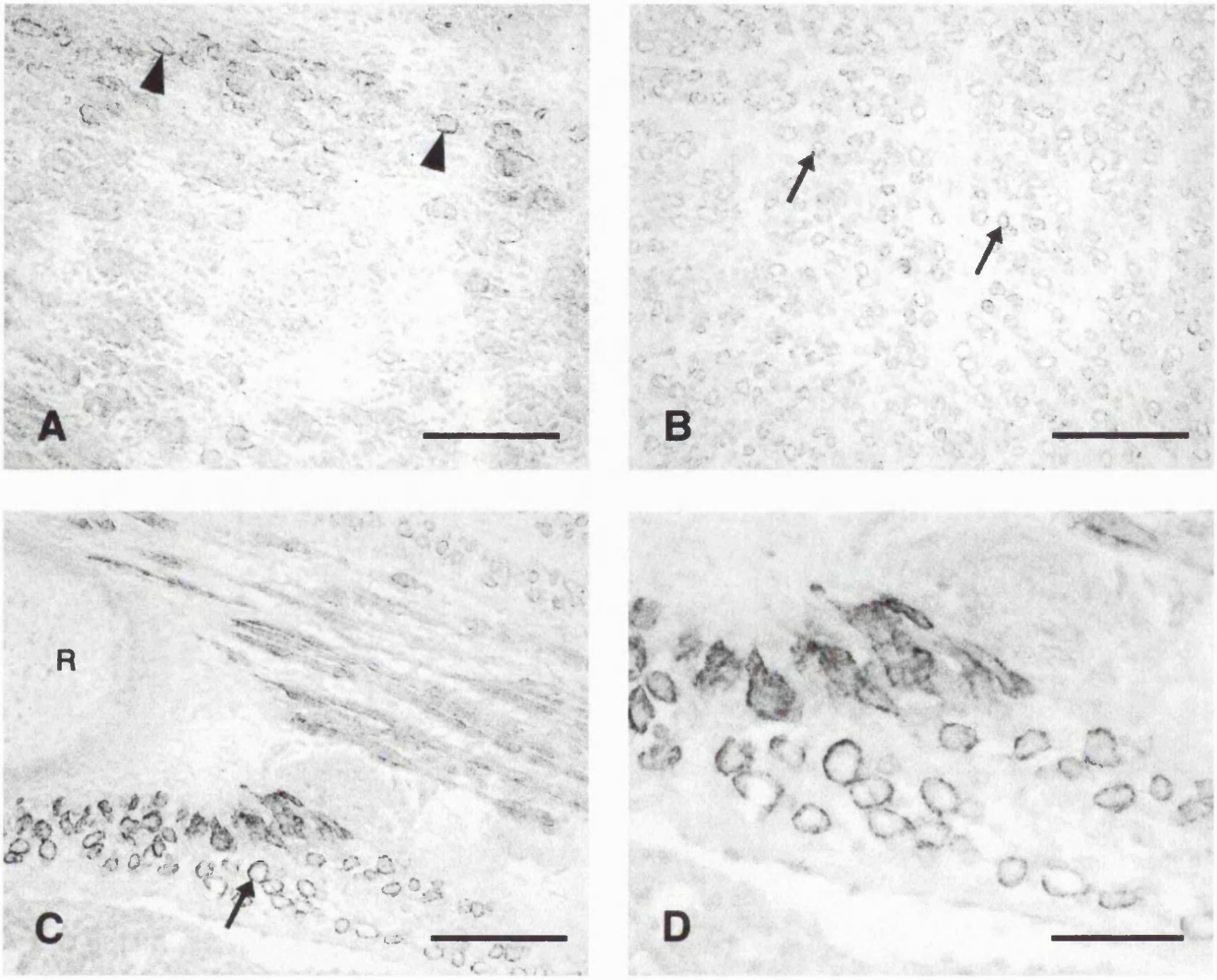


Fig. 4. Expression of $P2X_2$ in rat skeletal muscle at embryonic day (E) 18. **A:** In the lower limb muscle, shown in oblique section, $P2X_2$ immunoreactivity was restricted to a small number of cells (arrowheads). **B–D:** $P2X_2$ immunoreactivity, which was confined to cell membranes, was more widespread in the paravertebral muscles (B) and strongest in the intercostal muscles (C,D), shown in transverse section. R, rib cartilage. Scale bars = 100 μm in A–C, 50 μm in D.

secondary myotube formation. We detected no immunoreactivity for $P2X_5$ (the first receptor to be expressed) during at least the early stages of primary myotube formation (Ross et al., 1987). However, P2X receptors could be involved in the formation of secondary myotubes, which begins at E16 in the intercostal muscles (Kelly and Zacks, 1969), precisely the same time at which there is strongest immunoreactivity for $P2X_5$ and $P2X_6$.

Staining for $P2X_2$ begins in the intercostal muscles at E18, which is the peak of polyneuronal innervation (Dennis et al., 1981). It is well established that nerve-induced activity plays a crucial role in regulating the expression and distribution of receptors and channels on the muscle membrane, notably the acetylcholine receptor. Whereas developing myotubes express clusters of AChRs throughout the membrane, adult skeletal muscle is characterized by the striking concentration of receptors in the postsynaptic membrane and their virtual absence from the extrasynaptic membrane. This reorganization is in part activity-dependent (Vrbova et al., 1995; Sanes and Lichtman, 1999). In the case of rat intercostal muscles, synaptic inputs are first detected at E15 and produce clustering of AChRs by E16 (Dennis et al., 1981). This finding is confirmed in our results which show acetylcholine clusters in prospective endplate regions. However, on the basis of intracellular recordings in response to acetylcholine application (generally considered to be more sensitive than detection by α-bungarotoxin) the extrajunctional AChR density, particularly in regions close to the myotendinous junction, remains high after birth

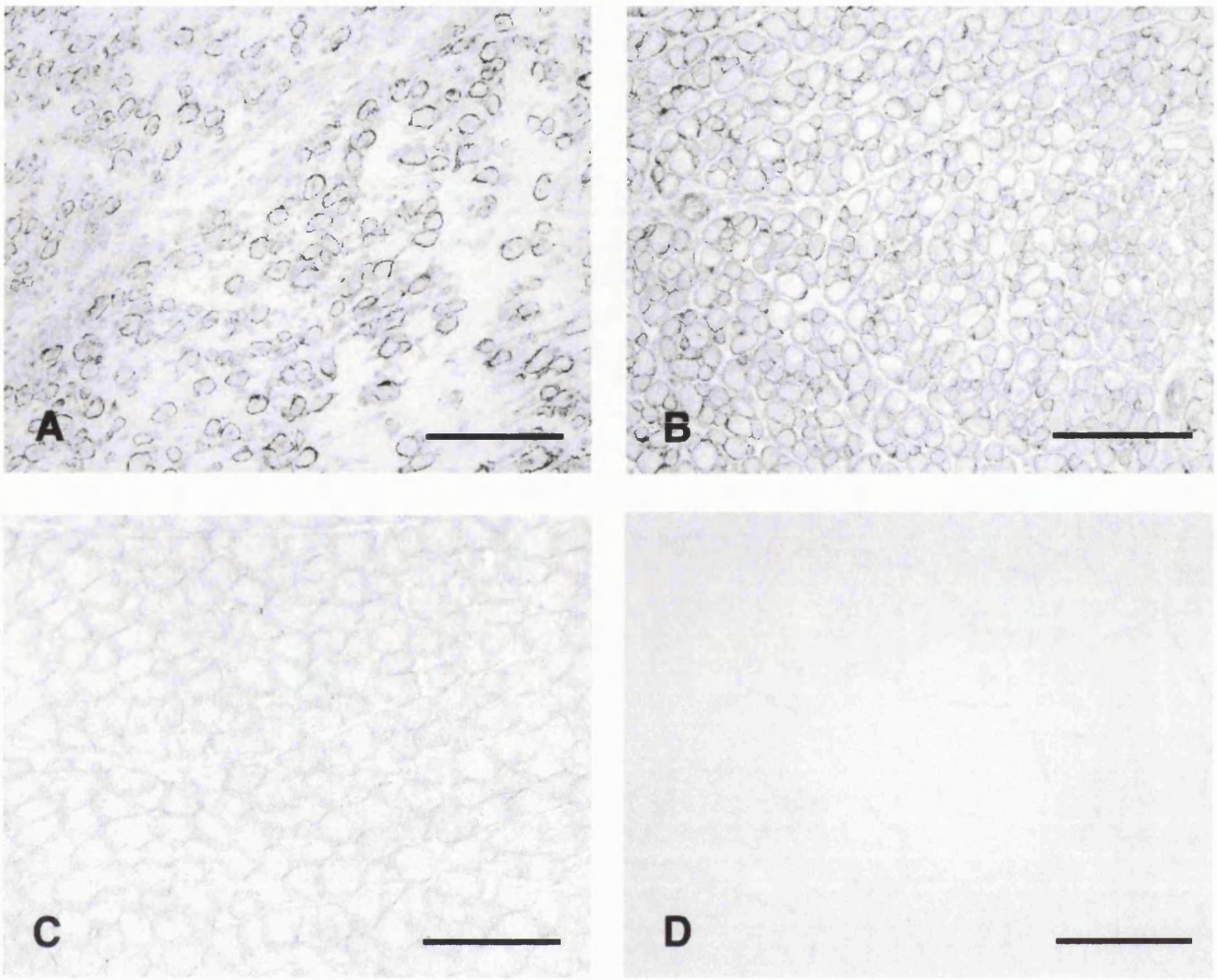


Fig. 5. Expression of $P2X_2$ in rat skeletal muscle at embryonic day (E) 20 and postnatally. **A:** At E20, strong $P2X_2$ immunoreactivity could be seen in the membranes of lower limb muscle cells. **B:** Strong immunostaining of lower limb muscles with $P2X_2$ remained postnatally at P7. **C:** $P2X_2$ immunoreactivity reduced in strength at P14. **D:** In adult tibialis anterior rat muscle immunostaining for $P2X_2$ was almost absent. All transverse sections. Scale bars = 100 μm in A–D.

and only declines during the first postnatal week (Diamond and Miledi, 1962). Changes in the expression of the P2X receptor subtypes, particularly $P2X_2$, mirrors and may even contribute to this process because $P2X_2$ receptor expression is also maintained during the first postnatal week and declines subsequently. In our dual-labeling experiments, extrajunctional AChRs were probably not visible due to the low detection sensitivity of α-bungarotoxin compared with intracellular recording. Interestingly, no expression of $P2X_2$ has been reported in chick skeletal muscle (Meyer et al., 1999). This may reflect differences in the development of mammalian as opposed to avian neuromuscular junctions.

The surprising abundance of these receptors raises the issue of what the potential sources of ATP in this developing system might be. It is well established that ATP is stored within the synaptic vesicles of presynaptic nerve terminals and is coreleased with acetylcholine (Silinsky and Hubbard, 1973). Muscles are also known to secrete substantial amounts of ATP in response to electrical activity (Landmesser and Morris, 1975). However, there are other important nerve-independent sources of extracellular ATP. Osteoblasts and chondrocytes, which are obviously closely associated with developing skeletal muscle, have been shown to be capable of releasing ATP (Bowler et al., 1998; Lloyd et al., 1999) and could be associated with the marked expression of $P2X_5$ at myotendinous junctions.

In conclusion, we show for the first time that three members of the P2X family, $P2X_2$, $P2X_5$, and $P2X_6$, are expressed in developing rat skeletal muscle. These re-

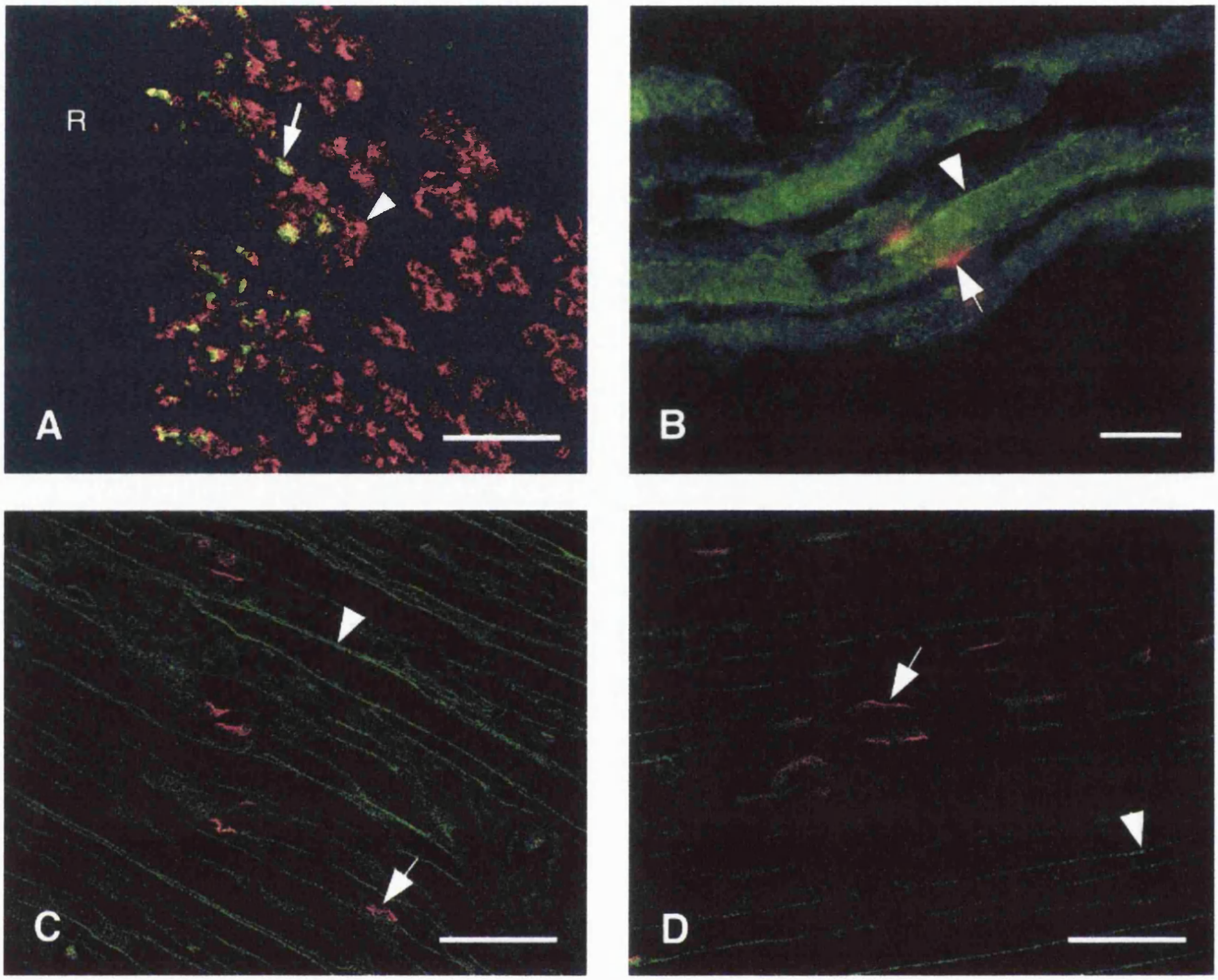


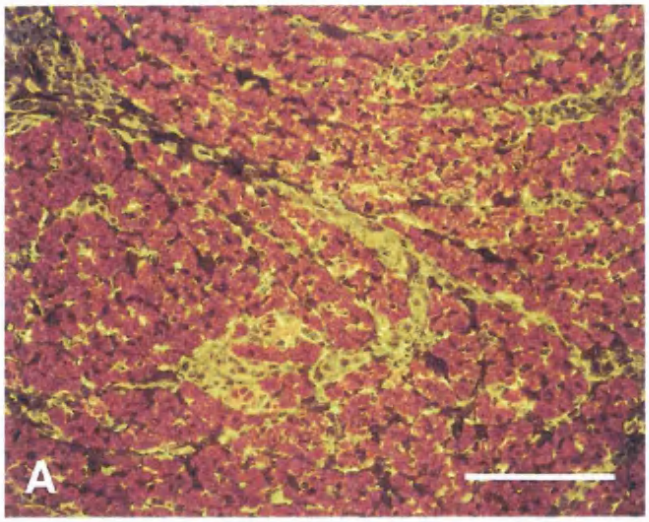
Fig. 6. **A:** Double labeling for $P2X_5$ (green, arrow) and skeletal myosin (red, arrowhead) demonstrated coexpression in areas close to the rib. **B–D:** Double labeling for $P2X_2$ (green, arrowheads) and acetylcholine receptors (AChRs) (red, arrows) on intercostal muscles at embryonic day (E) 20 (B) and tibialis anterior muscle at postnatal day (P) 7 (C) and P14 (D). Although there is strong staining for $P2X_2$ on the muscle membranes, it did not colocalize with AChR-clusters at any of the ages examined. R, rib cartilage. Scale bars = 50 μm in A, 20 μm in B, 100 μm in C,D.

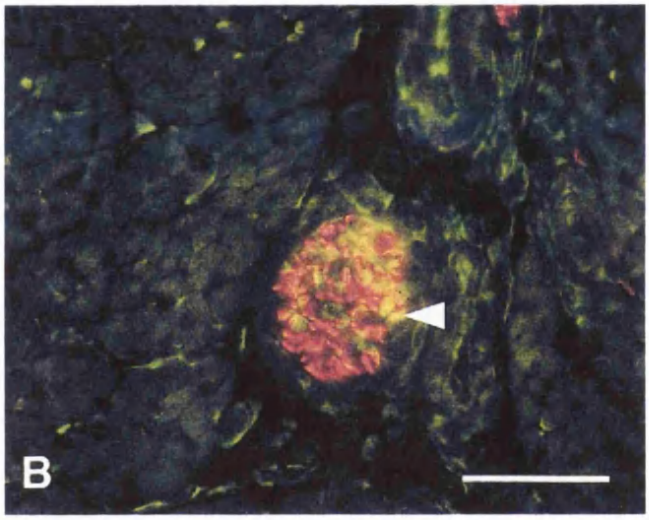
ceptors are expressed sequentially ($P2X_5$, followed by $P2X_6$, and finally $P2X_2$) and appeared to be developmentally regulated. The timing of receptor expression is closely related to key events in skeletal muscle development, notably the generation of secondary myotubes and the redistribution of acetylcholine receptors, suggesting that ATP may have trophic actions on developing mammalian skeletal muscle. However, the precise roles of the P2X receptor subtypes in skeletal muscle development remain to be investigated.

EXPERIMENTAL PROCEDURES

Mating of Rats and Embryo Preparation

For dated pregnancies, female Sprague-Dawley rats in estrus were placed overnight with primed male rats and examined in the morning for the presence of a vaginal plug. The day of finding the plug was designated embryonic day 0 (E0) and the day of birth, postnatal day 0 (P0). Pregnant rats, killed by a rising concentration of CO_2 and confirmed by cervical dislocation, were used to obtain embryos at E12, E14, E15, E16, E18, and E20. Once removed, some embryos were placed decapitated and intact onto a cork block, covered with OCT compound, and frozen in liquid nitrogen-cooled isopentane. Limb buds were dissected from the remaining embryos, mounted on cork blocks, and frozen separately in liquid nitrogen-cooled isopentane. Skeletal muscle samples were also taken from rat pups killed at P7, P14, and P21. Segments of rib cage and lower limb were removed and prepared as previously described. Cryostat sections were cut at 12 μm and collected on gelatinized slides.





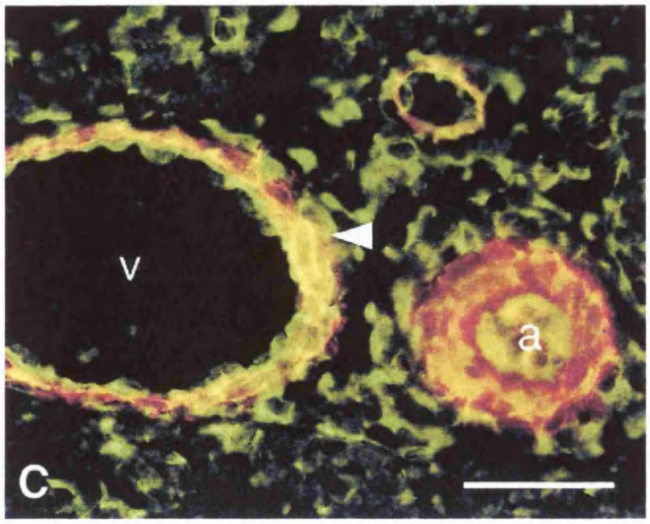


Fig. 7. **A:** At postnatal day (P) 7, double labeling for $P2X_5$ (green) and skeletal muscle myosin (red) showed the presence of $P2X_5$-immunopositive cells exclusively in between muscle fibers. **B:** Double labeling for $P2X_5$ (green) and neurofilament 200 (red) demonstrated limited coexpression (yellow, arrowhead) of these markers in fibers within a nerve bundle. **C:** Double labeling for $P2X_5$ (green) and smooth muscle actin (red) demonstrated limited coexpression (yellow) of these markers in both arteries and veins (arrowhead). Note that in C some $P2X_5$ (green) staining appears to be on endothelial cells. Because skeletal muscle develops from center to periphery, photographs taken from within the muscle bulk (B), which is more mature, demonstrate less immunoreactivity for $P2X_5$ than those taken at the less developed periphery (B). All transverse sections of lower limb muscle (tibialis anterior) at P7. a, artery; v, vein. Scale bars = 100 μm in A, 50 μm in B,C.

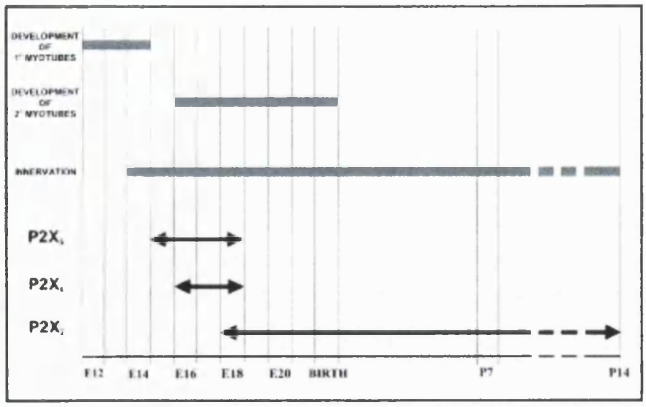


Fig. 8. This figure summarizes the time course of $P2X_{2,5,6}$ receptor expression for the rat intercostal muscles and demonstrates the possible overlaps with key events in skeletal muscle formation, namely development of primary and secondary myotubes and innervation (from the first detection of synaptic inputs to the development of the mature neuromuscular junction). The first receptor to appear is $P2X_5$ at embryonic day (E) 15, which disappears at E18; $P2X_6$ receptor expression starts at E16 and disappears after E18, whereas $P2X_2$ receptor expression only starts at E18 and gets significantly reduced after birth at postnatal day (P) 7.

Longitudinal sections of tibialis anterior muscle were prepared from animals at P7, P14, P21, and adult. Muscles were stretched and pinned on Sylgard, fixed in 4% paraformaldehyde for 2 hr at room temperature, and transferred to 20% sucrose in phosphate-buffered saline (PBS) and left overnight. Tissue was placed longitudinally and embedded in OCT on cork blocks. Cryostat sections were cut at 50 μm and collected in PBS.

Immunohistochemistry

The immunogens used for the production of polyclonal antibodies were synthetic peptides corresponding to the carboxy termini of the cloned rat P2X receptors, covalently linked to keyhole limpet hemocyanin. The peptide sequences are as follows. $P2X_1$: amino acids 385–399, ATSSTLGLQENMRTS; $P2X_2$: amino acids 458–472, QQDSTSTDPKGLAQL; $P2X_3$: amino acids 383–397, VEKQSTDSGAYSIGH; $P2X_4$: amino acids 374–388, YVEDYEQGLSGEMNQ; $P2X_5$: amino acids 437–451, RENAIVNVKQSQILH; $P2X_6$: amino

acids 357–371, EAGFYWRTKYEEARA; $P2X_7$: amino acids 555–569, TWRFVSQDMADFAIL.

The polyclonal antibodies were raised by multiple monthly injections of New Zealand rabbits with the peptides (performed by Research Genetics, Huntsville, AL). The specificity of the antisera was verified by immunoblotting with membrane preparations from CHO-K1 cells expressing the cloned $P2X_{1-7}$ receptors. The antibodies recognized only one protein of the expected size in the heterologous expression systems and were shown to be receptor subtype-specific (Oglesby et al., 1999). For immunostaining of cryostat sections, the avidin-biotin (ABC) technique was used according to the protocol developed by Llewellyn-Smith et al. (1992, 1993). Air-dried serial sections of the tissues were fixed in 4% formaldehyde and 0.2% of a saturated solution of picric acid in 0.1 M phosphate buffer (pH 7.4) for 2 min. After washing in PBS for 15 min, endogenous peroxidase activity was blocked by treating the sections with 0.4% H_2O_2 and 50% methanol for 10 min. Nonspecific binding sites were blocked by a 20-min preincubation in 10% normal horse serum (NHS) in PBS. Sections were incubated overnight at room temperature in 5 μg/ml of $P2X_{1-7}$ antibody or 2 μg/ml anti-skeletal myosin (Sigma, Poole, UK), in 10% NHS in PBS + 2.5% NaCl at room temperature. Subsequently, sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch Lab, West Grove, PA) diluted 1:500 in 1% NHS in PBS for 1 hr, and then with ExtrAvidin peroxidase (Sigma) diluted 1:1500 in PBS for 1 hr at room temperature. For color reaction, a solution containing 0.05% 3,3′-diaminobenzidine 0.04% nickel ammonium sulphate, 0.2% β-D-glucose, 0.004% ammonium nitrate, and 1.2U/ml glucose oxidase in 0.1 M PBS was applied for 8 min. Sections were washed three times with PBS after each of the above steps except after the preincubation. Control experiments were carried out with the primary antibody omitted from the staining procedure and the primary antibody preabsorbed with the peptides used to immunize the rabbits, according to the protocol described by Meyer et al. (1999).

For immunofluorescent staining, air-dried serial sections of tissue were fixed in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 min. After washing in PBS for 15 min, sections were treated as before with 10% NHS in PBS to block nonspecific binding. Sections were then incubated overnight at room temperature in 5 μg/ml of $P2X_5$ or $P2X_2$ antibody in 10% NHS in PBS + 2.5% NaCl. P2X expression was visualized by using Oregon-green–labeled goat anti-rabbit IgG (Jackson ImmunoResearch Lab) applied for 1 hr at 1:100. Double staining for acetylcholine receptors was performed by further incubation of sections with α-bungarotoxin-Texas Red at 1:1,000 for 1 hr at room temperature. For neurofilament 200 or smooth muscle actin, double staining was performed by incubation of sections with either mouse anti-neurofilament 200 IgG (Sigma) or mouse anti-smooth muscle actin (Sigma) at 1:100 and 1:1,000, respectively, overnight. Staining was visualized by incubating with TRITC-labeled goat anti-mouse antibody (Jackson ImmunoResearch Lab) at 1:100 for 1 hr.

In the case of double staining for $P2X_5$ and skeletal myosin, sections were incubated overnight at room temperature in 1 μg/ml of $P2X_5$ antibody in 10% NHS in PBS + 2.5% NaCl. This step was followed by incubation with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch Lab) diluted 1:500 in 1% NHS in PBS for 1 hr, ExtrAvidin peroxidase diluted 1:1500 in PBS for 1 hr, tyramide amplification for 8 min (Tyramide Amplification Kit, NEN Life Science Products, Boston, MA), and finally streptavidin-fluorescein (Amersham, UK) at 1:200 for 30 min. Sections were washed three times with PBS + Tween (0.05%) after each of the above steps. Staining for skeletal muscle myosin was performed by further incubation of sections overnight in 2 μg/ml rabbit anti-PM in 10% NHS in PBS + 2.5% NaCl, followed by visualization with goat anti-rabbit cy3 (Jackson ImmunoResearch Lab), 1:200 in 1% NHS in PBS for 1 hr. For the longitudinal 50-μm sections, photographs were generated by using confocal microscopy.

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