[Frontiers in Bioscience 3, d769-780, August 1, 1998]

G-PROTEIN COUPLED RECEPTORS IN BONE

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Received 1/16/98 Accepted 2/17/98

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1. ABSTRACT

The skeleton is a dynamic structure that undergoes continuous remodeling, a prerequisite to meeting the constant loading demands placed upon it. This process is controlled by a multitude of systemic and local factors which interact with receptors presented on the surface of both osteoblasts and osteoclasts; the osteogenic and osteolytic cells of bone. The seven transmembrane G-protein coupled superfamily of receptors are amongst the most important expressed by bone cells. Many local and systemic factors, including prostaglandins and parathyroid hormone, initiate cellular processes via interaction with members of this receptor family. The diversity of signals and signaling cross talk generated by activated G-protein receptor complexes, facilitates a huge range of downstream responses essential in the remodeling of the skeleton. Indeed, agonist-activated signaling crosstalk provides a mechanism for integrating the activities of local and systemic factors, an essential requirement of focal remodeling. This review has focused on those currently known seven transmembrane receptors expressed by bone cells that couple to G-proteins, and describes the nature of receptor-G protein interaction and the resultant functional consequences of effector activation within bone cells.

2. INTRODUCTION

Bone is a highly specialized tissue which undergoes cellular activity throughout life This is evident initially in the processes of growth and modeling which give rise to the mature skeleton. When longitudinal growth has ceased, cellular activity results in the processes of remodeling, renewed modeling and repair, all essential for the homeostasis of the mature skeleton. The principal cellular functions are bone formation, brought about by osteoblasts, and bone resorption, which is brought about by osteoclasts. The genesis, differentiation and activities of osteoblasts and osteoclasts are regulated by endocrine and paracrine factors which interact with specific receptors in cells (For review see (21)).

The G-protein coupled receptors which constitute the most important families of receptors in bone, are characterized by their ability to couple to catalytic heterotrimeric G-proteins, which through phosphorylation on serine/threonine residues, activate intracellular signalling cascades. Many important calciotrophic hormones including PTH and calcitonin, and local effectors such as PTHrP, ATP and prostaglandins initiate profound autocrine and paracrine events through the activation of G-protein-coupled receptors . As such this family of receptors provide the most propitious targets for pharmacological intervention in diseases of bone loss including osteoporosis.

The huge diversity of downstream cellular responses initiated upon G-protein coupled receptor-agonist binding is facilitated by a number of events. Alternate splicing of receptor mRNA can generate multiple subtypes

that differentially couple to one of the many characterized G-proteins, thereby activating specific intracellular cascades. The coupling specificity of receptor isoforms is the mechanism employed by locally released prostaglandins to differentially regulate osteoblast function. Differentiation stage specificity of signal transduction can occur through modulation of receptor-G-protein subtype coupling. Thus, the developmental status of bone cells can determine PTH receptor/G-protein interaction and therefore the activation of intracellular signaling cascades. Alternatively the interaction of multiple agonists provides a further layer of signaling diversity. Indeed, the interaction of local factors with those classically described hormones, has been forwarded as a means of localizing systemic responses in bone. In this review we describe in detail the molecular, and functional properties of the currently known G-protein coupled receptors expressed by osteoblasts and osteoclasts, and highlight the signaling diversity utilized by these receptors to maximize their functional impact.

3. SEVEN TRANSMEMBRANE DOMAIN RECEPTORS

The seven transmembrane domain receptors belong to a superfamily of functionally diverse receptors all sharing integral hydrophobic membrane spanning domains, separated by alternating extracellular and intracellular hydrophilic loops. Receptors belonging to this class respond to a variety of hormone and neurotransmitter agonists ranging from small biogenic amines such as adrenaline, to large peptides such as parathyroid hormone (PTH) and calcitonin. Ligand binding is facilitated by the formation of a pocket between specific` extracellular and transmembrane domains (16), within which conserved amino acid residues determine the ligand specificity and affinity for families of receptors. Binding of ligand to its receptor induces a conformational change resulting in the formation of a high affinity ligand-G-protein complex which catalyses guanine nucleotide exchange on the alpha subunit of the G-protein. The G-proteins dissociate from the receptor-ligand complex and transduce the extracellular signal from the receptor to a variety of intracellular signaling pathways. For excellent reviews on these processes see (23) and (84). The complex receptor-G-protein interaction occurs through a surface on the cytoplasmic side of the receptor composed of the ends of the transmembrane domains and the intracellular loops. This interaction predominantly involves the third intracellular loop, although mutational analyses have shown that specific residues in any of the intracellular loops or the C terminal tail contribute to the G-protein interaction (83). Receptor desensitization or downregulation occurs following repeated or sustained activation, providing a feedback mechanism limiting receptor activity. Such desensitization involves receptor phosphorylation by numerous kinases including, protein kinase A (PKA) and C (PKC) and by receptor specific kinases, including beta-adrenergic receptor kinase, BARK (3). Long term exposure to ligand can result in changes to transcriptional activity and ultimately receptor down regulation.

4. HETEROTRIMERIC G-PROTEINS

G-proteins are composed of three subunits (alpha, beta and the smaller gamma subunits). The alpha subunit shares homology with the GTPase family, possesses intrinsic GTPase activity and contains sites for myristoylation and palmitoylation (reviewed in (12)). All gamma subunits contain sites for isoprenylation (reviewed in (12)). These acetylations target G-proteins to the membrane compartment, the principal site for G-protein signaling.

Four G protein subfamilies have been identified and classified according to the known 23 alpha subunits (Gq/11, Gi/0 Gs and G12/13; (26)). There is a similar variety of beta (5) and gamma (11) subunits which are classed according to association with alpha subunits (19). These G protein subfamilies can be either stimulatory (Gq/11, Gi/0) or stimulatory and inhibitory (Gs). Furthermore, due to the host of interactions between alpha, beta and gamma subunits, many heterotrimeric species can be formed which confer increased specificity or flexibility of signaling.

The mechanism by which G-proteins convert receptor/ligand interaction to effector activation has been reviewed extensively elsewhere (23, 84). Briefly, ligand binding results in a conformational change in the receptor cytoplasmic domain that promotes association with an inactive GDP-bound heterotrimeric G-protein. This interaction enhances dissociation of GDP from the receptor/G-protein complex, facilitating GTP binding, alpha subunit activation, and dissociation from the receptor. Dissociation of G-protein from the receptor results in alpha subunit dissociation, facilitating its interaction with effector molecules. Intrinsic alpha subunit GTPase activity catalyses GTP hydrolysis causing inactivation, a process often enhanced or activated by effector binding. GDP-bound alpha subunit re-associates with beta and gamma subunits to form an inactive G protein heterotrimer which is once again capable of interacting with the receptor.

5. G-PROTEIN COUPLED RECEPTORS EXPRESSED BY OSTEOBLASTS

5.1. PTH/PTHrP receptor

Parathyroid hormone (PTH) is one of the cardinal regulators of bone and mineral homeostasis. The most conspicuous action of PTH, acting as a classical endocrine, is to regulate the concentration of calcium in the extracellular fluid (ECF). The hormone is secreted from the parathyroid glands in response to hypocalcemic challenge and acts on receptors in kidney and bone, to promote the reabsorption of calcium from the distal tubule and to promote resorption, respectively.

Molecular cloning has revealed that the PTH receptors expressed in bone and kidney are identical and belong to a subgroup of the seven transmembrane receptors which include receptors for calcitonin, secretin and VIP (1). These receptors are distinctive in the capacity for dual activation of two signal transduction pathways. The

receptors couple to Gs alpha, which activates adenylyl cyclase leading to intracellular accumulation of 3,5-cAMP and activation of the protein kinase A pathway. They also couple to Gq alpha which activates phospholipase C leading to the generation of phosphoinositides and activation of the protein kinase C cascade. This dual activation constitutes a complex system of regulation which allows for cross talk between the pathways.

One of the discernible actions of PTH in bone is to stimulate bone resorption and yet osteoclasts are devoid of PTH receptors. Paradoxically the receptors are expressed on cells of the osteoblastic lineage and PTH acts on these cells to promote bone-resorption by some as yet unidentified mechanism, possibly involving the release of a paracrine agent which stimulates the osteoclast directly (75). There is some evidence that the expression of PTH receptors in bone may be greatest in osteoblastic cells which are not involved in active matrix synthesis, including immature pre-osteoblasts or post-mature bone lining cells. Activation of PTH receptors in bone lining cells induces shape change and contraction which may allow access of osteoclasts to the bone matrix (36). It has also been proposed that PTH-induced secretion of matrix metalloproteases by osteoblastic cells may contribute to the resorptive process (15).

A second member of the PTH family designated parathyroid hormone-related protein (PTHrP) has been identified (89). PTHrP was first identified as a mediator of hypercalcemia of malignancy but was subsequently detected in many normal tissues including bone (93). The widespread expression of PTHrP and low circulating physiological levels indicate that this is a paracrine factor rather than a hormone. Nevertheless, PTHrP binds to the same receptor as PTH with similar affinity and activates the receptor with equal potency. Consequently, the receptor has been designated the PTH/PTHrP receptor. The interactions of PTH acting systemically, and PTHrP acting locally, have the potential to give rise to a complex regulatory network. This interaction is further complicated by the recent demonstration that activation of the osteoblast PTH/PTHrP receptor by PTH can induce the expression of PTHrP by human osteoblasts with the kinetics of an immediate early gene (94).

While the function of PTH in maintaining normocalcemia is well established, the physiological role of this hormone in bone is still poorly understood. It has been known for over sixty years that administered PTH can stimulate bone formation *in vivo*. (80). More recent studies have demonstrated that intermittent administration of PTH increases bone density and quality in experimental animals and man (for review see (71)). The PTH receptor in osteoblasts is therefore a major potential target for anti-osteoporotic therapy. Interestingly, one of the most successful agents known to increase bone mass is fluoride, which is a direct activator of Gs alpha (66). In addition to their role in bone remodeling, PTH receptors are important in the regulation of bone development including endochondral ossification. It is therefore not surprising that defects in bone growth are the most obvious manifestations of deletions or constitutive activation of the PTH receptor in inherited diseases or transgenic animals. A point mutation in the receptor in Jansen-type metaphyseal chondrodysplasia resulting in constitutive activation of Gs leads to hypercalcemia, delay in mineralization and short stature (78). Conversely, knockout of the PTH/PTHrP receptor leads to premature mineralization (46). Pseudohypoparathyroidism is a genetic disorder in which there is target organ resistance to PTH. The lack of response to PTH in kidney cells is demonstrated by a reduction in the nephrogenous cAMP response, but interestingly a normal response is observed in osteoblasts isolated from subjects with this disorder (34). This indicates that there may be tissue specific heterogeneity of adenyl cyclase isoforms or Gs alpha isoforms coupled to the PTH receptor.

Because of the complexity of PTH action on bone *in vivo*, researchers have turned to cell culture in order to elucidate the effects of receptor activation. PTH induces several phenotypic changes in osteoblasts which are consistent with an inhibitory effect on differentiation, including a reduction in osteoblastic markers such as alkaline phosphatase, osteocalcin and type I collagen (reviewed in, 22). PTH has been shown to have inhibitory or stimulatory effects on osteoblast proliferation depending on the cell type studied. The effect of PTH on human osteoblasts is to promote proliferation.(48), which may occur following induction of IGF1 and/or IGF binding proteins. Recent studies have demonstrated that the effects of PTH on osteoblasts are probably differentiation- and development-dependent (35).

The most important contribution that *in vitro* studies have made to understanding the biology of PTH receptors is in elucidating the signal tranduction pathways to which they couple. The primary observation is that PTH stimulates dual pathways, but perhaps of more significance are the observations that these stimulatory actions can be dissociated. The valine at position 2 in the PTH and PTHrP molecules is important for the cAMP response, hence, N-terminal truncated fragments, in which residues 1-2 are deleted, activate PKC but do not stimulate cAMP. These selective agonists are ineffective in stimulating bone resorption, but are mitogenic for osteoblasts *in vitro*. Activation of PKC is known to be an important signal in cell proliferation and it was initially thought that this pathway might be responsible for stimulation of bone formation. However, subsequent *in vivo* studies have confirmed that the complete N-terminal sequence, and thus activation of cAMP pathway, are required for anabolic actions. Further information linking peptide sequence to function has been obtained following the design of synthetic peptides including hPTH (1-31)NH2, which only activates PKC. Other fragments including PTH (39-84) and (53-84) appear to dissociate PKC from elevation of Ca^{2+}, while residues 28-34 are required for binding to the receptor. Interestingly, these regions are heterogeneous in the PTH and PTHrP peptide and yet both hormones bind to the receptor with equal affinity (74).

Recently, a second receptor for PTH, which has been designated PTH2 receptor, has been identified in placenta and uterine cells but not in bone (92). Finally, although the major functions of the PTH/PTHrP receptor are in bone and kidney, the receptor is more widely expressed, and has been detected on many cell types including tumor cells (11). Breast cancer cells express the receptor and proliferate in response to receptor activation (4). One of the tasks for future research will be to ask if the expression of the PTH/PTHrP receptor is a significant factor in the propensity of tumor cells to metastasize to bone.

5.2. Adenosine receptors

There are four cloned G-protein receptor subtypes termed A(1), A(2a), A(2b) and A(3) that bind and respond to adenosine. These receptors are coupled to both stimulatory (Gs) and inhibitory (Gi) G-proteins. The expression of these receptor subtypes has been widely characterized in many tissue types, however, little is known regarding subtype expression by osteoblasts. Functional data suggests that adenosine, through activation of Gs can stimulate osteoblast proliferation (64)(81), whilst a recent report has suggested that adenosine has no effect on bone formation (37). The expression of adenosine receptors by osteoblasts provides a further intriguing possibility. Adenosine, the breakdown product of ATP, may function in a negative feedback loop modulating the actions of extracellular nucleotides acting at P2-receptors.

5.3 Beta-adrenergic receptors

The beta-adrenergic receptors (BAR1, -2 and -3) are a family of seven transmembrane receptors that mediate the physiological responses of endogenous catecholamines (for review see (88)). The N-terminal region of these receptors is extracellular, glycosylated and of variable length, whilst the intracellular C terminal has consensus phosphorylation sites for both PKA and PKC, as well as for BARK. The BARs are coupled primarily to Gs and therefore adenylyl cyclase activation, although, recent evidence indicates that beta-3 receptors can also be negatively coupled through Gi. Following ligand binding and G protein activation, BARs are phosphorylated by PKA and BARK resulting in desensitization, which is further facilitated by the binding of the soluble protein beta-arrestin, which inhibits binding of Gs to the receptor (13) (62).

Extensive functional and molecular studies on a number of clonal osteoblastic cell lines has revealed expression of both beta-1 and beta-2 adrenergic receptors, with apparent differentiation-dependent regulation of these two subtypes (33), (57), (49), (41). The more tissue specific BAR3 appears not to be expressed by osteoblasts(41). BAR1 and BAR2 in osteoblasts are coupled to Gs, activation of which results in cAMP accumulation, PKA phosphorylation and stimulation of the immediate early gene *c-fos* (41). Heterodimers of *c-fos* with *c-jun* acting as the transcription factor AP-1, bind and regulate a wide variety of AP-1 responsive genes, including alkaline phosphatase, osteocalcin and collagen 1 (60, 82). It is therefore not surprising that BAR activation results in both skeletal anabolic effects and stimulatory effects on bone resorption (63, 57). Controversy exists regarding the exact receptor mediators of these cellular events, since enhanced resorption and stimulation of bone formation have been reported for both BAR agonists and antagonists. These inconsistencies probably reflect the complexity of adrenergic signaling and are compounded by ligand/ receptor subtype cross reactivity.

5.4. P2Y-receptors

The P2Y receptor subfamily of P2-receptors are amongst the smallest receptors in the superfamily of seven transmembrane domain receptors, with a short N-terminal domain at the extracellular surface, and a short third intracellular loop. The P2Y receptor family, of which their are currently seven reported subtypes (although controversy exists as to the true nature of the P2Y5 and P2Y7 receptors (97, 31, 47), are differentially responsive to extracellular adenine and uridine nucleotides. The P2Y1 receptor subtype has a conserved serine residue in the third intracellular loop that serves as a consensus phosphorylation site for protein kinase A, whilst the carboxy-terminal domain has three consensus phosphorylation sites for protein kinase C (for review see, 5).

Sequence comparisons between P2Y and adenosine receptors have revealed several positively charged amino acid residues in trans-membrane regions 3, 6 and 7, of the P2Y receptors only (5), leading to the suggestion that these residues are involved in the binding of negatively charged phosphate moieties presented by P2-receptor agonists. Perhaps more interesting, is the observation that replacement of the positive Lys289 with arginine, another positive residue, imparts enhanced potencies for ADP and UDP and markedly reduces the potencies of ATP and UTP. These data provide an insight into how differential responsiveness of receptors to nucleotides may be achieved. The exact nature of P2Y/G-protein interaction remains to be elucidated, however effector activation suggests coupling to multiple G-proteins, including Go, Gi and Gq/11 (5, 27, 18).

Kumagi *et al*, first proposed that osteoblasts respond to ATP through P2Y-receptor activation. Using the rat osteosarcoma cell line UMR-106 they demonstrated that ADP and ATP activation caused IP_3 mediated elevation of calcium from intracellular stores (45). Further to these initial observations, Shoefl *et al* demonstrated that primary human osteoblasts respond to ATP with a similar

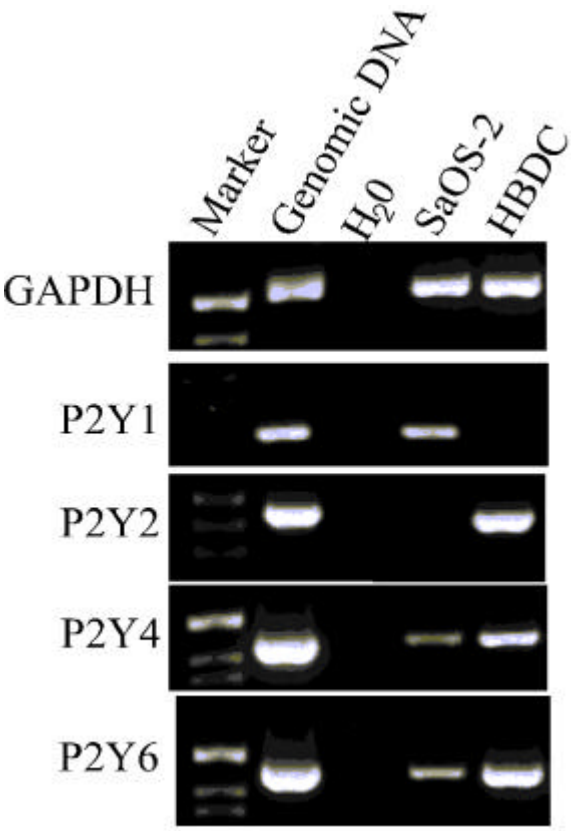

Figure 1. Amplification of P2Y1, P2Y2, P2Y4 and P2Y6 receptor transcripts using either SaOS-2 or human bone-derived cell (HBDC) cDNA as template. The specificity of amplified products was confirmed following sequence analysis. Human genomic DNA was used as a positive control, while water blank provided the negative control.

elevation of intracellular calcium (79). Conclusive evidence for osteoblastic P2 receptor expression came with the cloning and localization of P2Y2 receptor transcripts in primary human bone cells (6). Recent studies have demonstrated multiple P2Y subtype expression by a human osteoblastic cell line (figure 1), and indicate differentiation-dependent plasticity in P2Y receptor subtype expression, as described previously during the progression of human myeloid progenitors (17). It is therefore tempting to speculate that osteoblasts differentially express distinct P2 receptor subtypes during the processes of maturation and bone formation.

Although nucleotide stimulation of osteoblasts results in PLC activation and Ca^{2+} mobilization, there is no evidence to suggest that P2Y receptors are either positively or negatively coupled to adenylate cyclase, indicating that osteoblastic P2Y receptors couple to Gq. The exact functional consequence of individual P2Y subtype activation in osteoblasts remains largely to be elucidated, due to the presence of multiple receptor subtypes differentially activated by the complement of adenine and uridine nucleotide agonists, the existence of ectoenzymes on the surface of bone cells capable of initiating extracellular nucleotide inter-conversion (61), and the lack of specific antagonists for the panel of receptors expressed by osteoblasts. However, ATP and UTP are equipotent at up-regulating *c-fos* gene expression in primary human osteoblasts, data consistent with the activation of the P2Y2 receptor subtype (7). The *c-fos* gene has been implicated in many of the processes that govern skeletal tissue remodeling, including proliferation and osteoclastic recruitment (38). Indeed, ATP has been shown to stimulate the proliferation of osteoblastic MC3T3 cells (81).

In addition to the direct effects of nucleotides on cells, there is mounting evidence that extracellular nucleotides act in concert with other regulatory molecules (95). Consistent with findings in other cellular systems, extracellular nucleotides potentiate PTH-elevated intracellular calcium in rat UMR106 cells (39), and combine synergistically with PTH to induce *c-fos* expression in human osteoblasts (8). It therefore seems likely that extracellular nucleotides released locally in response to physiological loads or cellular trauma will regulate the responsiveness of surrounding bone cells to systemic factors like PTH, providing a mechanism that meets local modeling or remodeling requirements.

5.5. Prostaglandin receptors

The prostaglandins, including PGE_2, PGF_2 and PGI_2, are oxygenated metabolites of arachidonic acid that exert a wide range of local physiological effects through activation of G-protein-coupled prostaglandin receptors. These three prostaglandins have been demonstrated to be released by bone in both organ and cellular culture (70, 76), although PGE2 represents the major prostaglandin produced by bone cells (76), and until recently represented the most functionally relevant to bone.

There are currently four subtypes of seven transmembrane domain receptors that bind and respond to PGE2 (EP1, EP2, EP3 and EP4) (59). These four receptor types are coupled to either $[Ca^{2+}]_i$ mobilization, or stimulation or inhibition of adenylyl cyclase. Alternative splicing of EP3 receptor mRNA generates 4 subtypes which appear to be differentially coupled to one or more G-proteins, including Gi, Gs or Gq (44). Of the four PGE2 receptor types, bone cells appear to predominantly express EP1 and EP4 (86). Interestingly, EP2 receptors have been localized by *in situ* hybridization to pre-osteoblasts in neonatal rat calvaria (40), implicating this receptor in processes associated with an immature osteoblast phenotype.

The functional significance of PGE2 receptor activation in bone is diverse. Early reports demonstrated that PGE2 potently modulated bone resorption (42). A recent report suggests that this may occur as a result of increased osteoclastogenesis following EP4 receptor activation (77). Recently, anabolic effects of PGE2 have been described (96). In addition, these ligands exert effects on differentiation and cellular growth (85). Indeed, the EP2 receptor by virtue of its association with Gs and

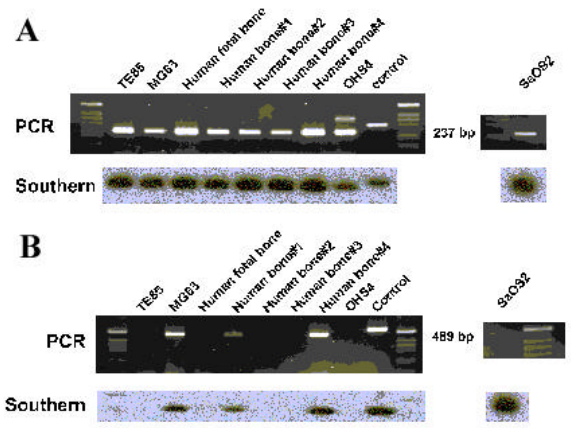

Figure 2. A: Amplification of angiotensin II type 1 receptor transcripts using as template a panel of cDNAs derived from both clonal and primary bone cell types. The specificity of amplified products was confirmed by Southern analysis. B: Amplification of angiotensin II type II receptor transcripts using as template a panel of cDNAs derived from both clonal and primary bone cell types. The specificity of amplified products was confirmed by Southern analysis.

cAMP accumulation has been implicated in mediating the proliferative effect of PGE2 on osteoblasts. To further complicate PG-stimulated signaling cascades, PGF2 has been shown to enhance both IL-6 production and osteoblastic proliferation, through the recently identified PGF2 alpha receptor coupled to Gq.

The local production and secretion of prostaglandins by bone cells occurs in response to many stimuli, including calciotropic hormones (53), cytokines (51), sex steroids (65), vitamin D metabolites (43) and shear stress (72). This observed systemic and environmental regulation of agonist, when combined with multiple receptor subtype expression and the diverse functional effects initiated upon ligand/receptor activation, clearly demonstrates the importance of prostaglandins as local autocrine and paracrine regulators of bone cell function.

5.6. Orphan receptors

Recent evidence suggests that during mouse embryo development a previously uncharacterized G protein coupled receptor with homology to the rat orphan receptor (65%) GPR-1, is expressed by the cells of developing bone, including osteoblasts and chondroblasts (52). This novel receptor also shares sequence homology with the rat angiotensin II receptor, the human C5alpha anaphylatoxin receptor and the human somatostatin receptor. The extent to which mature osteoblastic cells *in vitro* and *in vivo* express this receptor requires further investigation as does the nature of G-protein receptor interaction. Furthermore, the functional relevance of this receptor is unknown, hence its categorization as an orphan receptor.

5.7. Angiotensin II receptors

Angiotensin II acting at the angiotensin II receptors (AT) primarily regulates pressure and fluid osmolarity. There are two subtypes of receptor, the AT1 and AT2 types, of which the AT1 subtype is predominantly expressed in adult tissues, whilst the AT2 type appears to be expressed mainly during fetal development. The AT1 receptor gene encodes 5 exons which, through differential mRNA splicing generates two receptor isoforms which can be coupled to both Gq or Gi (For reviews see (91, 73). Interestingly, AT(1) and AT(2) exhibit unique signaling pathways among the superfamily of seven membrane-spanning receptors: i.e. the coupling of AT(1) to the Janus kinase-signal transducers and activators of transcription pathway and the coupling of AT(2) to phosphatase activation (54). Molecular studies have revealed expression of the AT1 receptor type by a panel of osteoblastic cell lines and by human adult and fetal bone *in vitro* (figure 2A).

The AT2 receptor subtype appears to display a more selective distribution as described previously in other tissue systems. Surprisingly, no expression was detected in the sample of fetal bone studied (Figure 2B). While osteoblasts clearly express angiotensin II receptors, the nature of G protein coupling in these cells remains to be investigated, as do the activated signal pathways following receptor-ligand interaction. Functionally, activation of these receptors by angiotensin II stimulates osteoclastic resorption in mixed bone cell suspensions, but not in purified osteoclastic populations, demonstrating that osteoblastic activation mediates this process (29).

5.8. Calcium Sensing Receptor

Based on current functional, biochemical and molecular studies there are three known receptor types that bind inorganic cations as traditional first messengers, of which the G protein-coupled calcium sensing receptor (CaR) cloned from parathyroid cells is the best characterised (10). This receptor exhibits a wide cellular distribution, possesses little homology to other members of the seven transmembrane metabotrophic receptors and appears to be coupled to the pertussis sensitive G-protein, Gi (reviewed in, 30). In parathyroid cells CaR activation follows receptor extracellular calcium binding. Physiologically, this event alerts cells to changes in systemic calcium homeostasis, to local cellular activity both related and unrelated to systemic fluid and electrolyte homeostasis, and probably represents a general paradigm for the sensing of inorganic cations.

Following the demonstration that extracellular cations can activate G-protein mediated second messenger cascades (67) (69), initiate early gene transcription (68), and stimulates DNA synthesis in osteoblasts (28), it was suggested that osteoblasts express a putative calcium sensing receptor. Cation specificities indicate that the calcium sensing receptor expressed by osteoblasts is functionally similar to the parathyroid CaR, however, molecular studies have failed to demonstrate expression of this receptor in a number of osteoblastic cell lines (68). Therefore, osteoblasts probably express a molecularly distinct G protein-coupled calcium sensing receptor, the nature of which has yet to be elucidated.

6. G-PROTEIN COUPLED RECEPTORS EXPRESSED BY THE OSTEOCLAST

6.1 Calcitonin receptor

The calcitonin receptor (CR) as described above is most closely related to the PTH and secretin receptors that form a distinct subfamily within the G-protein coupled superfamily. There are currently two well characterized CR subtypes that occur through alternative splicing of the CR gene, resulting in the addition or deletion of 16 aa in the first intracellular loop, between the first and second predicted transmembrane helices (56). Although CR display some tissue specificity, osteoclasts express both the insert positive and negative isoforms, however, Northern analysis studies suggest that the insert negative receptor is the predominant isoform (25, 32, 50).

The CR is coupled to both Gq and Gs, initiating pathways that activate both phospholipase C and adenylyl cyclase (100). As such, the peptide hormone calcitonin produced by the parafollicular cells of the thyroid, stimulates cAMP accumulation and induces intracellular calcium elevations in osteoclasts. Recent studies indicate that whilst the binding parameters for both insert negative and positive CR's are indistinguishable, there are striking differences in the ability of these two receptors to couple to second messenger pathways. Insert negative CR alone will mediate rises in intracellular calcium, whilst the ED50 for stimulation of adenylyl cyclase is 100 fold higher for the insert positive CR.

The main function of calcitonin is to negatively regulate calcium homeostasis. As such activation of CR expressed by osteoclasts results in an inhibition of resorption (reviewed in, 87). The mechanism of this inhibition involves both the arrest of osteoclast motility and a shape retraction, termed the Q and R effect respectively. Furthermore, the disruption of F actin ring structures formed at the clear zone of the resorbing osteoclast appears to be a primary mechanism of calcitonin-induced resorption inhibition. While intracellular calcium elevation appears to modulate the R effect (2), F actin disruption occurs following PKA activation (90). It is therefore probable that both the insert positive and negative CR's mediate the osteoclastic effects of calcitonin.

Multiple levels of regulation exists to facilitate CR desensitization. These include receptor-ligand internalization (specific to the insert positive subtype), uncoupling from signaling pathways, down regulation of receptor mRNA expression and loss of receptor binding from the cell surface (reviewed in (20). Specific C-terminal phosphorylation by both PKA, PKC and a non-second messenger dependent receptor kinase (GRK) have recently been reported for both the insert negative and positive CR's, suggesting a role for phosphorylation in the desensitization processes outlined above.

6.2. P2-receptors

It is becoming increasingly apparent that osteoclasts represent targets for extracellular nucleotide activation through G-protein-coupled P2Y receptors. The studies of Yu and Ferrier demonstrated an ATP-induced increase in $[Ca^{2+}]_i$ in isolated rabbit osteoclasts, an event attributed to the activation of two pathways; G-protein-mediated release of internal Ca^{2+} stores, and the opening of Ca^{2+} channels in the cell membrane facilitating Ca^{2+} influx (98, 99). However, the nature of the P2Y receptor responsible for this ATP-induced intracellular calcium elevation remains to be elucidated. Recently, Bowler *et al*, 1998, described the *in situ* localization of G-protein-coupled P2Y2 receptor transcripts in osteoclasts derived from a human giant cell tumor, however ATP and UTP, agonists at this receptor, were both ineffective at elevating intracellular calcium in isolated giant cells (9). The reasons for the discrepancy in ATP-induced signaling events described in the above studies are unclear, but suggest that in osteoclasts P2Y2 receptors are not coupled through Gq to Ca^{2+} mobilization, as found in osteoblasts. The coupling of P2Y receptors to signaling mechanisms distinct from PtdIns $(4,5)P_2$ hydrolysis has been reported in other cell types, and include adenylate cyclase, phospholipase D and the mitogen-activated protein kinase cascade (for review see, 5). Further studies are required to elucidate P2Y receptor expression by osteoclasts and to determine receptor/G protein and effector pathway interaction.

Similar uncertainty exists as to the functional role of G-protein-coupled P2Y-receptors expressed by osteoclasts. ATP initiates mechanisms that result in either inhibition or stimulation of bone resorption, depending upon agonist concentration (9, 58). The studies of Bowler *et al*, 1997, describing ATP, but not UTP-enhanced bone resorption by an enriched population of human giant cells derived from osteoclastoma, would suggest the involvement of an osteoclastic receptor other than the P2Y2 type (9). Indeed, there is mounting evidence that osteoclasts express ionotropic members of the P2-receptor family, including the ATP-responsive P2X4 receptor, although in the absence of receptor-specific antagonists it is difficult to attribute functional effects to activation of particular receptor types.

6.3. Calcium sensing receptor

When exposed to high extracellular Ca^{2+} concentrations, osteoclasts respond by rapidly elevating intracellular calcium (101). The physiological effects of this event on the osteoclast are profound; dramatic cell retraction followed by an extreme inhibition of resorption (14, 55). As described above a mechanism for sensing extracellular calcium ion concentration involving a G-protein-coupled receptor, the CaR, has been described in the cells of parathyroid and kidney (30). Less clearly defined is the receptor(s) expressed by osteoclasts that sense fluxes in extracellular ion concentration. It is clear from functional binding studies that these receptors are distinct from the molecularly characterized CaR (10), and the putative CaR expressed by osteoblasts (68). Indeed, recent evidence suggests that the sensing mechanism in osteoclasts involves a ryanodine-like calcium release channel (RyR), normally localized in microsomal membranes (102). However, it is unclear if the RyR molecule is physically coupled to and activated by a putative G-protein CaR or

alternatively if RyR itself can bind and gate divalent cations. The ability of osteoclasts to sense and functionally respond to rising calcium concentration in their extracellular environment, provides a negative feedback limiting further osteoclast activity, and an intriguing possibility would be the involvement of a molecularly distinct G-protein-coupled receptor.

7. PERSPECTIVE

The past five years have seen an explosion in the characterization and cloning of G-protein-coupled receptors expressed by bone cells, this list will inevitably expand, particularly as techniques to isolate and manipulate osteoclasts *in vivo* evolve. However, it is apparent from those receptors discussed here that G-protein-coupled receptor activation is vital to the functional viability of both osteoblasts and osteoclasts, which in part involves successful communication between cell types. The import of these receptors is further demonstrated in an ability to initiate multiple signals following activation of the same receptor, and their capacity to integrate signals generated following multiple receptor activation. The ability of members of this receptor superfamily to bind and respond to systemic ligands as well those produced locally by the cells of bone, probably facilitates the necessary signaling cross talk required to meet the focal requirements of the remodeling process. The ability of these receptors to so profoundly influence the anabolic or resorptive capacity of bone cells singles them out as potential therapeutic targets in disorders associated with a breakdown in the remodeling process. The selective manipulation of G-protein coupled receptors that target either bone formation or resorption is an exciting possibility for combating skeletal disorders such as osteoporosis, however, as indicated in this review, the complex network of signaling cross talk initiated by these activated receptors remains an obstacle to the development of effective therapeutic regimes.

8. REFERENCES

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Key words: G-protein, Receptor, Osteoblast, Osteoclast

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